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**ABSTRACTS  
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## **P001 More is not always better: Results of the randomized open-label GPOH trial NB2004-HR on the induction chemotherapy in high-risk neuroblastoma**

*Berthold, F.<sup>2</sup>, Faldum, A.<sup>3</sup>, Ernst, A.<sup>4</sup>, Boos, J.<sup>5</sup>, Dilloo, D.<sup>6</sup>, Eggert, A.<sup>7</sup>, Fischer, M.<sup>8</sup>, Frühwald, M.<sup>9</sup>, Henze, G.<sup>10</sup>, Klingebiel, T.<sup>11</sup>, Kratz, C.<sup>12</sup>, Kremens, B.<sup>13</sup>, Krug, B.<sup>14</sup>, Schmidt, M.<sup>15</sup>, Schmidt, R.<sup>16</sup>, Schumacher-Kuckelkorn, R.<sup>17</sup>, Von Schweinitz, D.<sup>18</sup>, Schilling, F.H.<sup>19</sup>, Theissen, J.<sup>20</sup>, Simon, T.<sup>21</sup>, Hero, B.<sup>22</sup>*

University of Cologne, Pediatric Oncology and Hematology, Köln <sup>2</sup>, University of Münster, Medical Statistics and Clinical Research, Münster<sup>3</sup>, University of Cologne, Medical Statistics and Computational Biology, Köln<sup>4</sup>, University of Münster, Pediatric Oncology and Hematology, Münster<sup>5</sup>, University of Bonn, Pediatric Oncology and Hematology, Bonn<sup>6</sup>, Charité Universitätsmedizin, Pediatric Oncology and Hematology, Berlin<sup>7</sup>, University of Cologne, Experimental Pediatric Oncology, Köln<sup>8</sup>, University Hospital, Pediatric Oncology and Hematology, Augsburg<sup>9</sup>, Charité Universitätsmedizin, Pediatric Oncology and Hematology, Berlin<sup>10</sup>, University of Frankfurt, Children and Adolescents, Frankfurt<sup>11</sup>, Medicinal University, Pediatric Oncology and Hematology, Hannover<sup>12</sup>, University of Essen, Pediatric Oncology and Hematology, Essen <sup>13</sup>, University of Cologne, Diagnostic and Interventional Radiology, Köln <sup>14</sup>, University of Cologne, Nuclear Medicine, Köln <sup>15</sup>, University of Münster, Medical Statistics and Clinical Research, Münster <sup>16</sup>, University of Cologne, Pediatric Oncology and Hematology, Köln <sup>17</sup>, University of Munich, Pediatric Surgery, München <sup>18</sup>, Ologahospital, Pediatric Oncology and Hematology, Stuttgart <sup>19</sup>, University of Cologne, Pediatric Oncology and Hematology, Köln <sup>20</sup>, University of Cologne, Pediatric Oncology and Hematology, Köln <sup>21</sup>, University of Cologne, Pediatric Oncology and Hematology, Köln <sup>22</sup>

### Background/Introduction:

Induction chemotherapy is a mainstay for treating patients with high-risk neuroblastoma.

### Aims:

This trial aimed to assess whether the addition of two courses of topotecan, etoposide and carboplatin compared to the GPOH standard induction chemotherapy is able to improve event free survival.

### Methods/Materials:

An open-label, multi-center, randomized prospective controlled trial was performed at 58 institutions in Germany and Switzerland. Patients with neuroblastoma diagnosed according to the INSS criteria either with stage 4 and age  $\geq 1$  - 21 years or with *MYCN* amplification and age  $\geq 6$  months - 21 years were eligible. The randomization was done at diagnosis by blocks and stratified by stage, LDH elevation, age, and *MYCN* amplification. The primary endpoint was event free survival (EFS). The patients were randomly assigned to standard induction chemotherapy consisting of six alternating courses N5 (cisplatin, etoposide, vindesine) and courses N6 (vincristine, dacarbazine, ifosfamide, doxorubicine) or to two courses consisting of topotecan, cyclophosphamide, and etoposide followed by standard induction chemotherapy (total eight courses). Induction chemotherapy was followed by high-dose chemotherapy with autologous hematopoietic stem cell transplantation and by nine courses of isotretinoin over 1 year. Radiotherapy to the primary tumor and/or mIBG therapy were applied only to patients with active tumor at the end of induction chemotherapy.

### Results:

Between October 12, 2004 and December 31, 2016 536 patients were enrolled and eligible for random allocations. Of 422 randomized patients (79%) patients, 211 were assigned to the control and 211 to the experimental arm (intent to treat cohort). Median follow-up time was 3.32 years (IQR 1.65-5.92). At the data lock (September 1, 2018), 138/211 patients of the experimental arm and 145/211 patients of the control arm had events. The 3 year event free survival proportions were 34% (95%-CI 28-40) and 32% (26-38) respectively ( $P = 0.258$ , 3-stage adaptive design, total observation period). The proportions for 3 year overall survival (OS) were 54% (95% CI 46-62; experimental arm) and 48% (40-56; control arm;  $P=0.558$ ). The response to induction chemotherapy after 2 courses and before

high-dose chemotherapy was not different between the arms. Patients of the experimental arm needed longer time for the induction chemotherapy (median 195 days, 95% CI 191-201) compared to patients of the control arm 134 days (130-138) ( $P<0.001$ ). Grade 3 and 4 thrombocytopenia was more frequently seen in the experimental arm (86% vs. 79%,  $P<0.001$ ) and grade 3 and 4 oral mucositis more in the control arm (6% vs. 3%,  $P=0.022$ ). The median number of non-fatal grade 3 and 4 toxicities per patient was higher in the experimental group (31; 95% CI 29-33) compared to the control group (22; 95% CI 22-23;  $P<0.001$ ). In both treatment arms two toxic deaths were caused by induction chemotherapy.

#### Summary/Conclusions:

The patients had no event free survival benefit from two additional chemotherapy courses. The longer chemotherapy period was associated with more grade 3 and 4 toxicities. The addition of chemotherapy courses consisting of topotecan, cyclophosphamide and etoposide to the standard induction chemotherapy cannot be recommended.

## **P002 Surveillance for Early Detection of Neuroblastoma Central Nervous System Relapse**

*Modak, S.<sup>2</sup>, Kushner, B.<sup>3</sup>, Cheung, N.<sup>4</sup>, Kramer, K.<sup>5</sup>*

Memorial Sloan Kettering Cancer Center, Pediatrics, New York <sup>2</sup>, Memorial Sloan Kettering Cancer Center, Pediatrics, New York<sup>3</sup>, Memorial Sloan Kettering Cancer Center, Pediatrics, New York<sup>4</sup>, Memorial Sloan Kettering Cancer Center, Pediatrics, New York<sup>5</sup>

### **Background/Introduction:**

Neuroblastoma relapsing in the central nervous system (CNS), though uncommon, is historically incurable. However, a recent multimodality approach that includes intra-ventricular radioimmunotherapy has significantly improved long term survival (J. Neurooncology 97:409, 2010). Timely detection of CNS relapse may reduce or prevent morbidity and mortality. In co-operative groups, surveillance for patients on or after treatment for high-risk neuroblastoma usually includes whole-body MIBG scans but does not require dedicated anatomical imaging of the brain. At Memorial Sloan Kettering Cancer Center (MSK), head MRI at 3-6 monthly intervals is standard-of-care.

### **Aims:**

The objective of this retrospective study was to determine the optimal imaging modality for the detection of CNS relapse in patients with neuroblastoma.

### **Methods/Materials:**

After MSK IRB approval, records of all patients with CNS neuroblastoma seen at MSK from 2004-2019 were evaluated. In most cases, relapse was diagnosed at other institutions before referral to MSK. Analyzed data included symptoms and radiological findings on brain CT/MRI and MIBG scans at CNS relapse. Data on available scans from neuroblastoma diagnosis until CNS relapse were also analyzed.

### **Results:**

153 patients, all with MIBG-avid disease at initial diagnosis were identified; 5 had CNS disease at diagnosis. For the remaining 148 patients, median time to CNS relapse from diagnosis was 18 (range 0-104) months. 102 (67%) patients experienced CNS relapse after achieving complete remission (CR) at a median of 9.8 (range 0.8-72) months later, while 43 patients had relapse without achieving CR. Of those relapsing post-CR, CNS was the initial site of relapse in 93 (90%). 97/153 (66%) patients had neurological symptoms at time of CNS disease; the rest (34%) were asymptomatic, relapse being detected on surveillance. Multiple (>1) parenchymal lesions were noted on imaging in 58 (38%), diffuse leptomeningeal disease without parenchymal involvement in 8 (5%) and solitary lesions in 87 (57%), located to cerebellum and cerebrum in 14 (16%) and 73 (84%), respectively. Median diameter of the largest lesion on imaging was 2.7 (range <0.5-6.8) cm. Anatomical imaging was performed with MRI (82; 54%), CT (14; 9%) or both (57;37%). Of those undergoing both scans, CNS relapse was missed on CT in 3/57 (5%) patients. 115 patients had MIBG scans prior to resection of CNS relapse. All sites of disease noted on MRI/CT were also positive by MIBG in 40 (35%) patients. However, MIBG scan was either totally or partly negative in 56 (49%) and 19 (15%) patients, respectively. Even for lesions  $\geq 2$ cm in diameter, MIBG was negative in 24/61 (39%). Of the 76 (50%) patient undergoing prior MR/CT of the brain (0.3-34 months before relapse), CNS disease was not detected in any; only 51/153 (33%) had MRI/CT surveillance within 6 months prior to relapse.

### **Summary/Conclusions:**

MIBG scan has poor sensitivity for the detection of CNS neuroblastoma, regardless of size or location. Although CT or MRI are both effective in detecting CNS relapse, the former can miss some lesions. Given that most relapses occur within 2 years of diagnosis, we recommend brain MRI for surveillance of high-risk neuroblastoma at regular intervals for  $\geq 3$  years after initial diagnosis.

### **P003 Case report of a metastatic VIP-secreting neuroblastoma in remission without postoperative chemotherapy**

*Ben fredj, D.<sup>2</sup>, Plantaz, D.<sup>3</sup>, Rubio, A.<sup>4</sup>, Freycon, C.<sup>5</sup>, Sartelet, H.<sup>6</sup>, Piolat, C.<sup>7</sup>, Combaret, V.<sup>8</sup>*

Grenoble Alpes University Hospital, Pediatric Onco-Immuno-Hematology, Grenoble <sup>2</sup>, Grenoble Alpes University Hospital, Pediatric Onco-Immuno-Hematology, Grenoble<sup>3</sup>, Grenoble Alpes University Hospital, Department of Pediatric Gastro-Enterology, Grenoble<sup>4</sup>, Grenoble Alpes University Hospital, Pediatric Onco-Immuno-Hematology, Grenoble<sup>5</sup>, Grenoble Alpes University Hospital, Anatomical pathologist, Grenoble<sup>6</sup>, Grenoble Alpes University Hospital, Surgery Pediatric, Grenoble<sup>7</sup>, Centre Leon Berard, Department of Genetic, Lyon<sup>8</sup>

#### Background/Introduction:

Introduction Neuroblastoma represents around 10% of malignant diseases in children, with 120-150 cases per year in France, with a peak of incidence at the age of 18 months. The mode of discovery is variable and often not very specific. In the metastatic stage, neuroblastomas lead in the majority of cases to heavy management by surgery, followed by high-dose chemotherapy, radiotherapy and immunotherapy.

#### Aims:

#### Methods/Materials:

#### Results:

The case We report here the case of a metastatic VIP-secreting neuroblastoma in remission without postoperative chemotherapy. This is a 15-month-old child who presented a chronic diarrhoea, for several months. Clinically he presented abdominal distension without mass, hepatomegaly and hypertension. Biologically there was an hypokalemia. Additional examinations revealed a calcified left adrenal mass and secondary pulmonary lesions. MIBG scintigraphy did not reveal any fixation. The dosage of urinary catecholamines was high and the VIP 10 times normal. Biopsy of the mass confirmed the diagnosis of differentiated neuroblastoma without *MYCN* amplification. The CGH analysis found: +3(p26.3 - p22.2), +14q11.2, +15(q22.2-q25.3), 15(q26.1 -qter)-. The child was included in the HLBL SIOP protocol, RAPID Cojec arm, treated with chemotherapy. Chemo-resistance of the tumour was noted, with no decrease in volume, neither resolution of diarrhoea or decreasing of metastatic lung lesions. Surgery was then performed by total left adrenalectomy, lumbo-aortic curage and resection of 2 pulmonary nodules. Anapathological analysis of these nodules confirmed the neuroblastic origin. Following surgery, the digestive symptoms disappeared and close surveillance was decided. Postoperative treatment with high dose chemotherapy has not been initiated. Clinical, biological and radio-logical monitoring notes no tumour and metastatic evolution. There is a continued quasi-normalisation of the VIP rate at 29 months of diagnosis.

#### Summary/Conclusions:

Conclusion In conclusion, this case is original by the unusual therapeutic development, because a clinical-biological remission was obtained without post-operative chemotherapy, despite the metastatic nature of the tumour. Metastases are most often bone, hepatic, lymph node or subhepatic, here it is a case with pulmonary metastases. The absence of fixation with MIBG can make us ask the question of a particular component of this tumour. In addition, genetic analysis in "classic" neuroblastoma can find chromosomal deletions, typically 1p, 3p, 4p, 6q, 11q or gains such as 1q, 2p or 17q, in our case the atypical CGH profile has given us leads to an in-depth molecular study, studies are underway.

Perspective: Metastatic neuroblastoma cases with Vaso-Intestinal-peptide secretion appears as a special entity among neuroblastic tumors, with a possible favourable prognosis, that might be related to the spontaneous maturation of the tumor and the metastases. A retrospective collection of such cases could help to confirm these data at the clinical, biological and therapeutic level.



## **P004 Contribution to metastasis of undifferentiated cells in neuroblastoma**

*Álvarez, A.A.<sup>2</sup>, Gómez Muñoz, M.A.<sup>3</sup>, Pardal Redondo, R.<sup>4</sup>, Vega Moreno, F.<sup>5</sup>*

Instituto de Biomedicina de Sevilla, Departamento de Biología Celular, Sevilla <sup>2</sup>, Instituto de Biomedicina de Sevilla, Departamento de Fisiología Médica y Biofísica, Sevilla<sup>3</sup>, Instituto de Biomedicina de Sevilla, Departamento de Fisiología Médica y Biofísica, Sevilla<sup>4</sup>, Instituto de Biomedicina de Sevilla, Departamento de Biología Celular, Sevilla<sup>5</sup>

### **Background/Introduction:**

Neuroblastoma (NB) is the most common extracranial solid tumour occurring in childhood. This disease arises from the components of the neural crest of the sympathetic nervous system and presents a high clinical heterogeneity that ranges from spontaneous regression to aggressive metastasis. Cellular heterogeneity has also been observed, which includes at least two types of tumour cells with divergent gene expression profiles and phenotypes: undifferentiated stem-like mesenchymal cells (MES) and compromised adrenergic cells (ADRN). The undifferentiated cell population seems to exhibit stem cell properties and it has been suggested that they contribute to NB malignization by evading intensive multimodal therapy. Therefore they might be the responsible of tumour relapses metastasis formation and poor clinical outcomes. The formation of these aggressive metastases takes place thanks to a multi-stage process in which cell migration is essential.

Migratory cancer cells undergo dramatic molecular and cellular changes by reshaping their cell-cell and cell-matrix adhesion, as well as their cytoskeleton. Changes in cell shape are translated and are indicative of biochemical signals that can modulate cell phenotype and biological properties. These shape changes are indicative of the behavior and evolution of the cells. In fact, it has been shown that cell morphology regulates biological processes, such as proliferation, differentiation and stem cells fate.

### **Aims:**

To study the specific adhesive and migratory properties of undifferentiated neuroblastoma cells.

### **Methods/Materials:**

Cultures are enriched in undifferentiated cells by tumorespheres in defined culture conditions and FACS sorting from NB cell lines and patient derived cells. Adhesion and spreading assay, cell morphology assay, migration analysis and membrane dynamics analysis, were performed.

### **Results:**

The results obtained show that undifferentiated NB cells have greater adhesion and spreading capacity than the rest of tumour cells. They also present defined morphological features (smaller area, less perimeter, greater form factor, greater solidity) and a lower number and length of neurites. In addition, they migrate at a faster speed, and more persistently. Undifferentiated cells also presented higher levels of actin membrane dynamics.

### **Summary/Conclusions:**

With the results obtained, it can be concluded that the most undifferentiated cells from the tumour, have great cellular plasticity, indicated by a particular cytoskeletal morphology and structure. In addition, they are characterized by having a greater migratory capacity than the cells forming the bulk of the tumour. This preliminary results suggests that undifferentiated NB cells could have important metastatic characteristics.

## **P005 Strong prognostic ability of LDH and serum ferritin in neuroblastoma: overall and in high-risk patients after 2009 - An International Neuroblastoma Risk Group (INRG) Project**

*London, W.B.<sup>2</sup>, Moroz, V.<sup>3</sup>, Machin, D.<sup>4</sup>, Hero, B.<sup>5</sup>, Ladenstein, R.<sup>6</sup>, Berthold, F.<sup>7</sup>, Kao, P.<sup>8</sup>, Obeng, Y.<sup>9</sup>, Pearson, A.D.J.<sup>10</sup>, Cohn, S.L.<sup>11</sup>*

Dana-Farber/Boston Children's Hospital Cancer & Blood Disorders Center, Pediatric Hematology/Oncology, Boston <sup>2</sup>, Cancer Research UK Trials Unit, University of Birmingham, Cancer Research UK Trials Unit, Birmingham<sup>3</sup>, University of Leicester, Leicester Cancer Research Centre, Leicester<sup>4</sup>, Children's Hospital, University of Cologne, Department of Pediatric Oncology and Hematology, Cologne<sup>5</sup>, St. Anna Children's Hospital, St. Anna Children's Hospital, Vienna<sup>6</sup>, Children's Hospital, University of Cologne, Department of Pediatric Oncology and Hematology, Cologne<sup>7</sup>, Dana-Farber/Boston Children's Hospital Cancer & Blood Disorders Center, Pediatric Hematology/Oncology, Boston, Ma<sup>8</sup>, Dana-Farber/Boston Children's Hospital Cancer & Blood Disorders Center, Pediatric Hematology/Oncology, Boston, Ma<sup>9</sup>, Institute of Cancer Research and Royal Marsden Hospital, Section of Paediatrics, Surrey<sup>10</sup>, The University of Chicago, Pediatrics, Chicago<sup>11</sup>

### Background/Introduction

Age, *MYCN* status, presence of metastases, and histology have been used as neuroblastoma risk factors for decades. Lactate dehydrogenase (LDH) and serum ferritin are reproducible, easily obtained from a blood sample, and prognostic of outcome, though not formally used in risk stratification except in one German trial. LDH is a marker of tissue injury, and ferritin is a marker of acute inflammatory reaction or cancer.

### Aims

To determine the prognostic strength of LDH and ferritin, overall, within high-risk (INSS stage 4 disease and were  $\geq 18$  months old at diagnosis), and by era, using the INRG Data Commons.

### Methods/Materials

Children diagnosed with neuroblastoma (1990–2016; all stages) were categorized into LDH (n=8,867) and ferritin (n=8,575) risk groups using event-free survival (EFS). Cox models compared the prognostic strength of LDH and ferritin to age, *MYCN* status, and presence of metastatic disease. Eras were defined based on major changes in therapeutic strategy (Era I: 1990-1996, Era II: 1997-2009, Era III: 2010-2016).

### Results

LDH and ferritin had non-linear relationships with outcome. The group with highest LDH had the worst EFS: overall cohort (5-year EFS) [100-899 IU/L: 76 $\pm$ 0.6%; (0-99 and 900-1399 IU/L): 60 $\pm$ 1.2%;  $\geq 1400$  IU/L: 36 $\pm$ 1.2%; p<0.0001], and high-risk cohort post-2009 (3-year EFS) [117-381 IU/L: 67 $\pm$ 8.9%; 382-1334 IU/L: 58 $\pm$ 4.4%; (0-116 and  $\geq 1335$  IU/L): 46 $\pm$ 3.9%; p=0.003]. The group with highest ferritin had the worst EFS: overall cohort (5-year EFS) [1-29 ng/mL: 87 $\pm$ 0.9%; (0 and 30-89 ng/mL): 74 $\pm$ 0.8%;  $\geq 90$  ng/mL: 48 $\pm$ 0.9%; p<0.0001], and high-risk cohort post-2009 (3-year EFS) [1-53 ng/mL: 71 $\pm$ 9.3%; (0 and 54-354 ng/mL): 55 $\pm$ 4.7%;  $\geq 355$  ng/mL: 34 $\pm$ 6.1%; p=0.0008]. In a multivariable model adjusting for age, *MYCN* status, and INSS stage 4 disease, LDH and ferritin maintained independent prognostic ability [p<.0001; adjusted hazard ratios (HRs) (95% CI): 1.7 (1.5-1.9), 2.3 (2.0-2.7), respectively].

EFS/OS improved steadily over time: 60 $\pm$ 0.7%/67 $\pm$ 0.7% in Era I, to 67 $\pm$ 0.5%/75 $\pm$ 0.5% in Era II, to 70 $\pm$ 1.0%/79 $\pm$ 1.0% in Era III. Over time, LDH maintained prognostic strength, attenuated slightly in Era III (HR=3.3, high-risk; HR=1.7, intermediate-risk) compared to Era I (HR=3.9, high-risk LDH; HR=1.8, intermediate-risk LDH). Ferritin gained prognostic strength over time, with larger HRs in Era III (HR=8.6, high-risk; HR=3.5, intermediate-risk) than in Era I (HR=5.6, high-risk; HR=2.4, intermediate-risk). Within the high-risk cohort over time, LDH had similar prognostic strength in all three Eras; similarly for ferritin.

### Summary/Conclusions

LDH and ferritin are strongly prognostic in neuroblastoma, overall, within high-risk in all eras, and within high-risk patients treated post-2009 with modern therapy. Risk stratification should utilize the most strongly prognostic factors, whatever they may be; to date LDH and ferritin have been overlooked. LDH and ferritin have maintained prognostic strength over time, possibly because treatment assignment has not been made on the basis of LDH and ferritin. LDH and ferritin show promise for a) identifying an ultra-high risk cohort; and, b) risk stratification in low-/middle-income countries who lack access to *MYCN* status and histology data. Our descriptive analysis provides justification for routine collection of LDH and ferritin on frontline studies. LDH and ferritin should be tested in future prognostic comparisons in evolving neuroblastoma risk stratification.

## **P007 A review of the 27K neuroblastoma PubMed abstracts: finding missing links of cellular heterogeneity with tumor origins through text-mining**

*Van Neste, C.<sup>2</sup>, Rogowski, M.<sup>3</sup>, Salhi, A.<sup>4</sup>, Gao, X.<sup>5</sup>*

,KAUST, Computational Bioscience Research Center, Thuwal 23955-6900 <sup>2</sup>, KAUST, Computational Bioscience Research Center, Thuwal 23955-6900<sup>3</sup>, KAUST, Computational Bioscience Research Center, Thuwal 23955-6900<sup>4</sup>, KAUST, Computational Bioscience Research Center, Thuwal 23955-6900<sup>5</sup>

### Background/Introduction:

A hallmark of neuroblastoma is its high cellular heterogeneity, both intra-tumor and inter-tumor despite the low mutational burden beyond copy number variants. *MYCN* is a key oncogene involved in this heterogeneity. One feature of the heterogeneity is the heavy metastasis burden for high-risk patients. The type of metastasis is very specific for neuroblastoma and cannot be directly compared to adult metastasized cancers. Neuroblastoma researchers might, therefore, be expected to communicate differently on concepts such as “metastasis” and “heterogeneity” in contrast to other (adult) cancer researchers.

### Aims:

Through the use of natural language processing (NLP), we aimed to map genes related to heterogeneity within neuroblastoma literature, to genes linked with the origin of the tumors. As a supporting aim, we aimed to extract genes discussed in the neuroblastoma literature, and link them to “factual” neuroblastoma transcriptional data.

### Methods/Materials:

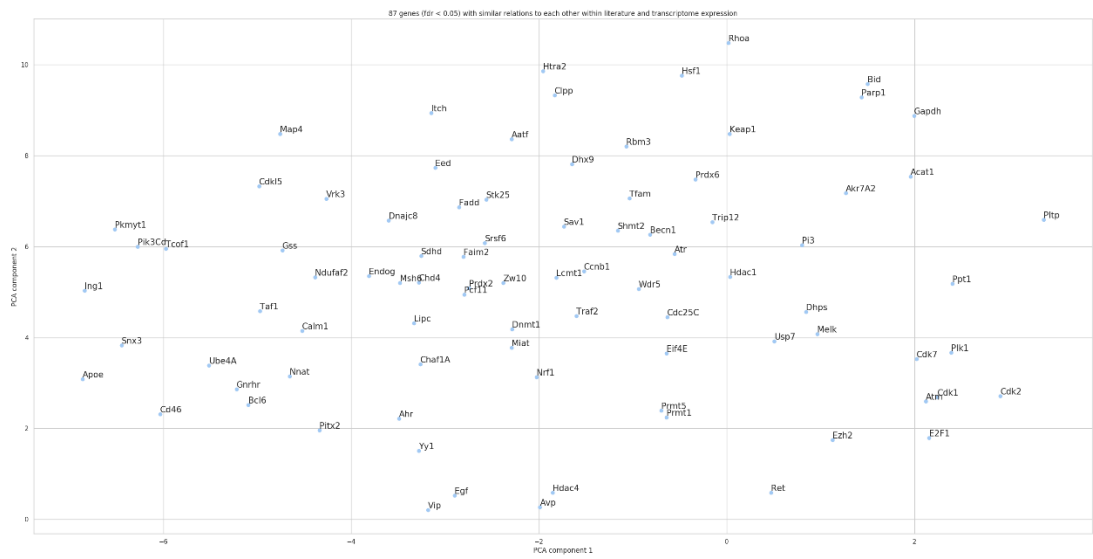
We collected 27,449 abstracts from a local PubMed repository and indexed it with the whoosh package. Using the gensim package, we generated embeddings for all words in abstracts containing “neuroblastoma”. In parallel, we processed transcriptomics from a neuroblastoma dataset. With the gene correlation-matrix, we formed a gene-gene network when a predefined minimum correlation was reached. We subsequently embedded the network with node2vec to vectors of the same size as the literature embeddings. Code for the analysis can be found in our broader text mining tool “sina” (search indirect nomenclature associations, <https://github.com/dicaso/sina>).

### Results:

In a multidimensional space, different word vector dimensions should correspond to different types of meaning (e.g. size of an entity, gender) that a computer algorithm can then subsequently use to make inferences. A well-known example within NLP is the ability to turn a “king”-vector into a “queen”-vector by subtracting the “man”-vector and adding a “woman”-vector. In analogy, what we have done is investigating the extent to which such relations can be extracted from the neuroblastoma literature or vice-versa to what extent factual experimental data is shaping the correlations that can be extracted through text-mining. From 845 genes for which we were able to construct neuroblastoma embedding vectors, we found 178 genes that had a p-value < 0.05 for correlation between literature association with other genes and correlations with those genes in the transcriptional dataset, of which 87 with FDR < 0.05. Top correlating genes between literature and transcriptional correlations were *WDR5*, *CLPP*, *CDK1*, *CDC25C*, and *PDRX2*. The abstract figure shows the gene associations within the neuroblastoma literature. Within the full set of significant genes, Enrichr shows enrichment for *SP1*, *E2F1*, and *TP53* targets. Subsequently, we linked these genes with significant embedded meaning, to heterogeneity and tumor origin of neuroblastoma using the literature embeddings.

### Summary/Conclusions:

Science progresses through analysis of correlations or mutual information between features such as genes, discovered in experimental results. Our work allows mapping the reported connections, directly back to experimental data to see which correlations are emphasized most in the literature. In the future, this might indicate underrepresented correlations worthy of further investigations.



Caption 1: 87 genes (fdr < 0.05) with similar relations to each other within literature and transcriptome expression

## **P008 Minimal residual disease evaluation by droplet digital PCR and quantitative real-time PCR in high-risk neuroblastoma patients**

*Nishimura, N.*<sup>2</sup>, *Ishida, T.*<sup>3</sup>, *Uemura, S.*<sup>4</sup>, *Thwin, K.K.M.*<sup>5</sup>, *Lin, K.S.*<sup>6</sup>, *Yamamoto, N.*<sup>7</sup>, *Nakatani, N.*<sup>8</sup>, *Tamura, A.*<sup>9</sup>, *Saito, A.*<sup>10</sup>, *Kishimoto, K.*<sup>11</sup>, *Mori, T.*<sup>12</sup>, *Hasegawa, D.*<sup>13</sup>, *Kosaka, Y.*<sup>14</sup>, *Nino, N.*<sup>15</sup>, *Takafuji, S.*<sup>16</sup>, *Iijima, K.*<sup>17</sup>

Kobe University Graduate School of Health Science, Department of Public Health, Kobe<sup>2</sup>, Kobe Children's Hospital, Department of Hematology and Oncology, Kobe<sup>3</sup>, Kobe University Graduate School of Medicine, Department of Pediatrics, Kobe<sup>4</sup>, Kobe University Graduate School of Medicine, Department of Pediatrics, Kobe<sup>5</sup>, Kobe University Graduate School of Medicine, Department of Pediatrics, Kobe<sup>6</sup>, Kobe University Graduate School of Medicine, Department of Pediatrics, Kobe<sup>7</sup>, Kobe Children's Hospital, Department of Hematology and Oncology, Kobe<sup>8</sup>, Kobe Children's Hospital, Department of Hematology and Oncology, Kobe<sup>9</sup>, Kobe Children's Hospital, Department of Hematology and Oncology, Kobe<sup>10</sup>, Kobe Children's Hospital, Department of Hematology and Oncology, Kobe<sup>11</sup>, Kobe Children's Hospital, Department of Hematology and Oncology, Kobe<sup>12</sup>, Kobe Children's Hospital, Department of Hematology and Oncology, Kobe<sup>13</sup>, Kobe Children's Hospital, Department of Hematology and Oncology, Kobe<sup>14</sup>, Kobe University Graduate School of Medicine, Department of Pediatrics, Kobe<sup>15</sup>, Kobe University Graduate School of Medicine, Department of Pediatrics, Kobe<sup>16</sup>, Kobe University Graduate School of Medicine, Department of Pediatrics, Kobe<sup>17</sup>

### **Background/Introduction:**

More than half of high-risk neuroblastoma (NB) cases experience tumor relapse/regrowth due to chemoresistant residual disease. To achieve the long-term survival of high-risk NB patients, the disappearance and/or inactivation of minimal residual disease (MRD) is essential. Although the detection of several sets of NB-associated mRNAs (NB-mRNAs) by quantitative real-time PCR (qPCR) has been used for monitoring MRD in NB patients, the accurate evaluation of MRD remains a clinical challenge. Droplet digital PCR (ddPCR) is an adaption of qPCR that potentially provides more simple and reproducible detection of low-level of mRNAs but remains tested for MRD detection in NB patients.

### **Aims:**

To compare ddPCR with qPCR for the accurate evaluation of MRD in high-risk NB patients.

### **Methods/Materials:**

Bone marrow (BM) and peripheral blood (PB) samples were collected from 20 high-risk NB patients treated based on the high-risk NB protocols (JN-H-07, JN-H-11, JN-H-15) of the Japanese Children's Cancer Group (JCCG) Neuroblastoma Committee (JNBSG) at Kobe Children Hospital and Kobe University Hospital between June 2011 and January 2018. Expression of CRMP1, DBH, DDC, GAP43, ISL1, PHOX2B, and TH mRNAs were determined by ddPCR using a QX200 ddPCR system (Bio-Rad Laboratories, Hercules, CA) and qPCR using an ABI 7500 Fast Real-time PCR system (Applied Biosystems, Foster City, CA, USA). HPRT1 and B2M, GAPDH, PGK1 were used as endogenous references for ddPCR and qPCR, respectively. Level of each NB-mRNA (each signature) and 7NB-mRNAs (combined signature) was defined as the relative copy number of each NB-mRNA and the weighted sum of 7 relative copy numbers, respectively. For a direct comparison between ddPCR and qPCR, level of 7NB-mRNAs was defined as the geometric mean of 7 relative copy numbers for ddPCR and of 7 delta threshold cycle (dCt) values for qPCR, respectively.

### **Results:**

Among 208 BM and 67 PB samples analyzed in the present study, level of 7NB-mRNAs determined by ddPCR varied between individuals and collection time points but was significantly correlated with the treatment response and disease burden. Of 208 BM samples, 73 samples were collected during post-treatment follow-up period and their level of 7NB-mRNAs were determined by both qPCR and ddPCR. 17 relapse/regrowth samples expressed significantly higher level of qPCR and ddPCR 7NB-

mRNAs than 56 non-relapse samples. To compare qPCR with ddPCR, their ROC curves were then plotted for level of 7NB-mRNAs. Whereas qPCR estimated area under curve (AUC) of 0.594 (<0.7), ddPCR did AUC of 0.706 (>0.7) with the significant accuracy.

Summary/Conclusions:

The present study suggests that level of 7NB-mRNAs detected by qPCR and ddPCR is significantly associated with tumor relapse/regrowth of high-risk NB patients and ddPCR provides a better prognostic value than qPCR.

## **P009 Targeting MIF/CXCR4 axis reduces neuroblastoma cell viability, invasion and chemoresistance in the bone marrow niche.**

*Garcia-Gerique, L.<sup>2</sup>, Torreadell, M.<sup>3</sup>, Mateo-Lozano, S.<sup>4</sup>, Pascual-Pasto, G.<sup>5</sup>, Garcia, M.<sup>6</sup>, Garrido-Garcia, A.<sup>7</sup>, Gómez-González, S.<sup>8</sup>, Salvador, N.<sup>9</sup>, Vila-Ubach, M.<sup>10</sup>, Suñol, M.<sup>11</sup>, Mora, J.<sup>12</sup>, Lavarino, C.<sup>13</sup>*

FSJD, Oncology, Barcelona<sup>2</sup>, HSJD, Oncology, Barcelona<sup>3</sup>, FSJD, Oncology, Barcelona<sup>4</sup>, FSJD, Oncology, Barcelona<sup>5</sup>, FSJD, Oncology, Barcelona<sup>6</sup>, FSJD, Oncology, Barcelona<sup>7</sup>, FSJD, Oncology, Barcelona<sup>8</sup>, FSJD, Oncology, Barcelona<sup>9</sup>, FSJD, Oncology, Barcelona<sup>10</sup>, HSJD, Oncology, Barcelona<sup>11</sup>, HSJD, Oncology, Barcelona<sup>12</sup>, FSJD, Oncology, Barcelona<sup>13</sup>

### Background/Introduction:

The receptor CXCR4 has a pivotal role for bone marrow (BM) homeostasis and BM metastasis in several cancers. In neuroblastoma (NB), CXCR4 is expressed in tumor cells; however, the oncogenic role of its ligand CXCL12 has shown contradictory results.

### Aims:

We investigated the role of Macrophage Inhibitory Factor (MIF), an alternative CXCR4 ligand, in BM environment and metastasis of NB. Using an *in vitro* model that recapitulates low oxygen levels and chemokine signaling, we explored whether MIF/CXCR4 signaling is critical for NB survival and proliferation in the BM niche. We also evaluated MIF inhibition as a therapeutic option for NB.

### Methods/Materials:

To develop a BM-based *in vitro* model, NB cells were cultured in different conditioned media (CMs) derived from supernatants of BM primary cells cultured alone (CM-BM), or in the presence of NB cells (CM-NB/BM; 1:1 NB:BM ratio), supernatant of NB cells cultured alone (CM-NB), or standard media (CM-CNT). To mimic BM oxygen levels, NB cells were cultured under hypoxia (1% O<sub>2</sub> as compared to cell culture normoxia (21% O<sub>2</sub>)). Cellular and chemokine characterization of BM cultures and CMs was performed using flow cytometry, cytokine array and ELISAs. *in vitro* functional studies were performed using MTS reagent, Transwell assays, flow cytometry and western blot with or without the covalent MIF inhibitor 4-IPP.

### Results:

Expanded BM cultures used to generate CMs contained a predominant cell population positive for mesenchymal stromal markers (49.2% CD90<sup>+</sup>, 29.9% CD105<sup>+</sup>). Cytokine arrays and ELISA assays of CMs revealed MIF as the highest released cytokine, whereas CXCL12 was not detected. CMs were used to perform functional cell viability, invasion, drug resistance and cell signaling studies. Under hypoxia, CM-BM and CM-NB/BM enhanced LAN-1 cell viability at 48, 72 and 96 h (P<0.01). This effect was reversed by adding sub-lethal concentrations of the MIF inhibitor 4-IPP. Using Matrigel-coated Transwells, CM-NB/BM chemoattracted and enhanced invasion of LAN-1 cells cultured under hypoxic conditions (P<0.01). Chemotaxis and invasion was reversed by preconditioning cells with 4-IPP (P<0.05). We also explored whether CMs affected response to chemotherapy. After treatment with doxorubicin and etoposide at IC<sub>50</sub> values (30 and 415 nM), LAN-1 cell viability increased 22% and 18% in CM-NB/BM compared to CM-CNT (P<0.05). In both cases, chemo-sensitivity was restored when 4-IPP was added to CM-NB/BM. We also evaluated whether CMs and hypoxia could increase expression of CXCR4 and CD74 (alternative MIF receptor), and activate intracellular signaling pathways. Under hypoxia, CM-BM and CM-NB/BM increased CXCR4 but not CD74 expression in LAN-1 cells by flow cytometry, and increased phosphorylated levels of AKT more than ERK measured by western blot.

### Summary/Conclusions:

We have generated an *in vitro* model of the BM microenvironment that recapitulates features such as hypoxia and chemokine signaling, which proved to enhance the pro-metastatic properties of NB cells. This BM-model enabled us to identify MIF/CXCR4 as a critical axis in NB chemotaxis and invasion, and demonstrate the *in vitro* efficacy of MIF inhibitors. Ongoing studies are being performed to elucidate the activity of MIF inhibitors *in vitro*.

## P010 BILATERAL ADRENAL NEUROBLASTOMA: CLINICAL AND BIOLOGICAL CHARACTERISTICS

*Andreeva, N.<sup>2</sup>, Kachanov, D.<sup>3</sup>, Shcherbakov, A.<sup>4</sup>, Zemcova, L.<sup>5</sup>, Druy, A.<sup>6</sup>, Likar, Y.<sup>7</sup>, Chaurasiya, K.<sup>8</sup>, Utalieva, D.<sup>9</sup>, Roschin, V.<sup>10</sup>, Ilyina, E.<sup>11</sup>, Shamanskaya, T.<sup>12</sup>*

Dmitry Rogachev National Research Center of Pediatric Hematology, Oncology, Clinical Oncology, Moscow<sup>2</sup>, Dmitry Rogachev National Research Center of Pediatric Hematology, Oncology, Clinical Oncology, Moscow<sup>3</sup>, Dmitry Rogachev National Research Center of Pediatric Hematology, Oncology, Radiology, Moscow<sup>4</sup>, Dmitry Rogachev National Research Center of Pediatric Hematology, Oncology, Cytogenetics, Moscow<sup>5</sup>, Dmitry Rogachev National Research Center of Pediatric Hematology, Oncology, Molecular Oncology, Moscow<sup>6</sup>, Dmitry Rogachev National Research Center of Pediatric Hematology, Oncology, Nuclear Medicine, Moscow<sup>7</sup>, Dmitry Rogachev National Research Center of Pediatric Hematology, Oncology, Nuclear Medicine, Moscow<sup>8</sup>, Dmitry Rogachev National Research Center of Pediatric Hematology, Oncology, Clinical Oncology, Moscow<sup>9</sup>, Dmitry Rogachev National Research Center of Pediatric Hematology, Oncology, Pathology, Moscow<sup>10</sup>, Dmitry Rogachev National Research Center of Pediatric Hematology, Oncology, Pediatrics, Moscow<sup>11</sup>, Dmitry Rogachev National Research Center of Pediatric Hematology, Oncology, Clinical Oncology, Moscow<sup>12</sup>

### Background/Introduction:

In 30-40% of cases neuroblastoma (NB) is localized in the adrenal gland. Bilateral adrenal involvement is extremely rare.

### Aims:

We aimed to compare clinical and biological characteristics of unilateral and bilateral adrenal NB.

### Methods/Materials:

The study included 285 patients with adrenal NB, ganglioneuroblastoma (GNB) and ganglioneuroma (GN) who received the treatment at Dmitry Rogachev NMRC PHOI from 01.2012 to 04.2019. The diagnosis was established based on the international criteria. The bilateral adrenal involvement was confirmed by different imaging modalities. Bilateral adrenal biopsy was done in selected cases. Patients were stratified and therapy was carried out according to the modified German NB2004 protocol.

### Results:

Of the 285 patients with peripheral neuroblastic adrenal tumors, 253 (88.8%) had unilateral and 32 (11.2%) had bilateral lesions. Patients with bilateral adrenal tumors were younger (1.7 months versus 17.6 months,  $p < 0.001$ ). Nine of 253 (3.6%) patients with unilateral adrenal gland lesions were diagnosed GN. The analysis of patients with malignant peripheral neuroblastic tumors (NB and GNB) showed that metastatic forms of the disease (4 and 4S stages) occurred more often in bilateral cases (27/32 (84%) vs. 113/244 (46%) in unilateral cases ( $p = 0.001$ ). Stage 4S were diagnosed more frequently in bilateral NB (19/32 (59.4%) vs. 34/244 (13.9%) in unilateral,  $p = 0.00001$ ). Metastatic pattern correlated with the laterality of the adrenal involvement. Bone (62/113 (54.9%),  $p = 0.0007$ ) and distant lymph node metastases (34/113 (30.1%),  $p = 0.01$ ) were significantly more often observed in unilateral diseases. Opposite, skin (3/27 (11.1%),  $p = 0.02$ ) and liver (26/27 (96.3%),  $p = 0.0001$ ) metastases were more common in bilateral cases. There was no statistical difference in the incidence of *MYCN* amplification and 1p deletion. Also, there was no statistical difference in the distribution to the risk groups between unilateral and bilateral cases. 3-year event-free survival (EFS) of patients with bilateral adrenal NB was 84% (95% confidence interval (CI) 71.8-97.0), compared with 69% (95% CI 63.0-75.6) of patients with unilateral adrenal NB ( $p = 0.12$ ). 3-year overall survival (OS) of patients with bilateral adrenal NB was 96% (95% CI 90.8-100.0), compared with 81% (95% CI 75.4-86.5) of patients with unilateral adrenal NB ( $p = 0.03$ ).

### Summary/Conclusions:

Bilateral adrenal NB is more common in children during the first months of life. There were no cases with GN and bilateral adrenal involvement. Bilateral adrenal NB more likely to have distant metastasis corresponding to stage 4S disease. Lack of the differences in the frequency of occurrence of



unfavorable cytogenetic markers was most likely due to the small number of patients with bilateral NB. OS was higher in the group of patients with bilateral adrenal NB in comparison to unilateral cases.

## **P011 A possible role for EPAS1/HIF2a in promoting a noradrenergic cellular state in neuroblastoma**

*Yuan, J.<sup>2</sup>, Liu, M.<sup>3</sup>, Bedoya Reina, O.<sup>4</sup>, Li, W.<sup>5</sup>, Shi, Y.<sup>6</sup>, Kogner, P.<sup>7</sup>, Schlisio, S.<sup>8</sup>, Holmberg, J.<sup>9</sup>*

Karolinska Institute, Cell and Molecular Biology, Solna<sup>2</sup>, Karolinska Institute, Cell and Molecular Biology, Solna<sup>3</sup>, Karolinska Institute, Microbiology Tumor and Cell Biology, Solna<sup>4</sup>, Karolinska Institute, Microbiology Tumor and Cell Biology, Solna<sup>5</sup>, Karolinska Institute, Cell and Molecular Biology, Solna<sup>6</sup>, Karolinska Institute, Women's and Children's Health, Solna<sup>7</sup>, Karolinska Institute, Microbiology Tumor and Cell Biology, Solna<sup>8</sup>, Karolinska Institute, Cell and Molecular Biology, Solna<sup>9</sup>

### **Background/Introduction:**

Neuroblastoma arises within the sympathetic nervous system and is the most frequent extracranial solid childhood cancer. It exhibits a high degree of clinical heterogeneity ranging from spontaneous regression to fatal progression. The hypoxia inducible factor, EPAS1/HIF2a, has been proposed to be NB oncogene. However, several studies challenge this concept and high expression levels of EPAS1 is associated with increased survival of neuroblastoma patients. To address the functional role of EPAS1/HIF2a in neuroblastoma we have performed analysis of single cell sequenced neuroblastoma and initiated gain- and loss-of-function experiments in neuroblastoma cells. Our analysis of single cell sequenced human neuroblastoma tumors revealed that EPAS1 is significantly enriched in cells from low-risk tumors characterized by high expression of sympathoadrenal markers such as PHOX2B and TH. This reflects the expression pattern of *Epas1* during development of the mouse sympathoadrenal lineage and suggests that EPAS1/HIF2a could play a role in promoting a differentiated sympathetic noradrenergic cellular state (NORAD), which have been shown to be more sensitive to chemotherapy. In contrast, neuroblastoma cells expressing markers defining the more chemoresistant mesenchymal cellular state (MES), e.g. PRRX1 and YAP1, exhibit lower levels of EPAS1/HIF2a. Crispr/Cas9 depletion of EPAS1 in SK-N-SH cells which harbor both MES and NORAD populations, resulted in rapid loss of PHOX2B and PHOX2A. Conversely, overexpression of EPAS1 in SH-EP2 neuroblastoma cells that only harbor the MES population, resulted in reduced YAP1 expression and altered morphology. This implies that high EPAS1/HIF2a levels are required to maintain the NORAD cellular state and that transition to a MES cellular states is associated with reduced EPAS1/HIF2a levels. Thus, our preliminary data suggest a novel role for EPAS1/HIF2a which is not associated with the response to hypoxia but rather as a determining factor of neuroblastoma heterogeneity.

## **P012 Fusion transcripts impact patient outcome in neuroblastoma**

*Bartenhagen, C.*<sup>2</sup>, *Ikram, F.*<sup>3</sup>, *Fernández-Cuesta, L.*<sup>4</sup>, *Roels, F.*<sup>5</sup>, *Lindner, S.*<sup>6</sup>, *Volland, R.*<sup>7</sup>, *Foll, M.*<sup>8</sup>, *Hertwig, F.*<sup>9</sup>, *Kahlert, Y.*<sup>10</sup>, *Cun, Y.*<sup>11</sup>, *Engesser, A.*<sup>12</sup>, *Rosswog, C.*<sup>13</sup>, *Fischer, J.*<sup>14</sup>, *Ackermann, S.*<sup>15</sup>, *Hero, B.*<sup>16</sup>, *Berthold, F.*<sup>17</sup>, *Schulte, J.H.*<sup>18</sup>, *Thomas, R.K.*<sup>19</sup>, *Peifer, M.*<sup>20</sup>, *Fischer, M.*<sup>21</sup>

University Children's Hospital of Cologne, Department of Pediatric Oncology and Hematology, Cologne<sup>2</sup>, University Children's Hospital of Cologne, Department of Pediatric Oncology and Hematology, Cologne<sup>3</sup>, International Agency for Research on Cancer, IARC-WHO, Genetic Cancer Susceptibility group, Section of Genetics, Lyon<sup>4</sup>, University Children's Hospital of Cologne, Department of Pediatric Oncology and Hematology, Cologne<sup>5</sup>, Charité University Medicine, Department of Pediatric Oncology and Hematology, Berlin<sup>6</sup>, University Children's Hospital of Cologne, Department of Pediatric Oncology and Hematology, Cologne<sup>7</sup>, International Agency for Research on Cancer, IARC-WHO, Genetic Cancer Susceptibility group, Section of Genetics, Lyon<sup>8</sup>, Charité University Medicine, Department of Pediatric Oncology and Hematology, Berlin<sup>9</sup>, University Children's Hospital of Cologne, Department of Pediatric Oncology and Hematology, Cologne<sup>10</sup>, University of Cologne, Center for Molecular Medicine Cologne (CMMC), Cologne<sup>11</sup>, University Children's Hospital of Cologne, Department of Pediatric Oncology and Hematology, Cologne<sup>12</sup>, University Children's Hospital of Cologne, Department of Pediatric Oncology and Hematology, Cologne<sup>13</sup>, University Children's Hospital of Cologne, Department of Pediatric Oncology and Hematology, Cologne<sup>14</sup>, University Children's Hospital of Cologne, Department of Pediatric Oncology and Hematology, Cologne<sup>15</sup>, University Children's Hospital of Cologne, Department of Pediatric Oncology and Hematology, Cologne<sup>16</sup>, University Children's Hospital of Cologne, Department of Pediatric Oncology and Hematology, Cologne<sup>17</sup>, Charité University Medicine, Department of Pediatric Oncology and Hematology, Berlin<sup>18</sup>, University of Cologne, Department of Translational Genomics, Cologne<sup>19</sup>, University of Cologne, Center for Molecular Medicine Cologne (CMMC), Cologne<sup>20</sup>, University Children's Hospital of Cologne, Department of Pediatric Oncology and Hematology, Cologne<sup>21</sup>

### **Background/Introduction:**

Chromosomal rearrangements and fusion transcripts provide a mechanism for activation of oncogenes and inactivation of tumor suppressor in cancer. Because of a lack of suitable techniques, the prevalence and clinical impact of these alterations in neuroblastoma may have been underappreciated in the past.

### **Aims:**

We aimed to systematically determine the pattern of expressed fusion transcripts and their potential relevance for neuroblastoma pathogenesis and clinical outcome. We also addressed the question whether fusion transcripts originate from genomic alterations or during RNA processing and investigated the association of fusion events with recurrent copy number alterations.

### **Methods/Materials:**

We analyzed 498 primary tumors by RNA-seq. Whole genome sequencing (WGS) data were available for 39/498 samples, and genomic copy number data for 239/498 samples. Fusion transcripts were detected by FusionHunter and TRUP, and were validated by dideoxynucleotide sequencing. Ectopic *FGFR2* expression and *FGFR2* knockdown was performed in JoMa1 neural crest progenitor cells and NBL-S neuroblastoma cells, respectively.

### **Results:**

We detected a total of 97 fusion transcripts in 78 tumors, involving 169 distinct genes. Fifty-two and 45 fusion transcripts were predicted to be potentially protein-coding and truncating, respectively. For 21/22 fusion transcripts, corresponding genomic rearrangements were identified by WGS, indicating that genomic events are the main cause for gene fusions. The spectrum of fusion transcripts was heterogeneous, with most fusions being private events. We observed, that chromosomes 2, 12, and 17 were significantly enriched for fusion partner genes. Furthermore, 58% of fusion breakpoints were

located in close vicinity to a breakpoint of a recurrent segmental copy number alteration, such as 1p loss, 11q loss, 17p gain and *MYCN* amplification. The number of segmental copy number alterations in tumors having fusion transcripts was significantly higher than that of tumors lacking fusions ( $p < 0.001$ ), indicating a general genomic instability in these tumors. Fusion transcripts were significantly enriched for genes annotated in the COSMIC ( $p = 0.002$ ) or the Mitelman database ( $p < 0.001$ ). These genes included *FOXR1*, *HACE1*, *FGFR2* and *TERT*, which have been previously associated with neuroblastoma and/or other cancers. We evaluated the functional effect of *FGFR2*, the full-length coding sequence of which was involved in a *KLHL13-FGFR2* fusion. Ectopic expression of *FGFR2* resulted in augmented clonogenic growth, while *FGFR2* knock-down significantly impaired proliferation. Finally, we observed that the outcome of patients whose tumors harbored fusion transcripts was significantly worse than that of patients without fusion transcripts, both in the entire cohort and in subgroups, such as *MYCN*-amplified or high-risk patients. In a subset of 4 tumors, we found that 5 of 6 fusion transcripts were also detected in the corresponding relapse samples, indicating that such alterations are mostly stable genetic events during neuroblastoma development and progression.

#### Summary/Conclusions:

We here demonstrate that fusion transcripts are regularly occurring events in primary neuroblastoma. Our data strongly suggest that the vast majority of fusion transcripts result from genomic alterations, and that they may represent a potential mechanism by which genomic alterations impact the clinical outcome of neuroblastoma patients.

## **P013 Segmental Chromosome Aberrations and Clinical Response Impact Outcome of INSS stage 3 Patients $\geq$ 18 months with Unfavorable Histology and without *MYCN* Amplification: A Children's Oncology Group (COG) Report**

*Pinto, N.*<sup>2</sup>, *Naranjo, A.*<sup>3</sup>, *Hibbitts, E.*<sup>4</sup>, *Ding, X.*<sup>5</sup>, *Tibbetts, R.*<sup>6</sup>, *Kennedy, R.*<sup>7</sup>, *Pfau, R.*<sup>8</sup>, *Hogarty, M.*<sup>9</sup>, *Kreissman, S.*<sup>10</sup>, *Irwin, M.*<sup>11</sup>, *Park, J.*<sup>12</sup>, *Asgharzadeh, S.*<sup>13</sup>

University of Washington/Seattle Children's Hospital, Pediatrics, Seattle<sup>2</sup>, Children's Oncology Group Data Statistics Center, Biostatistics, Gainesville<sup>3</sup>, Children's Oncology Group Data Statistics Center, Biostatistics, Gainesville<sup>4</sup>, University of Southern California, Pediatrics, Los Angeles<sup>5</sup>, University of Southern California, Pediatrics, Los Angeles<sup>6</sup>, University of Southern California, Pediatrics, Los Angeles<sup>7</sup>, Ohio State University, Institute for Genomic Medicine, Columbus<sup>8</sup>, University of Pennsylvania, Pediatrics, Philadelphia<sup>9</sup>, Duke University, Pediatrics, Durham<sup>10</sup>, Hospital for Sick Children, Pediatrics, Toronto<sup>11</sup>, University of Washington/Seattle Children's Hospital, Pediatrics, Seattle<sup>12</sup>, University of Southern California, Pediatrics, Los Angeles<sup>13</sup>

### Background/Introduction

Patients with INSS Stage 3 neuroblastoma represent a heterogeneous population with respect to disease presentation and prognosis and controversy exists regarding the most effective treatment algorithms. Patients  $\geq$ 18 months of age with INSS Stage 3 tumors that are unfavorable histology (UH) and *MYCN*-non-amplified (*MYCN*-NA) represent a small cohort of patients with an outcome intermediate of those with favorable histology tumors and *MYCN* amplified tumors. The presence of Segmental Chromosome Aberrations (SCA) may predict outcome; however, their impact specifically in this cohort of patients has not been reported.

### Aims

This analysis describes the event-free (EFS) and overall survival (OS) of patients with INSS Stage 3  $\geq$ 18mo patients with UH, *MYCN*-NA tumors. The impact of dinutuximab immunotherapy, clinical and biological factors including SCAs were evaluated for their impact on survival.

### Methods/Materials

Eligible patients enrolled on therapeutic protocols A3973 (n=34), ANBL0532 (n=27), and biology protocol ANBL00B1 (n=101 with 29 treated on A3973/ANBL0532) with Stage 3 disease, *MYCN*-NA, UH and age  $\geq$ 18 months at diagnosis were analyzed. Patients with available high-quality tumor DNA (n=65) underwent whole exome sequencing (WES). Copy number alterations and loss of heterozygosity (LOH) for loci at chromosomes 1p, 1q, 2p, 3p, 4p, 11p, 11q and 17q were scored for gains/losses by two independent reviewers. Additional structural events including mutation analyses were carried out using established bioinformatics pipelines.

### Results

The 5-year EFS/OS for children  $\geq$ 18 months with stage 3, *MYCN*-NA, UH disease treated on A3973 and ANBL0532 was 73.0 $\pm$ 8.1%/87.9 $\pm$ 5.9% and 61.4 $\pm$ 10.2%/ 73.0 $\pm$ 9.2%, respectively, with no statistical differences in EFS or OS between the two cohorts (p=0.1286 and p=0.2180, respectively). In the combined cohort of patients enrolled on A3973 and ANBL0532, statistically significant differences were found (p(s) <0.0001) in patients with CR/VGPR (n=39) and PR (n=13) having better outcomes than <PR (n=5) (5-year EFS: 74.0 $\pm$ 7.6% vs. 75.0 $\pm$ 12.5% vs. 0%; 5-year OS: 84.4 $\pm$ 6.2% vs. 100% vs. 20.0 $\pm$ 17.9%). There was no difference in EFS and OS for patients who received immunotherapy vs. did not receive immunotherapy (p=0.8390 and p=0.2575, respectively). WES revealed that subjects with chromosome 11q loss/LOH had an inferior outcome in comparison to those without 11q loss/LOH (10-year EFS: 44.4 $\pm$ 24.1% vs. 78.1 $\pm$ 9.4%, p=0.01; 10-year OS: 62.4 $\pm$ 15.9% vs. 85.9 $\pm$ 7.8%, p=0.02). Patients with 1p loss/LOH and 2p gain also showed trend towards worse event-free survival (p=0.086 and p=0.088, not statistically significant) but not in the overall-survival.

### Summary/Conclusions

High-risk therapy that included single myeloablative therapy led to an 81.6 $\pm$ 5.3% 5-year OS in patients  $\geq$ 18 months with UH and *MYCN*-NA Stage 3 neuroblastoma. Response to therapy is a powerful predictor of survival and the presence of chromosome 11q loss/LOH is also associated with inferior outcomes. These patients should continue to be treated on high-risk clinical trials.



## **P014 Multi-plex Imaging to dissect Bone Marrow Disseminated Disease in Neuroblastoma at the Single Cell Level**

*Lazic, D.<sup>2</sup>, Kromp, F.<sup>3</sup>, Mivalt, F.<sup>4</sup>, Rifatbegovic, F.<sup>5</sup>, Fischer, L.<sup>6</sup>, Ambros, I.M.<sup>7</sup>, Ambros, P.F.<sup>8</sup>, Taschner-Mandl, S.<sup>9</sup>, Ostalecki, C.<sup>10</sup>*

Children's Cancer Research Institute Vienna, Tumor Biology, Vienna<sup>2</sup>, Children's Cancer Research Institute Vienna, Tumor Biology, Vienna<sup>3</sup>, Children's Cancer Research Institute Vienna, Tumor Biology, Vienna<sup>4</sup>, Children's Cancer Research Institute Vienna, Tumor Biology, Vienna<sup>5</sup>, Software Competence Center Hagenberg, Knowledge-Based Vision Systems, Hagenberg<sup>6</sup>, Children's Cancer Research Institute Vienna, Tumor Biology, Vienna<sup>7</sup>, Children's Cancer Research Institute Vienna, Tumor Biology, Vienna<sup>8</sup>, Children's Cancer Research Institute Vienna, Tumor Biology, Vienna<sup>9</sup>, Universitätsklinik Erlangen, Hautklinik, Erlangen<sup>10</sup>

### **Background/Introduction:**

Disseminated tumor cells (DTCs) in the bone marrow (BM) are detected in the majority of stage M (metastatic) neuroblastoma patients who have a high risk of relapse and low survival rates.

Currently, automatic immunofluorescence (IF) imaging is state-of-the-art to evaluate treatment response and identify DTCs in the BM, however this method allows visualization of only up to three biomarkers on single cells, hampering a comprehensive spatially and time-resolved characterization of DTC heterogeneity and the local immune response in the BM microenvironment.

### **Aims:**

Thus, the aim was to identify as well as validate additional relevant biomarkers, visualize these by multi-plex imaging and thereby characterize DTCs and the metastatic BM microenvironment using machine-learning based methods.

### **Methods/Materials:**

Based on data mining of RNA-sequencing data-sets of stage M NB (tumors, DTCs, BM-derived non-tumor cells) and proteomics data (NB tumors, cell lines, fibroblasts), and guided by public databases, relevant biomarkers were selected. Sample preparation and IF-staining protocol were optimized to validate specificity of identified biomarkers and allow the assessment of protein expression profiles by multi-epitope ligand cartography (MELC). Single-cell morphological and fluorescence features were computed and validated on processed and segmented MELC images to be further used for exploration of distinct cell populations by dimensionality reduction based methods.

### **Results:**

Herein, three novel potential DTC biomarkers are proposed, namely DCLK1, FAIM2 and TAG1, which give new insight into NB heterogeneity. By addition and validation of further biomarkers to characterize the cell composition in the BM microenvironment and potential therapeutic targets, three individual 23 biomarker IF-panels were established. Based on machine learning methods, the qualification of computed features as single-cell descriptors was proven and the latter were employed for visualization of distinct cell populations, e.g. lymphocytes, myeloid cell types, stem/progenitor cells, mesenchymal and tumor cell phenotypes, by stochastic neighbor embedding approaches.

### **Summary/Conclusions:**

Our results demonstrate proof-of-principle for the assessment of cell composition in the metastatic BM microenvironment and illustrate tumor plasticity on a single cell level. This approach complements sequencing based approaches to study tumor heterogeneity and will help to refine diagnostics for stage M NB patients.

## **P015 Longitudinal ctDNA measurements in a multiply relapsed neuroblastoma patient detects disease progression earlier than image guided diagnostics**

*Gunnes, M.<sup>2</sup>, Forthun, R.B.<sup>3</sup>, Hoyseter, T.<sup>4</sup>, Hovland, R.<sup>5</sup>*

Haukeland University Hospital, Pediatric Oncology Department, Bergen <sup>2</sup>, Haukeland University Hospital, Department of Medicine, Bergen<sup>3</sup>, Haukeland University Hospital, Department of Medical Genetics, Bergen<sup>4</sup>, Haukeland University Hospital, Department of Medical Genetics, Bergen<sup>5</sup>

### Background/Introduction:

Neuroblastoma is the most common extracranial solid tumor occurring in the pediatric age group. There are increasing reports on the use of liquid biopsies for the mutation profiling, treatment monitoring and relapse detection in neuroblastoma

### Aims:

The aim of this study was to examine the accuracy and clinical usefulness of serial ctDNA (circulating tumor DNA) measurements for relapse surveillance and treatment response in a multiple relapsed neuroblastoma patient

### Methods/Materials:

A 5 year old girl initially treated for Stage Ms neuroblastoma at 4 months of age and recently treated for a second relapse of metastatic disease, without *MYCN* amplification or segmental chromosomal aberrations, was discovered to harbor a somatic activating ALK mutation (c.3824G>A, p.R1275Q) in her relapsed tumor sample. Due to some residual NBL cells in her bone marrow (complete remission by imaging) after end of relapse treatment, she was started on monotherapy with a first generation ALK-inhibitor, Crizotinib (280 mg/m<sup>2</sup> x 2), and entered into our liquid biopsy project for surveillance of treatment response and early relapse detection. EDTA blood was collected approximately every second month, and plasma was purified within two hours by centrifugation at 820 G, 10 min, followed by 10 000 G, 10 min and storage at -80°C. Cell free DNA was collected using the QIAamp Circulating Nucleic Acid kit (Qiagen), and ctDNA was evaluated in triplicate by digital droplet PCR (ddPCR) using the PrimePCR ddPCR Mutation Assay ALK p.R1275Q (dHsaMDV2516768, Bio-Rad) on a QX200 AutoDG Droplet Generator and QX200 Droplet Reader (Bio-Rad). PCR program was as follows: 95°C 10 min, 94°C 30 sec and 56°C 1 min for 40 cycles, 98°C 10 min, 4°C ∞, ramp rate was adjusted according to Bio-Rad's recommendations. Results were analyzed using Quantasoft version 1.7.4 (Bio-Rad), and fractional abundance (mutant DNA/total DNA) was reported. Samples having more than three positive droplets were defined as ctDNA-positive

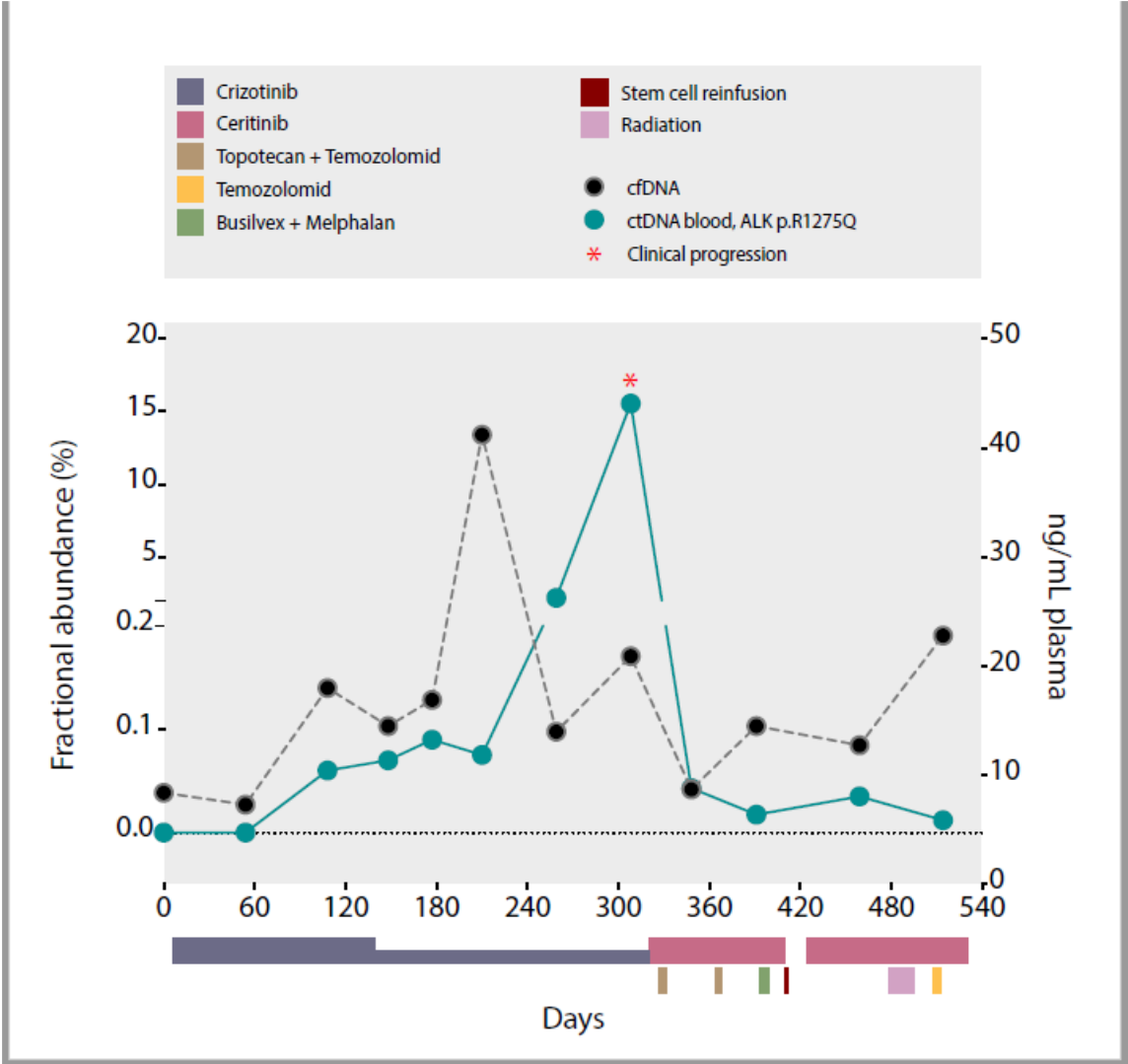
### Results

After 3.5 months of Crizotinib monotherapy, the ctDNA assay showed a slight increase in ctDNA (without clinical evidence of a new relapse) that stayed stable for about 3.5 months before showing a marked increase 1.5 months prior to a clinically evident relapse. The new relapse was treated aggressively with re-induction chemotherapy, followed by high dose chemotherapy with autologous stem cell support and change to a second generation ALK inhibitor. ctDNA was negative (0/3211 detected droplets) after the first course of re-induction chemotherapy and change of ALK inhibitor, and stays detectable but stable and low since, which is in accordance with imaging and clinical conclusion of continued complete remission.

### Summary/Conclusions:

Monitoring of serial ctDNA over a period of 17 months showed clear signs of a new relapse before it was clinically evident (Figure 1), allowing for treatment adjustment and avoiding the need for a re-biopsy of the third tumor relapse. The patient is now in radiological, clinical and biochemical remission after a fourth treatment regimen including maintenance treatment with a second generation ALK inhibitor and low dose metronomic oral chemotherapy, a total of 6 years after the primary presentation





Caption 1: Timeline of ctDNA, cfDNA and tumor treatment

## **P016 Metachronous bilateral adrenal neuroblastoma or a coincidence?**

*Ash, S.<sup>2</sup>, Jeison, M.<sup>3</sup>, Amar, S.<sup>4</sup>, Fienmesser, M.<sup>5</sup>*

Schneider Children's Medical Center of Israel, The Rina Zaizov Pediatric Hematology Oncology Division, Petach Tikva<sup>2</sup>, Schneider Children's Medical Center of Israel, The Ca-Cytogenetic and Molecular Cytogenetic Lab, Petach Tikva<sup>3</sup>, Schneider Children's Medical Center of Israel, The Rina Zaizov Pediatric Hematology Oncology Dept., Petach Tikva<sup>4</sup>, Rabin Medical Center, Pathology, Petach Tikva<sup>5</sup>

### **Background/Introduction:**

Bilateral adrenal neuroblastoma is very rare and occurs in infants. Its presentation suggests synchronous multifocal primaries rather than metachronous metastases. The therapeutic strategy is similar to that used against unilateral tumors.

### **Aims:**

We present a case of a 5.5 y old boy with Metachronous bilateral adrenal neuroblastoma.

### **Methods/Materials:**

SNParray Array analysis was performed on genomic DNA extracted from peripheral blood and bilateral adrenal tumors using Affymetrix CytoScan HD array.

### **Results:**

The patient was first diagnosed at the age of 3.5 years with a localized right adrenal *MYCN* not - amplified neuroblastoma. Two years after a complete resection of the right adrenal mass, an expanding mass was observed in the left adrenal. Metastatic work up was negative. The mass was completely resected. The pathology of the first adrenal tumor demonstrated a poorly differentiated neuroblastoma with diffuse necrosis, some calcifications, high cellularity, high mitotic activity and low MKI. The pathology of the second tumor demonstrated differentiating neuroblastoma, low mitotic activity, low MKI, and less necrosis.

Cytoscan HD (affymetrix) SNP array was done on both tumors and blood. In both tumors and blood samples we found several LOH regions (6p21.1-q15; 9q 22.-31.2), 11p15.4 deletion, 14q32.33 gain and more changes. We also found the following changes in both tumors: 12q24.12 LOH (PTPN11) and 19p13.2-13.3 deletion. However there were changes that were found only in the tumor in 2017: 2p(11.2-24.3) gain (NBAS, DOX1, PAM49A, SMC6); whole chromosome 7 gain; 17q(11.2-25.3) gain and the following changes only in the tumor in 2019: 7q(31.33-36.2) gain; 3q gain (GAP43). WES is ongoing on both tumors and blood.

### **Summary/Conclusions:**

According to these changes we assume that the patient has a background of predisposing syndrome and develop two different neuroblastoma tumors at two different time points. WES is ongoing and the results will be presented.

## P017 Insights into the biology of neuroblastoma exosomes

*Grimaldi, M.<sup>2</sup>, Lodrini, M.<sup>3</sup>, Wünschel, J.<sup>4</sup>, Astrahantseff, K.<sup>5</sup>, Münster-Wandowski, A.<sup>6</sup>, Kuenkele, A.<sup>7</sup>, Kirchner, M.<sup>8</sup>, Mertins, P.<sup>9</sup>, Eggert, A.<sup>10</sup>, Deubzer, H.E.<sup>11</sup>*

Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin<sup>2</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin<sup>3</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin<sup>4</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin<sup>5</sup>, Charité - Universitätsmedizin Berlin, Institute of Integrative Neuroanatomy, Berlin<sup>6</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin<sup>7</sup>, Max Delbrück Center for Molecular Medicine in the Helmholtz Association, Proteomics Platform, Berlin<sup>8</sup>, Max Delbrück Center for Molecular Medicine in the Helmholtz Association, Proteomics Platform, Berlin<sup>9</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin<sup>10</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin<sup>11</sup>

### Background/Introduction:

The interaction between the tumor and its microenvironment plays a key role in regulating neuroblastoma progression. In particular, neuroblastoma cells may create a supportive milieu contributing to tumor growth and metastasis through the secretion of tumor-derived factors, such as exosomes. Exosomes are the smallest extracellular vesicles of endocytic origin, released by both normal and cancer cells. Tumor-derived exosomes can affect the biological activities of recipient cells via transfer of their content, encouraging neoplastic transformation and survival. Moreover, the molecular composition and surface profile of exosomes is highly heterogeneous and dependent on the parental cell type, tumor stage and other parameters.

### Aims:

We set out to identify exosomal signatures specifically associated with genetic neuroblastoma subtypes.

### Methods/Materials:

Six human neuroblastoma cell lines reflecting the three major genetic subgroups of high-risk neuroblastoma were selected, including IMR5-75, BE(2)-C, LAN-6, SK-N-FI, GI-M-EN, CLB-GA. Exosomes were isolated from medium conditioned by the cell lines by OptiPrep density gradient ultracentrifugation (DGUC) followed by size-exclusion chromatography. Exosomes were characterized using transmission electron microscopy, nanoparticle tracking analysis and western blotting. The proteomes of exosome contents and surface profiles were investigated using liquid-chromatography tandem mass spectrometry and a novel multiplex bead-based flow cytometric assay, respectively.

### Results:

The workflow to isolate exosomes from conditioned medium was first established and optimized. A combination of DGUC and size-exclusion chromatography produced well-purified exosome samples. Neuroblastoma-derived exosomes exhibited the typical cup-shaped morphology in transmission electron micrographs, and showed an enrichment of exosome-specific markers (CD63, CD81, PDCD6IP, FLOT1). Absence of the endoplasmic reticular protein, calreticulin, demonstrated that our exosome preparation contained no contamination from apoptotic bodies or organelle derived-vesicles. Nanoparticle tracking analysis showed that vesicle size ranged from 30 to 150 nm, consistent with that of exosomes. Proteomic analysis and surface profile of IMR5-75 -derived exosomes revealed the presence of well-known exosome-associated proteins, as reported by the *ExoCarta* and *Vesiclepedia* databases. Proteins associated with the biological processes of cell adhesion, proliferation or the inflammatory response in the target cells were detected in exosomes. The proteomic contents of exosomes overlapped with the cell lines from which they were derived, and experiments are currently underway to compare the proteomic cargo among the six matched exosome/cell line pairs and define

exosome heterogeneity from one neuroblastoma cell line and among cell lines and genetic high-risk subtypes.

Summary/Conclusions:

An in-depth view on exosomes cargo and heterogeneity is crucial for a better understanding of the role of these vesicles in neuroblastoma pathogenesis. This may also produce new potential biomarkers and molecular targets for high-risk subtypes.

## **P018 Level of droplet digital PCR-detected seven neuroblastoma-associated mRNAs in peripheral blood stem cells predicts tumor relapse/regrowth of high-risk neuroblastoma patients**

*Nino, N.<sup>2</sup>, Ishida, T.<sup>3</sup>, Uemura, S.<sup>4</sup>, Mon Thwin, K.K.<sup>5</sup>, San Lin, K.<sup>6</sup>, Yamamoto, N.<sup>7</sup>, Nakatani, N.<sup>8</sup>, Tamura, A.<sup>9</sup>, Kishimoto, K.<sup>10</sup>, Mori, T.<sup>11</sup>, Hasegawa, D.<sup>12</sup>, Kosaka, Y.<sup>13</sup>, Takafuji, S.<sup>14</sup>, Iijima, K.<sup>15</sup>, Nishimura, N.<sup>16</sup>*

Kobe University Graduate School of Medicine, Department of Pediatrics, Kobe<sup>2</sup>, Hyogo Prefectural Kobe Children's Hospital, Department of Hematology/Oncology, Kobe<sup>3</sup>, Kobe University Graduate School of Medicine, Department of Pediatrics, Kobe<sup>4</sup>, Kobe University Graduate School of Medicine, Department of Pediatrics, Kobe<sup>5</sup>, Kobe University Graduate School of Medicine, Department of Pediatrics, Kobe<sup>6</sup>, Kobe University Graduate School of Medicine, Department of Pediatrics, Kobe<sup>7</sup>, Hyogo Prefectural Kobe Children's Hospital, Department of Hematology/Oncology, Kobe<sup>8</sup>, Hyogo Prefectural Kobe Children's Hospital, Department of Hematology/Oncology, Kobe<sup>9</sup>, Hyogo Prefectural Kobe Children's Hospital, Department of Hematology/Oncology, Kobe<sup>10</sup>, Hyogo Prefectural Kobe Children's Hospital, Department of Hematology/Oncology, Kobe<sup>11</sup>, Hyogo Prefectural Kobe Children's Hospital, Department of Hematology/Oncology, Kobe<sup>12</sup>, Hyogo Prefectural Kobe Children's Hospital, Department of Hematology/Oncology, Kobe<sup>13</sup>, Kobe University Graduate School of Medicine, Department of Pediatrics, Kobe<sup>14</sup>, Kobe University Graduate School of Medicine, Department of Pediatrics, Kobe<sup>15</sup>, Kobe University Graduate School of Health Sciences, Department of Public Health, Kobe<sup>16</sup>

### **Background/Introduction:**

More than 50% of high-risk neuroblastoma (NB) patients experience tumor relapse due to the activation of minimal residual disease (MRD). MRD is defined conceptually as residual tumor cells that persistently reside in cancer patients after local and systemic cancer therapies. In high-risk NB patients, MRD can be assessed in peripheral blood stem cell (PBSC) in addition to BM and PB because almost all of patients are subjected to autologous PBSC transplantation. Although the detection of several sets of NB-associated mRNAs (NB-mRNAs) by quantitative real-time PCR (qPCR) is reported to have a prognostic power in NB patients, the impact of NB-mRNAs detection PBSC is not clear. Conflicting results have been reported in the previous literatures. Whereas high level of B4GALNT1 or TH mRNA in PBSC samples did not affect survival, high level of CHGA, DCX, DDC, PHOX2B, or TH mRNA in PBSC samples was associated with worse outcome. In the present study, level of 7NB-mRNAs (CRMP1, DBH, DDC, GAP43, ISL1, PHOX2B, and TH mRNAs) were determined by droplet digital PCR (ddPCR) that is an adaption of qPCR and potentially provides more simple and reproducible detection of low-level of mRNAs.

### **Aims:**

To determine the impact of ddPCR MRD detection by 7NB-mRNAs in PBSC on tumor relapse/regrowth of high-risk NB patients.

### **Methods/Materials:**

PBSC samples were collected from 20 high-risk NB patients treated based on the high-risk NB protocols (JN-H-07, JN-H-11, JN-H-15) of the Japanese Children's Cancer Group (JCCG) Neuroblastoma Committee (JNBSG) at Kobe Children Hospital and Kobe University Hospital between June 2011 and January 2018. Expression of CRMP1, DBH, DDC, GAP43, ISL1, PHOX2B, and TH mRNAs were determined by ddPCR using a QX200 ddPCR system (Bio-Rad Laboratories, Hercules, CA). HPRT1 was used as an endogenous reference for ddPCR. Level of 7NB-mRNAs (combined signature) was defined as the weighted sum of 7 relative copy numbers.

### **Results:**

Among 20 high-risk NB patients included in the present study, 8 patients had tumor relapse/regrowth and 12 patients remained relapse/regrowth-free for more than 2 years. In 20 PBSC samples from 20

patients, 8 relapse/regrowth samples expressed significantly higher level of 7NB-mRNAs than 12 non-relapse/non-regrowth samples. Receiver operating characteristic (ROC) analysis was then performed for level of 7NB-mRNAs in 8 relapse/regrowth and 12 non-relapse/non-regrowth PBSC samples. Area under curve (AUC) was estimated to be  $0.7812 > 0.7$  with the significant accuracy.

#### Summary/Conclusions:

The present study suggests that level of ddPCR-detected 7NB-mRNAs in PBSC is significantly associated with tumor relapse/regrowth of high-risk NB patients.

## **P019 Longitudinal cell-free DNA analysis in patients with high-risk neuroblastoma is suitable to monitor treatment response and identify actionable targets**

*Sprüssel, A.,<sup>1</sup> Lodrini, M.<sup>2</sup>, Thole, T.<sup>3</sup>, Peitz, C.<sup>4</sup>, Astrahantseff, K.<sup>5</sup>, Grimaldi, M.<sup>6</sup>, Linke, R.<sup>7</sup>, Tiburtius, D.<sup>8</sup>, Jesse, P.<sup>9</sup>, Schmelz, K.<sup>10</sup>, Hundsdörfer, P.<sup>11</sup>, Eggert, A.<sup>12</sup>, Lodrini, M.<sup>13</sup>, Deubzer, H.E.<sup>14</sup>*

Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin<sup>1</sup>,  
Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin<sup>2</sup>,  
Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin<sup>3</sup>,  
Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin<sup>4</sup>,  
Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin<sup>5</sup>,  
Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin<sup>6</sup>,  
Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin<sup>7</sup>,  
Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin<sup>8</sup>,  
Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin<sup>9</sup>,  
Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin<sup>10</sup>,  
Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin<sup>11</sup>,  
Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin<sup>12</sup>,  
Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin<sup>13</sup>,  
Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin<sup>14</sup>

### Background/Introduction:

The invasive nature of surgical biopsies most often prevents their sequential application to monitor disease. Single biopsies also fail to reflect cancer dynamics, intratumor heterogeneity and drug sensitivities that most likely change during clonal evolution and under the selective pressure of therapy.

### Aims:

Detection and characterization of cell-free DNA (cfDNA) in peripheral blood and bone marrow samples from neuroblastoma patients may serve as a minimally invasive liquid biopsy approach.

### Methods/Materials:

Duplex digital droplet PCR (ddPCR) assays assessing *MYCN* and ALK copy number status as well as ALKF1174L and ALKR1275Q hotspot mutations were applied to longitudinally collected blood and bone marrow plasma samples during or after first-line therapy ( $\geq 3$  samples per patient) as well as corresponding primary tumor biopsies from 34 patients with high-risk neuroblastoma. Cell-free DNA was purified before ddPCR, and fragmentation of tumor-derived genomic DNA was achieved by direct enzymatic digestion in the ddPCR reaction mixture. Results were compared with data derived from clinical routine diagnostics including histopathological analyses of tumor samples, bone marrow cytology/immunocytology and imaging in patients (MIBG and MRI scans).

### Results:

Persistence of markers was observed in patients with mixed responses and progressive disease, while clearance of cfDNA markers from plasma correlated with complete remission and survival in patients. A decline in total cfDNA levels over time corresponded with favorable treatment response. In individual courses, *MYCN* amplifications and ALK mutations were detected when analyzing the cfDNA, but not the corresponding primary tumor biopsy, suggesting tumor heterogeneity and underlining the potential of cfDNA analysis for actionable target identification. Reappearance of a cfDNA marker in plasma preceded the diagnosis of a relapse based on established clinical parameters by > 3 months in one patient, supporting a future role for monitoring patient-specific cfDNA markers in clinical routine aftercare programs.

### Summary/Conclusions:

Our results indicate liquid biopsies in patients with suspected or diagnosed neuroblastoma may be superior to tumor biopsies for detecting all markers in heterogeneous tumors and superior to established clinical parameters for the early detection of relapse. Our data justify the further development and testing of molecular disease characterization using cfDNA in blood and bone marrow plasma samples from patients with neuroblastoma in trials.



## **P020 Outcome of patients with MIBG-positivity following multi-modality therapy for metastatic neuroblastoma**

*Usmani, N.<sup>2</sup>, Portwine, C.<sup>3</sup>, Deyell, R.<sup>4</sup>, Farfan, M.<sup>5</sup>, Morgenstern, D.<sup>6</sup>, Irwin, M.<sup>7</sup>*

McMaster University, Pediatric Hematology Oncology, Hamilton <sup>2</sup>, McMaster University, Pediatric Hematology Oncology, Hamilton<sup>3</sup>, University of British Columbia, Pediatric Hematology Oncology, Vancouver<sup>4</sup>, University of British Columbia, Pediatric Hematology Oncology, Vancouver<sup>5</sup>, Sick Kids Hospital, Pediatric Hematology Oncology, Toronto<sup>6</sup>, Sick Kids Hospital, Pediatric hematology oncology, Toronto<sup>7</sup>

### Background/Introduction:

Metaiodobenzylguanidine (MIBG) scans are used to detect neuroblastoma (NBL) metastatic lesions at diagnosis and during post-treatment surveillance. MIBG-positivity following induction chemotherapy for high-risk NBL correlates with poor outcome. However, there are reports of patients with progression-free survival despite MIBG-positivity. Factors distinguishing these survivors from patients who experience progression or relapse remain unclear. Although some publications have compared the sensitivity and specificity of PET and MIBG scans at diagnosis the roles for these scans during post-therapy surveillance are less well understood. We hypothesized that PET scans may help specifically identify less “active”, potentially differentiated, MIBG-avid lesions.

### Aims:

Our primary goal was to determine whether PET scan activity can distinguish which MIBG-positive lesions may remain quiescent and thus, can potentially be spared additional therapies. Our secondary aim was to determine whether any known clinical or biological factors at diagnosis are associated with end of therapy MIBG positivity and long-term survival. Understanding how to recognize these rare patients will allow tailoring of therapy and impact surveillance.

### Methods/Materials:

We conducted a multi-center retrospective analysis of pediatric patients diagnosed with INSS stage-4 NBL between 2002-2018. We identified patients with persistent MIBG positivity on their end-of-therapy scans without evidence of progression during treatment. We recorded patient characteristics including age, histopathology, biomarkers, treatment, and disease response.

### Results:

Ten patients (median age at diagnosis, 28.6 months) met inclusion criteria. All remain disease-free with a median time-off-therapy of 5 years. Six patients continued to show MIBG uptake up to 6.5 years following active therapy. All patients at diagnosis had MIBG-avid distant metastases and tumors that lacked *MYCN* amplification. Although all patients had confirmed INSS 4 disease at diagnosis, 4/10 of the patients had tumors with INPC favorable histology. Based on the COG risk classification criteria 3/10 patients were classified as intermediate and 7/10 as high risk at diagnosis. 8/10 patients had histologic evidence of pre-treatment marrow involvement. Six underwent at least one course of myeloablative chemotherapy with stem cell transplant. Seven patients had at least one PET scan performed and 7/7 PET scans were negative. Three patients had repeat biopsies of a positive MIBG lesion at some point following the end of upfront therapy. Differentiated NBL was reported for 2/3 biopsies and the third showed only fibrotic changes.

### Summary/Conclusions:

Our preliminary analysis suggests that persistent positive MIBG scans in a subset of INSS stage 4 patients in first response may not always lead to progression. In our cohort no patients had tumors that harboured *MYCN* amplification. Studies are needed to determine whether *MYCN* status together with other clinical or biological characteristics, and /or PET scans, may identify patients with residual “inactive” MIBG lesions that do not require further therapy.



## **P021 Resolving neuroblastoma heterogeneity via CD surface antigen codes**

*Pruszk, J.*<sup>2</sup>, *Menon, V.*<sup>3</sup>, *Ferlemann, F.*<sup>4</sup>, *Condurat, A.L.*<sup>5</sup>

Paracelsus Medical University (PMU), Institute of Anatomy and Cell Biology, Salzburg<sup>2</sup>, Former association: Institute of Anatomy & Cell Biology, University of Freiburg, Emmy Noether Group for Stem Cell Biology, Dept. Molecular Embryology, Freiburg<sup>3</sup>, Former association: Institute of Anatomy & Cell Biology, University of Freiburg, Emmy Noether Group for Stem Cell Biology, Dept. Molecular Embryology, Freiburg<sup>4</sup>, Former association: Institute of Anatomy & Cell Biology, University of Freiburg, Emmy Noether Group for Stem Cell Biology, Dept. Molecular Embryology, Freiburg<sup>5</sup>

### **Background/Introduction:**

Differential expression patterns of cluster-of-differentiation (CD) antigens have long been exploited to define cellular heterogeneity in immunology, hematopoiesis and hematological malignancies.

### **Aims:**

Having initiated the comprehensive characterization of CD surface molecules in neural cell types, our research is aimed at resolving neuroblastoma heterogeneity via combinatorial CD surface antigen codes.

### **Methods/Materials:**

Using a range of established neuroblastoma cell lines we determined the expression profiles of >240 CD antigens and applied SPADE (Spanning-tree Progression Analysis of Density-normalized Events) to automatically extract subpopulation clusters from multidimensional flow cytometry data (see Hindley *et al.*, 2016; Ferlemann *et al.*, 2017). Fluorescence-activated cell sorting (FACS), immunoblot and immunohistological expression analysis and *in vitro* functional assays were used for validation of distinct cellular subsets.

### **Results:**

Our work provides a comprehensive analysis of CD antigen expression patterns in neuroblastoma cell lines. Based upon this, we identified surface marker profiles specifically associated with NMYC-amplified tumor entities as well with YAP-TAZ expressing migratory neuroblastoma subtypes. Moreover, we demonstrated the utility of complex multicolor flow cytometric readout for small molecule screens towards identifying differential responsiveness of neuroblastoma subpopulations to established and novel pharmacological candidates.

### **Summary/Conclusions:**

Our work may contribute to expanding the neuro-histopathological arsenal of neuroblastoma subpopulation markers. Complementing transcriptomics and epigenetic analyses of neuroblastoma heterogeneity, CD surface antigen expression dynamics and cellular profiles may aid in refining more targeted therapeutic approaches including immunotherapeutic paradigms.

## **P022 *MYCN*-amplified neuroblastoma with vasoactive intestinal peptide syndrome and BRAF V600E mutation**

*Roberts, S.*<sup>2</sup>, *Shahid, S.*<sup>3</sup>, *Kushner, B.*<sup>4</sup>, *Modak, S.*<sup>5</sup>, *Basu, E.*<sup>6</sup>, *Rubin, E.*<sup>7</sup>

Memorial Sloan Kettering Cancer Center, Pediatrics, New York<sup>2</sup>, Memorial Sloan Kettering Cancer Center, Pediatrics, New York<sup>3</sup>, Memorial Sloan Kettering Cancer Center, Pediatrics, New York<sup>4</sup>, Memorial Sloan Kettering Cancer Center, Pediatrics, New York<sup>5</sup>, Memorial Sloan Kettering Cancer Center, Pediatrics, New York<sup>6</sup>, CHOC Children's Hospital, Pediatrics, Orange<sup>7</sup>

### Background/Introduction:

Neuroblastoma, the most common extracranial solid tumor in children, occasionally produces vasoactive intestinal peptide (VIP) resulting in secretory diarrhea (VIP-D). Patients with this paraneoplastic phenomenon usually have low-risk localized disease; resection is typically curative of both the neoplasm and the VIP-D. Rarely, patients present with metastatic high-risk neuroblastoma (HR-NB) and then develop VIP-D during induction. Chemotherapy is hypothesized to induce differentiation and VIP expression in these patients. Management is a major challenge because of the VIP-D in the setting of strongly myelosuppressive chemotherapy.

### Aims:

To report on two patients with HR-NB who developed VIP-D during induction chemotherapy.

### Methods/Materials:

With IRB approval, clinical and genomic information on two patients with VIP-secreting HR-NB treated at Memorial Sloan Kettering Cancer Center was retrospectively analyzed. Tumor sequencing was performed by CLIA-certified platforms: either MSK-IMPACT or Foundation One.

### Results:

Two females with *MYCN*-amplified HR-NB (abdominal primary with metastases to bones, bone marrow, and liver) acutely developed massive VIP-D after two cycles of induction chemotherapy with cyclophosphamide and topotecan as per ANBL0532. Both patients had a somatic BRAF V600E mutation. In patient #1 (age 19 months at diagnosis), severe VIP-D persisted despite chemotherapy, octreotide therapy, and tumor resection. However, secretory diarrhea promptly resolved and serum VIP levels decreased from >3200pg/mL to 438pg/mL within 14 days after starting dabrafenib (BRAF inhibitor). Four weeks later, the patient relapsed in brain with BRAF wild-type neuroblastoma. Dabrafenib was stopped; diarrhea recurred and VIP levels rose to 879pg/mL. After management of the hemorrhagic brain metastases, trametinib (MEK inhibitor) was added and dabrafenib restarted. Although the diarrhea improved, the neuroblastoma rapidly progressed leading to death 13 months post-diagnosis. Patient #2 (age 11 months at diagnosis) had prompt resolution of diarrhea after starting dabrafenib and trametinib with induction cycle five. Serum VIP levels decreased from 1416pg/mL to 209pg/mL within six days of starting the tyrosine kinase inhibitors (TKIs). These agents were discontinued after six cycles of post-induction chemo-immunotherapy without return of diarrhea or elevation of VIP levels (remained <50pg/mL). Subsequent therapy included anti-GD2 antibody and anti-neuroblastoma vaccine. She remains progression-free 27 months post-diagnosis. At the time of primary tumor resection, metastatic sites (liver and lymph nodes) showed the BRAF mutation, but the adrenal neuroblastoma that was previously biopsied and BRAF-positive no longer had the BRAF mutation.

### Summary/Conclusions:

Patients with HR-NB who develop VIP-D during induction chemotherapy may have an association with somatic BRAF V600 mutations. Treatment with BRAF inhibitors leads to rapid improvement in symptoms and facilitates continuation of anti-neuroblastoma therapy. Both patients demonstrated tumor heterogeneity, suggesting that combination therapy with BRAF and MEK inhibitors is warranted. Concurrent therapy with TKIs and anti-neuroblastoma chemotherapy was found to be safe and

feasible in patient #2. Tumor sequencing should be performed in all patients with HR-NB, especially those with VIP-D since timely treatment with appropriate TKIs can contribute to a favorable outcome in these rare patients.

## **P023 Application of minimally invasive surgery techniques in treatment of neuroblastoma**

Wieczorek, A.<sup>2</sup>, Labuz, P.<sup>3</sup>, Taczanowska-Niemczuk, A.<sup>4</sup>, Maslanka, M.<sup>5</sup>, Gorecki, W.<sup>6</sup>

Institute of Pediatrics, Jagiellonian University Medical College, Pediatric Oncology and Hematology, Kraków<sup>2</sup>, Institute of Pediatrics, Jagiellonian University, Medical College, Pediatric Surgery, Kraków<sup>3</sup>, Institute of Pediatrics, Jagiellonian University, Medical College, Pediatric Surgery, Kraków<sup>4</sup>, Institute of Pediatrics, Jagiellonian University, Medical College, Pediatric Surgery, Kraków<sup>5</sup>, Institute of Pediatrics, Jagiellonian University, Medical College, Pediatric Surgery, Kraków<sup>6</sup>

### Background/Introduction:

Major goals of surgical treatment of neuroblastoma are completion of resection and safety of patient. Simultaneously minimally invasive techniques are broader used in modern surgery. Application of minimally invasive techniques may fulfill both goals of treatment in neuroblastoma.

### Aims:

Analysis of single center experience in treatment of neuroblastoma with minimally invasive surgery techniques.

### Methods/Materials:

Between 2010-2019, 85 children underwent surgery for neuroblastoma. 12 of these were operated with usage of minimally invasive surgery techniques (9 with laparoscopy and 3 with thoracoscopy). These techniques were mostly used in infants and children under age of 4 with low grading of primary lesion up to 4cm of size, without IDRf, mostly situated in suprarenal area. After resection tumor was removed by mini-laparotomy or mini-thoracotomy.

### Results:

There were no intraoperative or post operative complication. Resections were macroscopically complete. Histopathology showed two cases of ganglioneuroma and ten cases of neuroblastoma. N-myc amplification was detected in one case. In 6 patients surgery was the only treatment, the follow up is 6 months up to 5 years. One patient (7%) occurred with local recurrence. One patient (7%) suffered of metastatic disease. One patient (7%) with a tumor in the chest and abdomen, a local recurrence appeared in the second body cavity

### Summary/Conclusions:

Minimally invasive surgery techniques are safe. Their effectiveness in treatment of neuroblastoma is still matter of reaserch but they seem to be used with success in some cases.

## **P024 Non-invasive treatment monitoring of neuroblastoma patients using copy number and methylation profiling of cfDNA**

Vandeputte, C.<sup>2</sup>, Van Paemel, R.<sup>3</sup>, De Wilde, J.<sup>4</sup>, Schuppers, R.<sup>5</sup>, De Koker, A.<sup>6</sup>, Callewaert, N.<sup>7</sup>, Menten, B.<sup>8</sup>, Laureys, G.<sup>9</sup>, De Wilde, B.<sup>10</sup>, Lammens, T.<sup>11</sup>, Vandesompele, J.<sup>12</sup>, Speleman, F.<sup>13</sup>, Van Roy, N.<sup>14</sup>, De Preter, K.<sup>15</sup>

, , <sup>1</sup>, Ghent University, Department of Biomolecular Medicine; Cancer Research Institute Ghent, Ghent  
<sup>2</sup>, Ghent University, Department of Biomolecular Medicine; Cancer Research Institute Ghent, Ghent<sup>3</sup>,  
Ghent University, Department of Pathology; Cancer Research Institute Ghent, Ghent<sup>4</sup>, Ghent  
University, Department of Biomolecular Medicine; Cancer Research Institute Ghent, Ghent<sup>5</sup>, Ghent  
University, Center for Medical Biotechnology, Flemish Institute Biotechnology (VIB), Ghent<sup>6</sup>, Ghent  
University, Center for Medical Biotechnology, Flemish Institute Biotechnology (VIB), Ghent<sup>7</sup>, Ghent  
University, Department of Biomolecular Medicine; Cancer Research Institute Ghent, Ghent<sup>8</sup>, University  
Hospital Ghent, Department of Biomolecular Medicine; Cancer Research Institute Ghent, Ghent<sup>9</sup>,  
Ghent University, Department of Biomolecular Medicine; Cancer Research Institute Ghent, Ghent<sup>10</sup>,  
University Hospital Ghent, Department of Internal Medicine and Pediatrics, Ghent<sup>11</sup>, Ghent University,  
Department of Biomolecular Medicine; Cancer Research Institute Ghent, Ghent<sup>12</sup>, Ghent University,  
Department of Biomolecular Medicine; Cancer Research Institute Ghent, Ghent<sup>13</sup>, Ghent University,  
Department of Biomolecular Medicine; Cancer Research Institute Ghent, Ghent<sup>14</sup>, Ghent University,  
Department of Biomolecular Medicine; Cancer Research Institute Ghent, Ghent<sup>15</sup>

### Background/Introduction:

The genetic work up of neuroblastoma (NB) patients is currently based on the examination of tissue biopsies that only reflect a spatially limited snapshot of the primary tumor. Given that most tumors are heterogeneous, subclones may be overlooked. Furthermore, additional surgical sampling during therapy to monitor disease evolution is not possible for NB patients. Non-invasive sampling of blood (liquid biopsy) can resolve these issues and opens new possibilities for more accurate genetic work up and serial sampling for therapy response monitoring.

### Aims:

In this study, we aimed to investigate the DNA copy number and methylation profiles of cfDNA retrieved from plasma samples both at diagnosis and during follow-up of NB patients, in order to investigate the potential of cfDNA to measure therapy response and to detect early relapses.

### Methods/Materials:

We have collected cfDNA from diagnostic and follow-up samples from 7 patients and performed shallow whole genome sequencing (sWGS) as reported by Van Roy, *et al.* (2017). For methylation profiling, we applied a recently published method by De Koker, *et al.* (2019), that enables reduced representation bisulfite sequencing of cfDNA (cfRRBS). Patient 1, 2, 3 and 5 were sampled at diagnosis and during treatment; patient 4, 6 and 7 were sampled at diagnosis and at progression.

### Results:

DNA copy number alterations were successfully detected in the tissue and the liquid biopsy, except for 1 patient. Using cfRRBS, a strong decrease in the estimated tumor fraction was observed during treatment in patients 1, 2, 3 and 5. cfRRBS detected for all cfDNA samples a small tumor fraction, however this fraction was lower than 10% in half of the cases and further investigation in order to define a reliable cut-off is needed. Shallow whole genome sequencing did not identify DNA copy number aberrations in 6 out of 10 of these follow-up samples. In patient 2, a small increase in tumor signal was noted, which might be indicative of poor therapy response. In the patients where both samples at diagnosis and at progression were available (pt 4, 6 and 7), a high tumor fraction was still detectable around 250 days after diagnosis. Furthermore, CNAs were detected at tumor progression of patient 4 and 7.

#### Summary/Conclusions:

Our study shows that sWGS and cfRRBS can be applied on cfDNA as non-invasive approach to monitor therapy response and relapse prediction in NB patients, providing complementary information. We are currently expanding our cohort of patients and samples to further explore the power and accuracy of these methods.



## **P025 Neurologic and Orthopedic Manifestations of Spinal Cord Compression in Intermediate-risk Neuroblastoma: A report from the Children's Oncology Group (COG) study ANBL0531**

*Katzenstein, H.<sup>2</sup>, Naranjo, A.<sup>3</sup>, Tenney, S.<sup>4</sup>, London, W.<sup>5</sup>, Handler, M.<sup>6</sup>, Schmidt, M.L.<sup>7</sup>, Shimada, H.<sup>8</sup>, Hogarty, M.<sup>9</sup>, Gastier-Foster, J.<sup>10</sup>, Park, J.<sup>11</sup>, Cohn, S.<sup>12</sup>, Maris, J.<sup>13</sup>, Twist, C.<sup>14</sup>*

Nemours Children's Specialty Care/Wolfson Children's Hospital, Pediatric Hematology/Oncology, Jacksonville<sup>2</sup>, Children's Oncology Group Statistics and Data Center, Biostatistics, Gainesville<sup>3</sup>, Children's Oncology Group Statistics and Data Center, Biostatistics, Gainesville<sup>4</sup>, Dana Farber/Boston Children's Cancer and Blood Disorders Center, Pediatrics, Boston<sup>5</sup>, The Children's Hospital, Neurosurgery, Denver<sup>6</sup>, University of Illinois at Chicago College of Medicine, Pediatrics, Chicago<sup>7</sup>, Lucille Packard Children's Hospital/Stanford University, Pathology, Palo Alto<sup>8</sup>, Children's Hospital of Philadelphia/Perelman School of Medicine, Pediatrics, Philadelphia<sup>9</sup>, Baylor College of Medicine, Pediatrics, Houston<sup>10</sup>, Seattle Children's Hospital, Pediatrics, Seattle<sup>11</sup>, University of Chicago, Pediatrics, Chicago<sup>12</sup>, Children's Hospital of Philadelphia/Perelman School of Medicine, Pediatrics, Philadelphia<sup>13</sup>, Roswell Park Cancer Institute, Pediatrics, Buffalo<sup>14</sup>

### Background/Introduction:

Epidural or intradural tumor extension occurs in 5-15% of patients with neuroblastoma (NB), and may occur with or without neurologic impairment. Neurologic outcome may be impacted by degree of disability at diagnosis and clinical management.

### Aims:

The ANBL0531 Phase 3 trial included a secondary objective to examine the clinical symptoms and neurologic/orthopedic outcome of intermediate-risk NB patients with paraspinal or intraspinal tumors.

### Methods/Materials:

Between 2007 and 2011, 404 evaluable patients were enrolled on study, including 138 with paraspinal +/- intraspinal tumors. Baseline assessment included duration of neurologic symptoms, degree of motor deficit (Grade 1= mild, Grade 2= moderate, Grade 3= severe), sensory deficit, pain, and orthopedic abnormalities. Follow-up neurologic and orthopedic evaluations were obtained at 6, 12, 24, and 36 months off therapy and then annually. Treatment was biology- and response-based, as previously described. For patients with intraspinal tumors, the criteria to stop therapy included both the protocol-defined treatment endpoint (partial response of the primary tumor) as well as sufficient resolution of the intraspinal tumor component to allow for observation with minimal risk of neurologic compromise. In this circumstance, additional cycles of chemotherapy, rather than surgical decompression with laminectomy, was recommended.

### Results:

The median age for all 138 patients with any paraspinal/intraspinal tumor involvement at diagnosis was 9.1 months (range= 0.2-86.2 months) and was no different than for the 54 patients with symptomatic cord compression at diagnosis. Disease stages included n=20 (INSS stage 2A/2B), n=72 (stage 3) n=42 (stage 4) and n=4 (stage 4S). Tumor biology features included n=125 with favorable histology, n=112 with hyperdiploidy, and n=18 with either 1p36 and/or 11q23 loss of heterozygosity. Symptomatic cord compression was reported at baseline in 54 patients as Grade 1 (n=11, 20.4%), Grade 2 (n=15, 27.8%), or Grade 3 (n=28, 51.9%) motor deficit. Bladder or bowel dysfunction were reported at baseline in 31 of the 54 symptomatic patients (57.4%). Sensory deficits were noted in 24 patients (44.4%) and 13 (24.1%) reported pain. Orthopedic abnormalities were reported at baseline in 15 of the 54 (27.8%) symptomatic patients, with scoliosis in 8 patients (14.8%) and 1 (1.9%) with kyphosis. At diagnosis, 8 of the 138 patients underwent >90% tumor resection, 15 underwent ≥50% to 90% resection, and 111 had a biopsy only or <50% resection of the tumor; surgical data was not available for 4 patients. Fifty-eight patients (42.0%) had a second operation. Surgical complications at any timepoint were reported in 34 patients (24.6%), 18 of which were present at baseline and 16 resulting following surgery. Fifty-five patients (39.9%) received more chemotherapy cycles than initially

assigned. Overall response at end of therapy was complete response in 11 (8.4%), very good partial response in 49 (37.4%), partial response in 54 (41.2%), and stable disease in 11 (8.4%).

**Summary/Conclusions:**

Excellent tumor response was achieved using a biology- and response-based treatment algorithm for patients with non high-risk NB presenting with paraspinal+/-intraspinal disease extension. The role of additional cycles of chemotherapy, type of surgical intervention, and neurologic and orthopedic outcomes will be reported.

Clinical trial information: NCT00499616.

## **P026 Schwann cell plasticity regulates neuroblastic tumor cell differentiation**

*Taschner-Mandl, S.*<sup>2</sup>, *Weiss, T.*<sup>3</sup>, *Bileck, A.*<sup>4</sup>, *Rifatbegovic, F.*<sup>5</sup>, *Dodig, H.*<sup>6</sup>, *Kauer, M.*<sup>7</sup>, *Frech, C.*<sup>8</sup>, *Windhager, R.*<sup>9</sup>, *Gerner, C.*<sup>10</sup>, *Ambros, P.F.*<sup>11</sup>, *Ambros, I.M.*<sup>12</sup>

Children's Cancer Research Institute, Tumour Biology, Vienna<sup>2</sup>, Children's Cancer Research Institute, Tumour Biology, Vienna<sup>3</sup>, University of Vienna, Department of Analytical Chemistry, Vienna<sup>4</sup>, Children's Cancer Research Institute, Tumour Biology, Vienna<sup>5</sup>, Children's Cancer Research Institute, Tumour Biology, Vienna<sup>6</sup>, Children's Cancer Research Institute, Bioinformatics, Vienna<sup>7</sup>, Children's Cancer Research Institute, Bioinformatics, Vienna<sup>8</sup>, Medical University of Vienna, Department of Orthopedic Surgery, Vienna<sup>9</sup>, University of Vienna, Department of Analytical Chemistry, Vienna<sup>10</sup>, Children's Cancer Research Institute, Tumour Biology, Vienna<sup>11</sup>, Children's Cancer Research Institute, Tumour Biology, Vienna<sup>12</sup>

### **Background/Introduction:**

Schwann cells develop along neuroblasts from trunk neuronal crest stem cells in embryonic development. The remarkable plasticity of Schwann cells is essential for nerve regeneration but also contributes to neuropathies and cancer. Stromal, tumor-associated Schwann cells are characteristically found within ganglioneuroma, where the presence of stromal Schwann cells is associated with a favorable tumor behavior and increased patient survival, whereas aggressive subtypes of neuroblastic tumors lack stromal Schwann cells. It has not been investigated yet whether the adaptive potential of Schwann cells is manifested in ganglioneuroma.

### **Aims:**

This enigma prompted us to investigate (1) the molecular wiring and functional state of stromal versus injury associated repair Schwann cells and thereby determine whether the presence of stromal Schwann cells in benign neuroblastic tumors is an expression of Schwann cell plasticity, and (2) how Schwann cell signals could be leveraged as therapeutics.

### **Methods/Materials:**

We here performed transcriptome and high-resolution mass spectrometry profiling of human neuroblastic tumors, rich and poor in Schwann cell stroma, as well as human injured nerves, rich in repair Schwann cells. Further we established a novel direct and trans-well co-culture model, recapitulating some aspects of ganglioneuroma development *in vitro* which was combined with functional and biochemical assays.

### **Results:**

We provide evidence that stromal Schwann cells in ganglioneuroma exhibit a repair Schwann cell characteristic gene expression signature. In turn, primary repair Schwann cells had a pro-differentiating and anti-proliferative effect on neuroblastoma cell lines and this was mediated by soluble factors. Within the pool of secreted stromal/repair Schwann cell factors, we identified EGFL8, a matricellular protein with so far undescribed function, to induce neuronal differentiation of aggressive neuroblastoma cells. EGFL8 expression further correlated with favorable tumor stage and increased patient survival.

### **Summary/Conclusions:**

Our findings suggest that stromal Schwann cells exert nerve repair associated functions in the tumor-environment and underline the therapeutic value of Schwann cell-derived factors for aggressive, Schwann cell stroma-poor neuroblastoma.

## **P027 Clinical and Biological Factors associated with Refractory disease in UK patients with High Risk Neuroblastoma**

*Basta, N.<sup>2</sup>, Herd, F.<sup>3</sup>, Gabriel, A.S.<sup>4</sup>, Hawley, J.<sup>5</sup>, Bown, N.<sup>6</sup>, Whiteman, S.<sup>7</sup>, Pearson, A.<sup>8</sup>, Moreno, L.<sup>9</sup>, Elliott, M.<sup>10</sup>, Gaze, M.<sup>11</sup>, Morgenstern, D.A.<sup>12</sup>, Feltbower, R.<sup>13</sup>, Stiller, C.<sup>14</sup>, Makin, G.<sup>15</sup>, Juliet, J.<sup>16</sup>, Wheeler, K.<sup>17</sup>, Ng, A.<sup>18</sup>, Howell, L.<sup>19</sup>, Johnston, R.<sup>20</sup>, Mark, C.<sup>21</sup>, Yeomanson, D.<sup>22</sup>, King, D.<sup>23</sup>, McNally, R.J.Q.<sup>24</sup>, Tweddle, D.A.*

Newcastle University, Population Health Sciences Institute, Newcastle Upon Tyne <sup>2</sup>, Department of Paediatric Oncology, Royal Aberdeen Children's Hospital, Department of Paediatric Oncology, Aberdeen<sup>3</sup>, Newcastle University, Wolfson Childhood Cancer Research Centre, Newcastle Centre for Cancer, Newcastle Upon Tyne<sup>4</sup>, The Great North Children's Hospital, Department of Paediatric Oncology, Newcastle Upon Tyne<sup>5</sup>, Newcastle University, Northern Genetics Service, Biosciences Institute, Newcastle Upon Tyne<sup>6</sup>, Newcastle University, Wolfson Childhood Cancer Research Centre, Newcastle Centre for Cancer, Newcastle Upon Tyne<sup>7</sup>, The Royal Marsden NHS Foundation Trust and Division of Clinical Studies and Canc, Children and Young People's Unit and The Institute of Cancer Research, Sutton<sup>8</sup>, Vall d'Hebron Hospital, Paediatric Haematology & Oncology, Barcelona<sup>9</sup>, Leeds Teaching Hospitals NHS Trust, Paediatric Oncology and Haematology Department, Leeds<sup>10</sup>, University College London Hospitals NHS Foundation Trust, 9University College London Hospitals NHS Foundation Trust, London<sup>11</sup>, The Hospital for Sick Children, Division of Paediatric Haematology/Oncology, Toronto<sup>12</sup>, School of Medicine, University of Leeds, Leeds Institute for Data Analytics, Leeds <sup>13</sup>, Public Health England, National Cancer Registration and Analysis Service, England <sup>14</sup>, University of Manchester, Division of Cancer Sciences, Manchester <sup>15</sup>, University Hospital Southampton, Department of Paediatric Oncology, Southampton <sup>16</sup>, Oxford Children's Hospital, Department of Paediatric Oncology, Oxford <sup>17</sup>, University hospitals Bristol, Department of Paediatric Oncology, Bristol <sup>18</sup>, Alder Hey Children's NHS Foundation Trust, Department of Paediatric Oncology, Liverpool <sup>19</sup>, Royal Belfast Hospital for Sick Children, Children's Haematology Unit, Belfast <sup>20</sup>, Royal Belfast Hospital for Sick Children, Children's haematology Unit, Belfast <sup>21</sup>, Sheffield Children's NHS Foundation Trust, Department of Paediatric Oncology, Sheffield <sup>22</sup>, Sheffield Children's NHS Foundation Trust, Department of Paediatric Oncology, Sheffield <sup>23</sup>, Newcastle University, Population Health Sciences Institute, Newcastle Upon Tyne <sup>24</sup>, Wolfson Childhood Cancer Research Centre, Newcastle Centre for Cancer, Newcastle, Wolfson Childhood Cancer Research Centre, Newcastle Upon Tyne <sup>25</sup>

### **Background/Introduction:**

Despite advances in neuroblastoma (NB) therapy, around 20% of high risk cases will develop primary refractory disease, and fail to achieve an adequate response to induction chemotherapy to allow surgical resection of the primary tumour, consolidation with myeloablative therapy, radiotherapy and minimal disease therapy. Patients in the UK diagnosed with high risk NB, from 2002-2017 were treated on the European HR-NBL1/SIOPEN trial (NCT01704716).

### **Aims:**

To describe the clinical and biological factors associated with primary refractory disease in high risk NB patients defined as patients who came off the SIOPEN HR-NBL-1 trial due to insufficient response, the treatment received, subsequent clinical course and outcome.

### **Methods/Materials:**

This is a retrospective study, which links epidemiological, clinical, genetic, and treatment data of refractory patients identified from 21 Paediatric Oncology Principal Treatment centres, in the UK and Ireland. Refractory patients were defined as those who came off the HRNBL-1 trial because of insufficient response, defined as > 3 positive bone lesions on mIBG scan or a positive bone marrow aspirate / trephine, after first line therapy with COJEC or modified N7 chemotherapy and up to 2 cycles of topotecan, vincristine and doxorubicin (TVD). The clinical, biological and treatment factors are described and chi-square and fisher-exact tests used to compare the refractory cases

characteristics with 166 high risk UK NB HRNBL-1 trial cases, identified for a genetic study using high-density single nucleotide polymorphism arrays.

#### Results:

In this interim data analysis from 10/21 centres, 37 refractory disease cases were identified so far. All were stage 4, 24% *MYCN* amplified, 12/20 (60%) 11q deleted, median age at diagnosis 3.6 years (range 1.4 – 20.4 years, mean = 5.1 years; 3% aged <18 months, 68% 18 months-5 years and 29% >5 years). In comparison, there were 19% of 166 high risk NB cases <18 months, 69% 18 months-5 years, 12% >5 years ( $P<0.01$ ); 48% *MYCN* amplified ( $P=0.01$ ) and 40% had 11q deletions ( $P<0.01$ ). Treatment for refractory disease included additional cycles of TVD in 25/37 (68%) cases (range 1-4 cycles), temozolomide alone or with irinotecan in 13/37 (35%) cases, BEACON trial treatment (NCT02308527) in 8/18 (44%) cases diagnosed after 2013 (24%). Response to second line or subsequent treatments for refractory disease assessed by the International Neuroblastoma Response Criteria (INRC) was 2/37 (5%) complete response, 8/37 (22%) very good partial response, 7/37 (19%) partial response, 5/37 (14%) mixed response, 7/37 (19%) stable disease and 7/37 (19%) progressive disease (1 unknown). Subsequently 11/37 (30%) patients received myeloablative therapy and 10/37 (27%) patients immunotherapy. The median follow-up period was 3.4 years (IQR 1.8 – 4.5 years) with 10/37 (27%) alive disease free, 8/37 (22%) alive with disease and 19/37 (51%) cases have died from disease.

#### Summary/Conclusions:

Refractory patients are more likely to be non-*MYCN* amplified and older at diagnosis compared with standard high risk patients. These early data show that some refractory patients can achieve subsequent responses and complete standard treatment for high risk NB. Once completed, this study will form a useful historical comparator group for forthcoming clinical trials for refractory NB such as the VERITAS trial (NCT03165292).

## **P028 Quantitative and highly sensitive detection of neuroblastoma minimal residual disease based on *MYCN* amplicon breakpoints**

*Szymansky, A.*<sup>2</sup>, *Hertwig, F.*<sup>3</sup>, *Winkler, A.*<sup>4</sup>, *Witthauer, M.J.*<sup>5</sup>, *Fuchs, S.*<sup>6</sup>, *Overath, M.D.*<sup>7</sup>, *Hero, B.*<sup>8</sup>, *Deubzer, H.E.*<sup>9</sup>, *Helmsauer, K.*<sup>10</sup>, *Barz, M.J.*<sup>11</sup>, *Ahrens, K.*<sup>12</sup>, *Heukamp, L.*<sup>13</sup>, *Heuckmann, J.M.*<sup>14</sup>, *Astrahantseff, K.*<sup>15</sup>, *Schmelz, K.*<sup>16</sup>, *Künkele, A.*<sup>17</sup>, *Menon, R.*<sup>18</sup>, *Hundsdoerfer, P.*<sup>19</sup>, *Eggert, A.*<sup>20</sup>, *Henssen, A.G.*<sup>21</sup>, *Fischer, M.*<sup>22</sup>, *Eckert, C.*<sup>23</sup>, *Toedling, J.*<sup>24</sup>, *Schulte, J.H.*<sup>25</sup>

Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin<sup>2</sup>, Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin<sup>3</sup>, Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin<sup>4</sup>, Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin<sup>5</sup>, Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin<sup>6</sup>, Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin<sup>7</sup>, University Hospital Cologne, Department of Pediatric Hematology and Oncology, Cologne<sup>8</sup>, Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin<sup>9</sup>, Experimental and Clinical Research Center (ECRC) of the Charité and MDC, Clinical research, Berlin<sup>10</sup>, Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin<sup>11</sup>, Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin<sup>12</sup>, Institute for Hematopathology Hamburg, Division of Molecular Biology, Hamburg<sup>13</sup>, PearlRiver Bio GmbH, Executive Team, Dortmund<sup>14</sup>, Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin<sup>15</sup>, Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin<sup>16</sup>, Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin<sup>17</sup>, NEO New Oncology GmbH, Molecular Diagnostics, Cologne<sup>18</sup>, Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin<sup>19</sup>, Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin<sup>20</sup>, Experimental and Clinical Research Center (ECRC) of the Charité and MDC, Clinical research, Berlin<sup>21</sup>, University Hospital Cologne, Department of Pediatric Hematology and Oncology, Cologne<sup>22</sup>, Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin<sup>23</sup>, Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin<sup>24</sup>, Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin<sup>25</sup>

### Background/Introduction:

More than half of the patients with high-risk neuroblastoma relapse and succumb to their disease despite initial remission in response to intensive treatment, implying the survival of neuroblastoma cells as minimal residual disease (MRD). *MYCN* amplification is a major driver of high-risk neuroblastoma, and a molecular criterion for treatment classification. Since the *MYCN* amplicon is stable during progression, the genomic breakpoints of the *MYCN* amplicon will largely persist in all tumor cells.

### Aims:

PCR-based assays to detect MRD levels are already incorporated in the clinical routine for patients with leukemia. We hypothesized that risk stratification for patients with neuroblastoma would benefit from individual PCR-based MRD assays utilizing the *MYCN* amplicon chromosomal breakpoints. Here we show that PCR-based tracking of unique genomic *MYCN* amplicon breakpoints can be used to sensitively and specifically detect MRD in *MYCN*-amplified neuroblastoma.

### Methods/Materials:

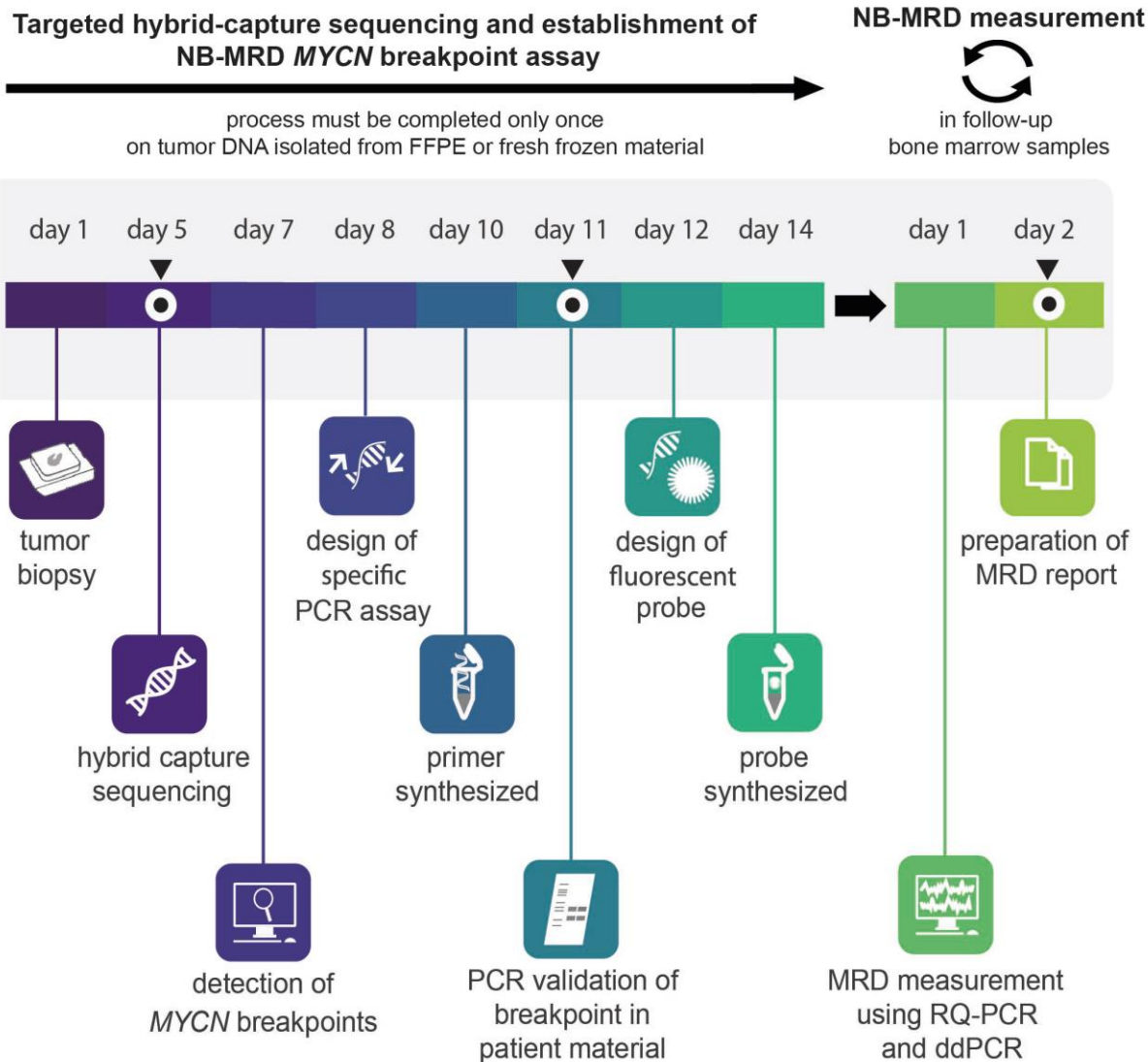
We employ a custom neuroblastoma hybrid capture-sequencing approach for high-coverage sequencing (>1000x) of the *MYCN* amplicon sequence from formalin-fixed, paraffin-embedded and fresh-frozen primary tumor material. Patient-specific PCR primers are designed from the *MYCN* amplicon chromosomal breakpoints identified by panel sequencing. After successful validation of the *MYCN* amplicon breakpoint via classical PCR and Sanger sequencing, the personalized MRD assay applies dual-labeled fluorescent hydrolysis probes within RQ-PCR or droplet digital PCR techniques.

## Results:

As a proof of concept, we performed targeted sequencing in seven *MYCN*-amplified neuroblastoma cell lines and successfully recovered all identified *MYCN* amplicon breakpoints (n=20) by classical PCR and Sanger sequencing. To estimate the detection limits of our MRD assay, eight *MYCN* breakpoints in cell lines underwent further analysis by RQ-PCR and droplet digital PCR. *MYCN* chromosomal breakpoint PCR amplicons were detectable in single tumor cells among up to  $10^6$  reference cells. Targeted sequencing of 13 primary tumor samples revealed 35 *MYCN* amplicon breakpoints that were also validated for eight patients. *MYCN* breakpoints detected in primary neuroblastomas remained stable in relapsed neuroblastomas. In total, patient-individual RQ-PCR and digital droplet PCR assays were developed for all 13 patients with the sensitivity to detect a single tumor cell in  $10^4$  to  $10^6$  normal cells. Using our strategy for personalized MRD monitoring, we could track tumor cells with *MYCN* amplicon breakpoints in bone marrow aspirates at diagnosis and during therapy, which was feasible within the clinical treatment schedule and revealed MRD persistence until relapse and good response to the first treatment course. Moreover, the MRD assay detected bone marrow infiltration below the detection limits of the currently used cytology and GD2 immunohistochemistry assays.

## Summary/Conclusions:

PCR-based tracking of patient-specific *MYCN* amplicon breakpoints can be used as a highly sensitive and specific assay detecting neuroblastoma MRD in high-risk cases. Robust MRD testing has the potential to support MRD-based therapy adaptations and pave the way for analyzing clinical significance of changes in neuroblastoma MRD levels in clinical trials. Particularly future trials testing when to integrate particular immunotherapies, would benefit from integrating MRD testing and MRD-based criteria in patient trials.



Caption 1: Outline of the clinical implementation of [simplequote]Neuroblastoma-*MYCN*-breakpoint-MRD[simplequote] detection.



## **P029 Haddad Syndrome and Neuroblastoma: a case report**

Tondo, A.<sup>2</sup>, Carra, F.<sup>3</sup>, Trevisan, F.<sup>4</sup>, Cuzzubbo, D.<sup>5</sup>, Azzali, A.<sup>6</sup>, Perrone, A.<sup>7</sup>, Favre, C.<sup>8</sup>

A.Meyer" Children's Hospital", Hematology Oncology and HSCT Unit, Florence <sup>2</sup>, A.Meyer" Children's Hospital", Hematology Oncology and HSCT Unit, Florence<sup>3</sup>, A.Meyer" Children's Hospital", Hematology Oncology and HSCT Unit, Florence<sup>4</sup>, A.Meyer" Children's Hospital", Hematology Oncology and HSCT Unit, Florence<sup>5</sup>, A.Meyer" Children's Hospital", Hematology Oncology and HSCT Unit, Florence<sup>6</sup>, A.Meyer" Children's Hospital", Radiology Unit, Florence<sup>7</sup>, A.Meyer" Children's Hospital", Hematology Oncology and HSCT Unit, Florence<sup>8</sup>

### Background/Introduction:

Haddad Syndrome (HS) is a very rare disease including Congenital Central Hypoventilation Syndrome (CCHS) and Hirschsprung disease (HSCR). We report a case of HS complicated by neuroblastoma.

### Aims:

### Methods/Materials:

Case description: Second child born at full-term with perinatal asphyxia. During hospitalization, she presented respiratory failure, needing assisted ventilation and tracheostomy. Genetic studies showed a *de novo* heterozygous mutation (C.780dupT) of PHOX2B gene, confirming CCHS. At 1 month of age HSCR was diagnosed, with subsequent segment resection and coloanal anastomosis. The presence of CCHS and HSCR led to the diagnosis of Haddad Syndrome. As CCHS and HSCR may be associated with Peripheral Neuroblastic Tumor, a close follow up was performed. At 13 months of age a right surrenalic mass was found, associated with an increment of urinary VanillylMandelic Acid (25.94 mg/gr urinary creatinine). Magnetic Resonance Imaging showed a voluminous thoraco-abdominal mass, from D5 to L2, with predominant left paravertebral thoracic development, inhomogeneously hyperintense in TR sequences and positive in DWIBS (mm 108 x 36 x 37); IDRF were present. MIBG-Scintigraphy confirmed the presence of the tumor in the thoraco-abdominal region. The ultrasound-guided ago-biopsy showed fragments with proliferation of neuronal cellular elements with maturation phenomena and calcifications, low MKI and MYCN-non-amplified. Findings were compatible with neuroblastic tumors not further classifiable. Bone marrow biopsy were negative. During hospitalization, the patient presented recurrent life-threatening event with hypoglycemia, hyperglycemia and metabolic acidosis. Therefore, because of the complex clinical presentation of the baby and her metabolic instability, in agreement with the family, we chose a wait and see approach.

### Results:

### Summary/Conclusions:

We highlight the importance of a multidisciplinary follow up for CCHS patients in order to early detect associated illnesses such as HSCR and tumors derived from the neural crest and to plan the best treatment for the patient.

## **P030 Intratumor heterogeneity and single cell changes induced by traditional chemotherapy**

*Keller, K.M.<sup>2</sup>, Kholosy, W.M.<sup>3</sup>, Lijnzaad, P.<sup>4</sup>, Koster, J.J.B.<sup>5</sup>, Tas, M.<sup>6</sup>, Langenberg, K.P.S.<sup>7</sup>, Schild, G.G.<sup>8</sup>, Margaritis, T.<sup>9</sup>, Van der Linden, R.<sup>10</sup>, Van den Boogaard, T.L.<sup>11</sup>, Holstege, F.C.P.<sup>12</sup>, Dolman, M.E.M.<sup>13</sup>, Molenaar, J.J.<sup>14</sup>*

Prinses Máxima Centrum, Research, Utrecht<sup>2</sup>, Prinses Máxima Centrum, Research, Utrecht<sup>3</sup>, Prinses Máxima Centrum, Research, Utrecht<sup>4</sup>, Amsterdam UMC, Research, Amsterdam<sup>5</sup>, Prinses Máxima Centrum, Research, Utrecht<sup>6</sup>, Prinses Máxima Centrum, Research, Utrecht<sup>7</sup>, Prinses Máxima Centrum, Research, Utrecht<sup>8</sup>, Prinses Máxima Centrum, Research, Utrecht<sup>9</sup>, Hubrecht Institute, Research, Utrecht<sup>10</sup>, Prinses Máxima Centrum, Research, Utrecht<sup>11</sup>, Prinses Máxima Centrum, Research, Utrecht<sup>12</sup>, Prinses Máxima Centrum, Research, Utrecht<sup>13</sup>, Prinses Máxima Centrum, Research, Utrecht<sup>14</sup>

### **Background/Introduction:**

With the advent of bulk DNA and RNA sequencing, our knowledge of the genomic and transcriptomic landscapes of neuroblastoma (NB) has increased drastically. This has helped create a stronger fundamental understanding of the disease; however, it has not provided great insights into new therapeutic approaches. This can be partially attributed to shortcomings in bulk sequencing techniques. Using bulk techniques, we remain unable to answer crucial questions about tumor heterogeneity and the tumor evolution in response to treatment. Ultimately this limits our understanding of neuroblastoma and thus our ability to develop of novel, more effective therapies for neuroblastoma.

### **Aims:**

In this study, we will use single cell RNA sequencing of tumor samples to gain a greater insight into intratumor heterogeneity and intratumor transcriptomic changes over the course of standardly given chemotherapy. With a greater understanding of tumor evolution in response to therapy, we aim to identify and validate more effective therapeutic approaches for NB.

### **Methods/Materials:**

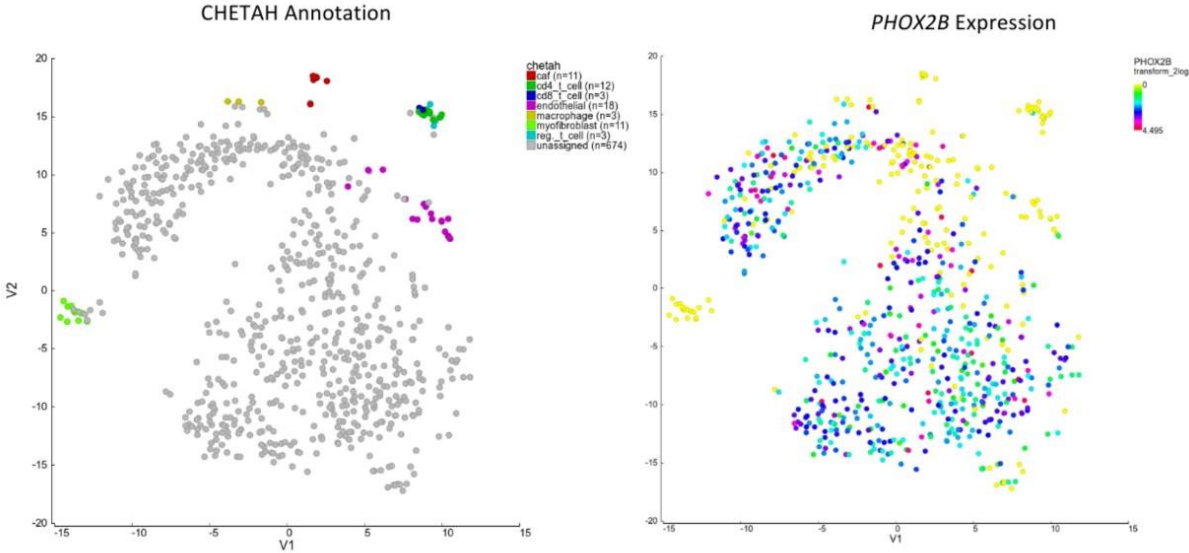
Tumor biopsies from patients at diagnosis and after chemotherapy are collected from surgery and immediately processed. Following mechanical digestion, samples are enzymatically dissolved to achieve a cell suspension. Cells are then stained and FACS sorted for single cell sequencing using the CELL-Seq2 protocol. After data processing and parsing, results are visualized using R2 (genomics analysis and visualization platform).

### **Results:**

Due to the clinical procedure of collecting diagnostic (or primary) NB samples, samples received are typically needle biopsies, offering very limited material for analysis. Initial FACS strategies for single-cell sorting resulted in poor sorting quality due to the overall low number of cells. However, incorporation of a nuclear stain with a viability stain allowed for elimination of debris and more efficient sorting of tumor material for single-cell RNA sequencing. Using the CHETAH (Characterization of cELL Types Aided by Hierarchical clustering) algorithm, preliminary results of a tumor sample reveal that diagnostic NB samples are mainly composed of tumor cells (verified via PHOX2B expression) with small populations of fibroblasts, immune cells, macrophages and endothelial cells. Tumor cells cluster based on expression of apoptosis-associated genes which could be intrinsic to the tumor, however, more tumor samples need to be evaluated to rule out processing artifacts. Upon collection of more single-cell sequencing data, matched untreated and treated tumor samples will be compared to elucidate therapeutic targets.

### **Summary/Conclusions:**

Single cell sequencing of limited diagnostic material offers a high-resolution overview to investigate intratumor heterogeneity. In order to further investigate tumor changes induced by chemotherapy, matched patient samples need to be collected and sequenced.



Caption 1: Single cell RNA sequencing data of primary, untreated NB.

## **P031 Issues on a rare case of ganglioneuroblastoma**

*Tondo, A.*<sup>2</sup>, *Carra, F.*<sup>3</sup>, *Trevisan, F.*<sup>4</sup>, *Cuzzubbo, D.*<sup>5</sup>, *Perrone, A.*<sup>6</sup>, *Olianti, C.*<sup>7</sup>, *Sementa, A.R.*<sup>8</sup>, *Buccoliero, A.M.*<sup>9</sup>, *Favre, C.*<sup>10</sup>

A.Meyer" Children's Hospital", Hematology Oncology and HSCT Unit, Florence<sup>2</sup>, A.Meyer" Children's Hospital", Hematology Oncology and HSCT Unit, Florence<sup>3</sup>, A.Meyer" Children's Hospital", Hematology Oncology and HSCT Unit, Florence<sup>4</sup>, A.Meyer" Children's Hospital", Hematology Oncology and HSCT Unit, Florence<sup>5</sup>, A.Meyer" Children's Hospital", Radiology Unit, Florence<sup>6</sup>, AOU Careggi, Nuclear Medicine Department, Florence<sup>7</sup>, Istituto Giannina Gaslini, Pathology Unit, Genoa<sup>8</sup>, AOU Careggi, Pathology Unit, Florence<sup>9</sup>, A.Meyer" Children's Hospital", Hematology Oncology and HSCT Unit, Florence<sup>10</sup>

### **Background/Introduction:**

Ganglioneuroblastoma is a very rare histological subgroup of peripheral neuroblastic tumors (pNTs) in the pediatric population; its clinical behavior is extremely heterogeneous.

### **Aims:**

### **Methods/Materials:**

**Case Presentation:** Patient G.L., male, aged 3 years, was admitted to our Department with a left supraclavicular adenopathy that was noted from the parents about 3-4 weeks earlier. He had no history of pain, fever, weight loss or other comorbidities. His family and personal pathological history was insignificant.

At admission, physical examination revealed good general status except for a 2 cm left supraclavicular adenopathy. Laboratory tests did not detect anemia or thrombocytopenia and the vanillylmandelic acid was at upper reference values (12.23 mg/gr urinary creatinine). Ultrasound of the abdomen was normal. A chest X-ray was performed, which showed a solid nodular mass and calcifications in the posterior superior mediastinum. Thoracic computed tomography (CT) scan showed a left apical thoracic tumor mass (6.4 x 4.7 x 6.7 cm) involving the left costo-vertebral groove (D1-D2) and displacing the subclavian vessels that appeared partially stenotic. A CT-guided biopsy of the tumor without complications was performed. The first pathological assessment was ganglioneuroblastoma, intermixed type, Schwannian stroma-rich, *MYCN* non-amplified and presence of segmental chromosomal aberrations.

A whole body MRI showed an hyperintense signal area in D8 vertebra on STIR and DWIBS sequences, without signs of bone erosion. The same abnormalities were found at MIBG scintigraphy. Bone marrow aspirate from bilateral iliac crest was negative.

A multidisciplinary discussion to perform a resection of tumor was done and surgery was planned and approached using left thoracotomy under general anesthesia with the complete excision of the tumor and the supraclavicular adenopathy. Histopathologic exam of the surgical samples were more consistent with composite neuroblastoma (intermixed-nodular ganglioneuroblastoma), *MYCN* non-amplified; this histological pattern was confirmed in a more careful evaluation of the previous bioptic samples.

The patient underwent 4 cycles of chemotherapy (SIOPEN NB HR); a good response was evidenced in the next radiological exams by minimal abnormal signal in the vertebral lesion on MRI DWIBS without MIBG uptake on SPECT.

### **Results:**

### **Summary/Conclusions:**

Ganglioneuroblastoma is a rare subtype of pNTs; its histopathological diagnosis sometimes has some issues but a proper diagnosis is of utmost importance because of the clinical, therapeutic and

prognostic significance among the different subgroups. Although if the multimodal imaging could guide the diagnostic approach, a correct assessment of the histologic samples is the cornerstone; the identification of nodular lesions in the second samples changed the prognosis and the therapeutic approach.

Clinicians and pathologists should be aware of the various clinical presentations of pNTs, and the appropriate use of multimodal imaging and pathologic investigations is crucial for the diagnosis and therapy.

## **P032 Outcomes of children with ganglioneuroblastoma and ganglioneuroma, a single centre case series**

*Jain, N.<sup>2</sup>, Barone, G.<sup>3</sup>, Sebire, N.<sup>4</sup>, Rampling, D.<sup>5</sup>, Anderson, J.<sup>6</sup>*

Great Ormond Street Hospital, Paediatric Oncology, London, United Kingdom <sup>2</sup>, Great Ormond Street Hospital, Paediatric Oncology, London, United Kingdom<sup>3</sup>, Great Ormond Street Hospital, Department of Histopathology, London, United Kingdom<sup>4</sup>, Great Ormond Street Hospital, Department of Histopathology, London, Great Ormond Street Hospital<sup>5</sup>, Institute of Child Health, Experimental Paediatric Oncology, London, United Kingdom<sup>6</sup>

### **Background/Introduction:**

Peripheral neuroblastic tumors are the most common extracranial solid tumors in childhood. These tumors are a heterogeneous group with varying degrees of differentiation and malignant potential. They are divided into four broad categories; neuroblastoma (NBL), ganglioneuroma (GN), ganglioneuroblastoma intermixed (GNBi) and ganglioneuroblastoma nodular (GNBn). Historically, GN and GNBi are more benign, GNBn can be divided into favourable and unfavourable based on characteristics such as grade of differentiation, mitosis-karyorrhexis index (MKI), diploidy and age of child, while NBL is considered malignant. Genomic characterisation with *MYC-N* amplification and segmental chromosomal alterations (SCA) (1p loss, 11q loss, 17q gain) has been added to risk stratification in infant NBL and GNBn but is not widely used for GN or GNBi.

### **Aims:**

We report a retrospective case review, which highlights patients whose clinical course does not correlate with the historically good prognosis associated with GN, GNBi and GNBn. We also describe the genomic alterations and treatment received by this group of patients.

### **Methods/Materials:**

The database of histopathology reports from 2000 to 2018 issued at Great Ormond Street Hospital were searched for the terms ganglioneuroma and ganglioneuroblastoma. A total of 314 histopathology reports were reviewed. 26 reports containing other final diagnoses were excluded. The remaining 288 reports from 153 patients were included in the analysis.

### **Results:**

From the 153 patients reviewed, the final treatment diagnosis was GN for 25% (n=38), GNBi for 31% (n=47), GNBn for 3% (n=5) and NBL with evidence of differentiation for 41% (n=63). Clinical information was available for 72 patients with ganglioneuroma and ganglioneuroblastoma. Of these 72 patients, all except 3 patients had localised disease, 2 with GNBi and 1 with GNBn. 45 patients had genomic information available for analysis. *MYC-N* amplification was present in 1 case only, a child with GNBn. SCA were found in 2 patients with GN, 11 patients with GNBi and 2 with GNBn. Only 1 patient with GN received chemotherapy and all were well and alive at last clinical follow-up. All 4 children with GNBn with clinical information available received chemotherapy. The remaining 3 children with GNBn are well at time of last review. 11 patients with GNBi were treated systemically, including single agent treatment with isotretinoin. 6 children with GNBn showed evidence of disease progression during their treatment or follow-up period, 5 of who had SCA present. There were a total of 3 deaths in our case series, 2 related to disease progression. 1 child treated for Stage M GNBn with no *MYC-N* amplification and unknown SCA, had slowly progressive disease, was treated with several lines of therapy but eventually died from disease progression seven years after initial diagnosis. 1 child had localised progressive GNBi with 1p loss.

### **Summary/Conclusions:**

Overall, children with ganglioneuroma and ganglioneuroblastoma have a favourable outcome. However children diagnosed with GNBi who have SCA can show disease progression and we describe one child who died as a result of their progressive disease. We also describe a single case of

a child with GNBn with slowly progressive disease over seven years, unresponsive to several treatments.

## **P033 Modelling the First Step of Metastasis; 3D Culture Assays of Neuroblastoma Invasion.**

*Gavin, C.*<sup>2</sup>, *Geerts, N.*<sup>3</sup>, *Ewald, A.*<sup>4</sup>, *Reynolds, P.*<sup>5</sup>, *Piskareva, O.*<sup>6</sup>, *Haynes, M.*<sup>7</sup>

Royal College of Surgeons in Ireland, School of Pharmacy and Biomolecular Sciences, Dublin, Ireland<sup>2</sup>, Royal College of Surgeons in Ireland, School of Pharmacy and Biomolecular Sciences, Dublin, Ireland<sup>3</sup>, Johns Hopkins University, Department of Cell Biology, Baltimore, USA<sup>4</sup>, Texas Tech University Health Science Centre, Children's Oncology Group, Lubock, Texas Tech University Health Science Centre<sup>5</sup>, Royal College of Surgeons in Ireland, Department of Anatomy and Regenerative Medicine, Dublin, Ireland<sup>6</sup>, Johns Hopkins University, Department of Cell Biology, Baltimore, USA<sup>7</sup>

### Background/Introduction:

Neuroblastoma (NB) is a highly heterogenous paediatric malignancy that accounts for approximately 7% of all childhood cancers in the United States. However, the same disease is responsible for about 15% of paediatric oncology deaths (1). This disparity is due to the fact that half of the patients have metastatic spread at the time of diagnosis, which represents the most aggressive form of the disease. For this group of high-risk patients, treatment options remain poor resulting in a dismal 5-year survival rate of approximately 50% (2). Therefore, elucidating the molecular basis of the metastatic process may guide novel strategies for drug discovery and therapy.

### Aims:

The aims of this study were to 1.) model the first step of metastasis by imaging neuroblastoma cell lines and organoids in real-time as they invade 3D hydrogels representing the *in vivo* extracellular matrix 2.) to characterise the different migration strategies employed by NB cells in response to local ECM composition.

### Methods/Materials:

NB cell lines as well as organoids isolated from patient-derived xenografts (*MYCN* and non-*MYCN* amplified) were cultured in hydrogels of three different compositions. Collagen I was used to model the stromal ECM and Matrigel was used to model the basement membrane. We also cultured in a hydrogel composed of 50% collagen I and 50% Matrigel. We used 3D invasion assays, as described by our collaborator Professor Ewald (3), coupled with DIC time-lapse microscopy and confocal imaging to directly observe the migration strategies employed by NB during invasion of the local microenvironment.

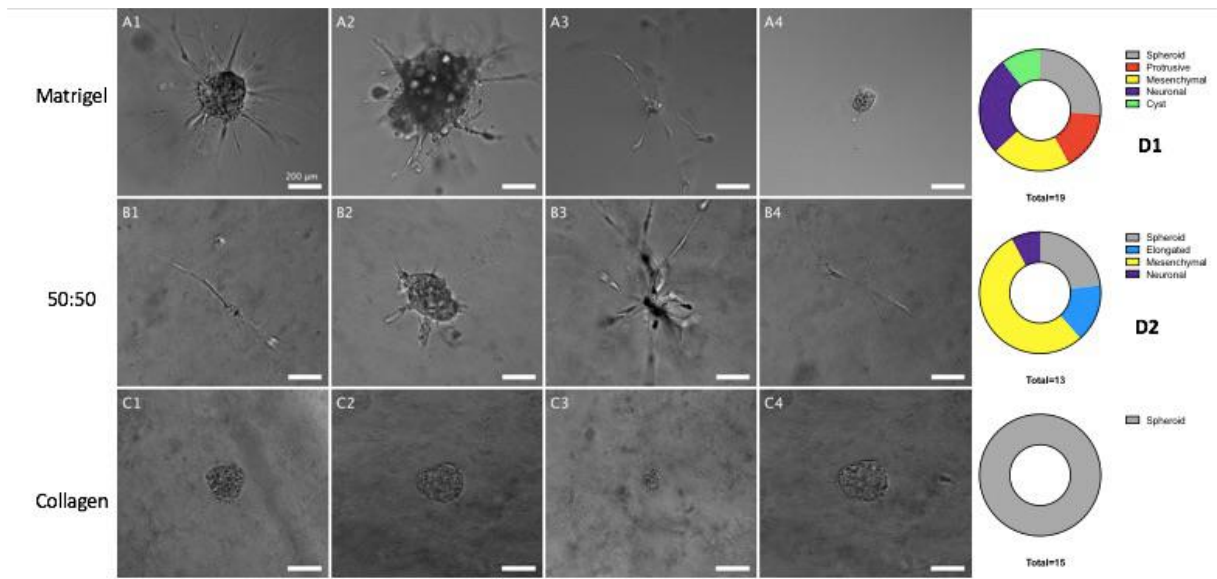
### Results:

When cultured in 3D hydrogels the migration strategies employed by NB cell lines is not influenced by the local microenvironment. In contrast, NB organoids isolated from patient-derived xenografts (PDXs) employ distinct migration strategies that are dependent on the ECM. In collagen I hydrogels, NB organoids predominantly form multicellular spheroid colonies that do not invade the ECM (C1-C4). However, in hydrogels containing Matrigel, organoids invade the ECM using four distinct migration strategies that we have termed elongated (B1, B4), collective mesenchymal (A2, B2), protrusive (A1), and neuronal (A3). We have also identified a non-invasive bi-layered structure containing a lumen, termed a cyst (A4).

### Summary/Conclusions:

Our results show that, *in vitro* NB organoids isolated from PDXs maintain the heterogenous characteristics of native neuroblastomas, regardless of their *MYCN* status. We have demonstrated that the molecular properties of the ECM microenvironment dictate invasion strategies in NB organoids but not cell lines. Furthermore, we have validated the pairing of 3D hydrogel culture and invasion assay as a useful tool for the study of NB migration, invasion and dissemination.





**Figure.1 Local microenvironment dictates invasion strategies of NB organoids.** Representative images of organoids isolated from Felix-PDX in Matrigel (A1-A4), 50:50 (B1-B4) and collagen I (C1-C4) hydrogels. D1-D3 Indicates proportion of organoids employing specific invasion strategies in respective matrices.

**D3**

## **P034 Association of heterogeneous *MYCN* amplification with clinical features, biological characteristics, and outcomes in Neuroblastoma: A Report from the Children's Oncology Group**

*Campbell, K.*<sup>2</sup>, *Dubois, S.*<sup>3</sup>

Dana-Farber Cancer Institute, Pediatric Oncology, Boston, USA <sup>2</sup>, Dana-Farber Cancer Institute, Pediatric Oncology, Boston, USA<sup>3</sup>

### Background/Introduction:

*MYCN* amplification (MNA) is associated with poor outcomes in children with neuroblastoma. In most cases, *MYCN* amplification is seen homogeneously throughout tumor cells, typically with high-level amplification (>4-fold ratio of *MYCN* signals to reference probe signals). With the advent of fluorescent in situ hybridization (FISH) methods to assess *MYCN* status, it has become clear that MNA may be heterogeneous, defined as amplification in a minority ( $\leq 20\%$ ) of tumor cells in a sample.

### Aims:

1. To describe the clinical characteristics and biologic features of patients with heterogeneous *MYCN* amplification. 2. To compare clinical outcomes of patients with heterogeneous MNA to patients with either homogeneous MNA or *MYCN* wild-type tumors.

### Methods/Materials:

In this retrospective cohort study from the Children's Oncology Group Neuroblastoma Biology Study (ANBL00B1), we categorized neuroblastoma patients as having tumors with *MYCN* wild-type, homogeneous MNA or heterogeneous MNA. We used chi-squared or Fisher's exact tests to compare features between groups. We used log-rank tests and Cox models to compare event-free survival (EFS) and overall survival (OS) between groups.

### Results:

*MYCN* status was ascertained in diagnostic tumor samples from 5,975 patients, including 4,937 (82.6%) with *MYCN* wild-type tumors, 981 (16.4%) with homogeneous MNA, and 57 (1%) with heterogeneous MNA. Clinical and biological features differed significantly between patients whose tumors had heterogeneous and homogeneous MNA, including stage, risk group, thoracic primary site, LDH, ploidy, 1p LOH, histology, MKI, grade, and *MYCN* copy number. Despite these differences, there was no statistically significant difference in EFS between patients with heterogeneous MNA compared to patients with homogeneous MNA [5-year EFS 57.5% (95% confidence interval (CI) 39.7-75.3%) vs. 50.7% (95% CI 46.3-55.2%), respectively;  $p=0.3537$ ]. However, comparing outcomes for patients with heterogeneous MNA vs. wild-type *MYCN*, EFS was significantly higher for patients with wild-type *MYCN* [5-year EFS 78.4% (95% CI 76.6-80.1%;  $p<0.0001$ )]. Further, controlling for other key prognostic factors (age and INSS stage), Cox proportional hazards model for EFS comparing heterogeneous MNA to homogeneous MNA with *MYCN* status as the variable of interest found no difference in EFS (hazard ratio of 0.96;  $p=0.8453$ ). In a separate model using heterogeneous MNA vs. wild-type *MYCN* and controlling for age and stage, EFS for patients with heterogeneous MNA remained significantly inferior to patients with wild-type *MYCN* (hazard ratio of 1.61;  $p=0.0284$ ).

### Summary/Conclusions:

Neuroblastoma with heterogeneous MNA demonstrates significantly different biological and clinical patterns compared to homogeneous MNA. Prognosis is similar between these two groups. These results support current practice that treats patients with heterogeneous MNA similarly to patients with homogeneous MNA.

## **P035 Single-cell comparison of neuroblastoma stages with mouse embryonic neural crest and human adrenal gland**

*Bedoya-Reina, O.C.<sup>2</sup>, Li, W.<sup>3</sup>, Arceo, M.<sup>4</sup>, Plescher, M.<sup>5</sup>, Kharchenko, P.<sup>6</sup>, Adameyko, I.<sup>7</sup>, Deng, Q.<sup>8</sup>, Pui, H.<sup>9</sup>, Larsson, C.<sup>10</sup>, Juhlin, C.<sup>11</sup>, Kogner, P.<sup>12</sup>, Schlisio, S.<sup>13</sup>*

Karolinska Institutet, MTC, Stockholm, Sweden <sup>2</sup>, Karolinska Institutet, MTC, Stockholm, Sweden<sup>3</sup>, Karolinska Institutet, MTC, Stockholm, Sweden<sup>4</sup>, Karolinska Institutet, MTC, Stockholm, Karolinska Institutet<sup>5</sup>, Harvard Medical School, Biomedical Informatics, Boston, USA<sup>6</sup>, Karolinska Institutet, Department of Physiology and Pharmacology, Stockholm, Sweden<sup>7</sup>, Karolinska Institutet, Department of Physiology and Pharmacology, Stockholm, Sweden<sup>8</sup>, Karolinska Institutet, Department of Physiology and Pharmacology, Stockholm, Sweden<sup>9</sup>, Karolinska Institutet, Department of Oncology and Pathology, Stockholm, Sweden<sup>10</sup>, Karolinska Institutet, Department of Oncology and Pathology, Stockholm, Sweden<sup>11</sup>, Karolinska Institutet, Department of Women's and Children's Health, Stockholm, Sweden, <sup>12</sup>, Karolinska Institutet, MTC, Stockholm, Sweden <sup>13</sup>

### Background/Introduction:

Intra-tumor heterogeneity in neuroblastoma (NB) and the presence of resistant clones represent a challenge for therapy. To understand the heterogeneity and “differentiation-like” status of cancer cells, we need to determine the origin and differentiation paths of cancer-originating cells. We explored intra-tumor heterogeneity using single-nuclei sequencing (Smart-seq2) and examined clinical heterogeneity by sequencing tumors from different stages of favorable or unfavorable outcome. A total of 3,853 tumor cells were sequenced, with 1,412 million reads mapped to annotated genes. Tumors clustered differently depending on stage and outcome. By using a cell type enrichment approach, we identified cell clusters that can be broadly classified as 1) neural, 2) undifferentiated, 3) immune, 4) mesenchymal stroma (MSC), and 5) endothelial. We compared cell populations of the tumors with mouse neural-crest populations from different embryonic ages at E9.5, E10.5, E12.5 and E13.5. We identified gene signatures shared between the sympatho-adrenal lineage of mouse E12.5 and E13.5 and the neural populations of NB. We further compared tumor populations with human normal adrenal gland populations, and determined commonalities in gene expression between cell clusters.

**P036 Stage 4S Neuroblastomas: Molecular, Histological and Immunohistochemical Characteristics and Presence of 2 Distinct Patterns of *MYCN* Protein Expression: A Report from the Children's Oncology Group.**

*Shimada, H.*<sup>1</sup>, *Kawano, A.*<sup>2</sup>, *Kawano, A.*<sup>3</sup>, *Hazard, F.K.*<sup>4</sup>, *Naranjo, A.*<sup>5</sup>, *LaBarre, B.*<sup>6</sup>, *London, W.B.*<sup>7</sup>, *Hogarty, M.D.*<sup>8</sup>, *Cohn, S.L.*<sup>9</sup>, *Maris, J.M.*<sup>10</sup>, *Park, J.R.*<sup>11</sup>, *Gastier-Foster, J.M.*<sup>12</sup>, *Ikegaki, N.*<sup>13</sup>

Stanford University, Pathology, Stanford, USA<sup>1</sup>, Chiba University of Graduate School of Medicine, Pathology, Chiba, Japan<sup>2</sup>, Chiba University of Graduate School of Medicine, Pathology, Chiba, Japan<sup>3</sup>, Stanford University, Pathology, Stanford, USA<sup>4</sup>, University of Florida, Biostatistics, Gainesville, University of Florida<sup>5</sup>, University of Florida, Biostatistics, Gainesville, USA<sup>6</sup>, Boston Children's Hospital, Pediatrics, Boston, USA<sup>7</sup>, The Children's Hospital of Philadelphia, Division of Oncology, Philadelphia, USA<sup>8</sup>, University of Chicago, Pediatrics, Chicago, USA<sup>9</sup>, The Children's Hospital of Philadelphia, Division of Oncology, Philadelphia, USA<sup>10</sup>, Seattle Children's Hospital, Pediatrics, Seattle, USA<sup>11</sup>, Baylor College of Medicine, Pediatrics, Houston, USA,<sup>12</sup> University of Illinois at Chicago, Anatomy and Cell Biology, Chicago, USA<sup>13</sup>

**Background/Introduction:**

Stage 4S neuroblastoma (4SNB: <1yr at diagnosis, INSS stage 1 or 2 primary, metastasis limited to liver, skin and/or bone marrow) is often associated with spontaneous regression and a good clinical outcome. *MYCN* oncogene amplification and augmented expression of MYC-family (*MYCN*/*MYC*) protein indicate aggressive clinical behavior in NBs.

**Aims:**

Molecular, histological and immunohistochemical characteristics of 4SNB tumors were analyzed along with clinical outcomes of the patients.

**Methods/Materials:**

A total of 185 4SNBs filed at the COG NB Pathology Reference Laboratory were available in this study. Their *MYCN* oncogene status [Non-amplified(NA) vs. Amplified(A)] by FISH, MYC-family protein expression [no-overexpression(-)/(+/-) vs. overexpression(+)] by immunohistochemistry(IHC), and histopathology by INPC [Favorable Histology(FH) vs. Unfavorable Histology(UH)] with a special attention to the nucleolar hypertrophy [NH, supporting increased protein expression, (-) vs. (+)] were correlated with patient survival.

**Results:**

As shown in the Table, *MYCN* oncogene status (165NA;20A) distinguished favorable and unfavorable clinical outcomes of the patients. The vast majority of tumors showed *MYCN* NA, MYC-family protein(-)/(+/-), FH, NH(-), and excellent survivals (conventional NA tumors). Among *MYCN*-NA tumors, however, 11 demonstrated *MYCN* protein(+) with uniform and moderate intensity: they were FH(10/11), NH(-) and one showed MYC protein(+). Also found were 5 MYC protein(+) and *MYCN*(-)/(+/-) tumors: they were FH without NH(4/5). Among the *MYCN*-A tumors, 18 had *MYCN* protein(+) with heterogeneous and strong intensity regardless of INPC [UH(9, conventional A tumors)/FH(9)] and 15 of them had NH(+). Two tumors had *MYCN* protein(-)/(+/-) despite *MYCN*-A; both were FH and NH(-).

**Summary/Conclusions:**

In most of the 4S NBs (89%), all or majority of the factors tested in this study were favorable, indicating an excellent prognosis of the patients. However, rare tumors (11%) were *MYCN*-A, and had a poor prognosis when *MYCN* protein was (+). Two patterns of *MYCN* protein(+) were distinguished: (1) In *MYCN*-A tumors, the expression was associated predominantly with NH, showed a heterogeneous and strong pattern, and indicated a poor prognosis and (2) Protein expression in *MYCN*-NA tumors was mostly not associated with NH, showed a uniform and moderate pattern, and indicated an excellent prognosis. The definition of 4SNB should be refined to adhere to the biological

characteristics of tumors, especially to the *MYCN* status, expression pattern of the *MYCN* protein and the nucleolar morphology.

MYCN oncogene	MYCN protein	MYC protein	INPC	NH	3-year EFS (SE)%	3-yr OS (SE)%
<b>Non-Amplified (165)</b>						
<b>Conventional (147)</b>	{-}/(+/-)	{-}/(+/-)	FH	{-}	88.5 (3.1)	94.1 (2.3)
<b>Others (10)</b>	{+}*	{-}/(+/-)	FH(9);UH(1)	{-}	100 (0)	100 (0)
<b>(1)</b>	{+}*	{+}	FH	{-}	100 (0)	100 (0)
<b>(5)</b>	{-}/(+/-)	{+}	FH	4{-};1{+}	100 (0)	100 (0)
<b>(2)</b>	{-}/(+/-)	{-}/(+/-)	UH	{-}	100 (0)	100 (0)
<b>Amplified (20)</b>						
<b>Conventional (9)</b>	{+}**	{-}/(+/-)	UH	2{-};7{+}	44.4 (23.4)	44.4 (23.4)
<b>Others (9)</b>	{+}**	{-}/(+/-)	FH	1{-};8{+}	68.6 (19.2)	68.6 (19.2)
<b>(2)</b>	{-}/(+/-)	{-}/(+/-)	FH	{-}	N/A	N/A

MYCN expression by IHC: \*Moderately and uniformly positive; \*\*Strongly and heterogeneously positive.

## **P037 *In vitro* 3D bioprinted neuroblastoma: A tunable platform to assess extracellular matrix impact on growth and phenotypic cell behavior**

*Monferrer, E.*<sup>2</sup>, *Martín-Vañó, S.*<sup>3</sup>, *García-Lizarribar, A.*<sup>4</sup>, *Samitier, J.*<sup>5</sup>, *Navarro, S.*<sup>6</sup>, *Noguera, R.*<sup>7</sup>

Medical School, University of Valencia-INCLIVA, Pathology Department, Valencia, Spain<sup>2</sup>, Medical School, University of Valencia-INCLIVA, Pathology Department, Valencia, Spain<sup>3</sup>, University of Barcelona / Institute for Bioengineering of Catalonia, (IBEC), Electronics and Biomedical Engineering Department, Barcelona, Spain<sup>4</sup>, University of Barcelona / Institute for Bioengineering of Catalonia, (IBEC), Electronics and Biomedical Engineering Department, Barcelona, University of Barcelona / Institute for Bioengineering of Catalonia, (IBEC)<sup>5</sup>, Medical School, University of Valencia-INCLIVA, Pathology Department, Valencia, Spain<sup>6</sup>, Medical School, University of Valencia-INCLIVA, Pathology Department, Valencia, Spain<sup>7</sup>

### Background/Introduction:

The composition of extracellular matrix (ECM) and its structural and mechanical properties can control cancer cell behavior, developing a tumor microenvironment that promotes cell migration and invasion. We have previously described in human biopsies that ECM stiffness is related to poor outcome in neuroblastoma (NB) patients. 3D bioprinted *in vitro* models can imitate in a simplified and controlled way the ECM stiffness, which makes them a useful tool for the study of ECM-dependent tumor cell behavior.

### Aims:

This work aims to study the effect of ECM stiffness over the time on several parameters which characterize cell tumor behavior using different bioprinted 3D NB models.

### Methods/Materials:

Hydrogels were made of 5% of methacrylated gelatin and different percentages of methacrylated alginate (from 0% to 2%). Cell populations were incorporated during hydrogel fabrication and were together bioprinted applying air pressure extrusion system with 3DDiscovery BioSafety printer. Four types of matrices were generated regarding cell composition, differentiated by the incorporation of SK-N-BE(2) or SH-SY5Y neuroblastic cells and the presence (in a proportion of 10%) or absence of SW10 Schwann cells. The 3D bioprinted hydrogels were cultured from 2 to 6 weeks. Models were paraffin-embedded, sectioned, and stained according to the parameter to be analyzed. Hydrogel porosity, cell cluster density, cell cluster occupation, cell nucleus size and staining intensity, cell proliferation (anti-Ki67 antibody), mRNA activity (anti-PTBP1 antibody) and vitronectin expression (anti-VN antibody) were quantitatively analyzed in digitalized hydrogel stained sections applying Panoramic Viewer software modules. Mitosis Kariorrhesis Index (MKI), stem cell markers (anti-SOX2 and anti-OCT4 antibodies), antiapoptosis (anti-BCL2 antibody) and apoptosis (anti-BAX antibody) were qualitatively analyzed by the pathologist and two researchers. All analyses were based on the presence, distribution and intensity of the staining and differences were related to the different time and stiffness conditions.

### Results:

SK-N-BE(2) cells grew in all hydrogel conditions while SH-SY5Y presented some difficulties. The patterns of growth and behavior presented differences with time and stiffness in both cell lines with or without SW10 cells. Some parameters remained with less variability (cell cluster occupation, mRNA activity, MKI, cell nucleus size and staining intensity), but others, and depending on the analyzed model, inverted their tendency with SW10 cells incorporation, leading to a less aggressive cell behavior.

### Summary/Conclusions:

Tunable *in vitro* 3D bioprinted cell culture scaffolds can replicate the NB pathophysiology, which allows to achieve deeper knowledge of cell behavior related to the tumor microenvironment.



## **P038 Immunosuppression mechanisms in 11q-deleted neuroblastomas support increased anti-GD2 response when combined with dual immunotherapy**

*Coronado, E.*<sup>2</sup>, *Yañez, Y.*<sup>3</sup>, *Rubio, L.*<sup>4</sup>, *Vera-Sempere, V.S.*<sup>5</sup>, *Panadero, J.*<sup>6</sup>, *Cañete, A.*<sup>7</sup>, *Castel, V.*<sup>8</sup>, *Font de Mora, J.*<sup>9</sup>, *Ladenstein, R.*<sup>10</sup>

Health Research Institute Hospital La Fe, Laboratory of Cellular and Molecular Biology.Clinical and Translational Research, Valencia, Spain <sup>2</sup>, Health Research Institute Hospital La Fe, Laboratory of Cellular and Molecular Biology.Clinical and Translational Research, Valencia, Spain<sup>3</sup>, La Fe University Hospital, Department of Pathology, Valencia, Spain<sup>4</sup>, La Fe University Hospital, Department of Pathology, Valencia, La Fe University Hospital<sup>5</sup>, Health Research Institute Hospital La Fe, Genomics Unit, Valencia, Spain<sup>6</sup>, La Fe University Hospital, Clinical and Translational Research in Cancer. Pediatric Oncology Unit, Valencia, Spain<sup>7</sup>, La Fe University Hospital, Clinical and Translational Research in Cancer. Pediatric Oncology Unit, Valencia, Spain<sup>8</sup>, Health Research Institute Hospital La Fe, Laboratory of Cellular and Molecular Biology.Clinical and Translational Research, Valencia, Spain<sup>9</sup>, St. Anna Children's Hospital, St. Anna Children's Hospital, Vienna, Austria<sup>10</sup>

### **Background/Introduction:**

Post-consolidation treatment with anti-GD2 immunotherapy has become the standard of care for patients with high-risk neuroblastoma (NB). However, the relapse rate of patients exceeds 50% with fatal outcome. Dissecting molecular hallmarks within high-risk NB subgroups will identify actionable targets for new treatment options.

### **Aims:**

To evaluate the response of 11q-deleted NB subgroup to standard anti-GD2 immunotherapy and define potential new targets for tailored therapies.

### **Methods/Materials:**

Response to maintenance therapy was evaluated in 84 high-risk (M stage) NB patients. Primary endpoints were 3-year event-free survival (EFS) and 3-year overall survival (OS). Patients received either standard anti-GD2 immunotherapy (n=51) or conventional retinoic acid-based therapy (n=33). Differences in EFS and OS between treatment regimens were compared with the log-rank test. Immune and genetic profiles of high-risk NB were analyzed to examine their relationship with immunotherapy response. CIBERSORT was used to quantify immune cell infiltration. Segmental chromosome aberrations (SCAs) were analyzed by SNP arrays (CytoScan HD, Affymetrix). Gene Set Enrichment Analysis (GSEA) with gene-targeted 11q-miRNAs was performed to explore the effect of 11q miRNAs haploinsufficiency. Differences in immune cell infiltration, gene expression profiles, and SCA profiles between subgroups were tested using one-way analysis of variance (one-way ANOVA).

### **Results:**

Anti-GD2 immunotherapy improved outcome in the overall high-risk NB cohort with regard to EFS (52.9 vs 30.3, p=0.013) and OS (74.2 vs. 51.5, p=0.023) at 3 years. Similarly, 11q-deleted subgroup showed improvement in EFS (54.2% vs. 10%, p=0.006) and OS (74.2% vs. 50%, p=0.169), although survival rates are still sub-optimal. Noteworthy, several approaches reveal that 11q-deleted NB exhibit various immune inhibitory mechanisms: higher SCAs, dysregulation of immunosuppressive miRNAs, increased regulatory T cells infiltration (Tregs, p=0.035), and higher expression of several immune checkpoint biomarkers: programmed death-ligand 1 (PD-L1, p=0.029), interleukin-10 (IL-10, p=2.47e-03), transforming growth factor-beta 1 (TGF-β1, p=0.016) and indoleamine 2,3 dioxygenase 1 (IDO1, p=0.021).

### **Summary/Conclusions:**

NB patients with 11q-deletion display similar outcome as those with 11q-undeleted NB to anti-GD2 immunotherapy. However, 11q-deleted tumors display a distinct immune suppression profile, supporting the combination treatment of anti-GD2 with immune checkpoint inhibitors to circumvent immune checkpoint blockade. This combination may be a more efficient therapeutic strategy for this tumor subtype.





## **P039 Use of the genetic basis of Beckwith-Wiedemann to reveal the genetic variation associated with the development of sporadic neuroblastoma**

*Cantalupo, S.*<sup>2</sup>, *Formicola, D.*<sup>3</sup>, *Vaksman, Y.*<sup>4</sup>, *Diskin, S.*<sup>5</sup>, *Maris, J.*<sup>6</sup>, *Iolascon, A.*<sup>7</sup>, *Capasso, M.*<sup>8</sup>

Università degli Studi di Napoli "Federico II", Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Naples, Italy <sup>2</sup>, Università degli Studi di Napoli "Federico II", Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Naples, Italy<sup>3</sup>, Children's Hospital of Philadelphia, Division of Oncology, Philadelphia, USA<sup>4</sup>, Children's Hospital of Philadelphia, Division of Oncology, Philadelphia, Children's Hospital of Philadelphia<sup>5</sup>, Children's Hospital of Philadelphia, Division of Oncology, Philadelphia, USA<sup>6</sup>, Università degli Studi di Napoli "Federico II", Dipartimento di Medicina Molecolare e Biotecnologie Mediche,, Naples, Italy<sup>7</sup>, Università degli Studi di Napoli "Federico II", Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Naples, Italy<sup>8</sup>

### Background/Introduction

Beckwith–Wiedemann syndrome (BWS) is an overgrowth disorder characterized by perinatal overgrowth, macroglossia, exomphalos, hemihyperplasia, and postnatal hypoglycemia, and associated with an increased risk to develop embryonic tumors like neuroblastoma (NB). Patients with BWS who develop NB, in 2,8% of cases have mutations in CDKN1C, a well-known tumor suppressor. Thus we speculated that common genetic variants affecting CDKN1C could be associated with risk of NB development.

### Aims

Our aim was to identify single nucleotide polymorphisms (SNPs) in CDKN1C predisposing to NB.

### Methods/Materials

We performed genotype imputation of the CDKN1C locus using GWAS data from 2101 NB cases and 4202 controls of European ancestry. The variants were filtered based on the allelic frequency (MAF > 5% classified as "high-frequency (HF)"; 1% > MAF >= 5% classified as "low-frequency (LF)") and the statistical significance (p < 0.05). A replication study was conducted on 455 cases and 969 controls of Italian origin.

### Results

After imputation, we identified 313 SNPs in the CDKN1C gene locus. We selected 231 HF SNPs and 66 LF SNPs. Between these, only 38 HF variants and 3 LF variants have p < 0.05. Among HF and LF SNPs, rs12293538 and rs143992355 were the most significant, respectively (p = 1.77x 10<sup>-4</sup>, OR: 1.16; p = 0.02, OR: 1.28). We have validated the association of rs12293538 in the Italian population (p = 0.077, OR: 1.16). Using public ChIP-seq data, we observed that the SNP rs12293538 falls in a region characterized by H3K27Ac in the SKNBE cell lines while the SNP rs143992355 falls in a region characterized by both H3K27Ac and H3K4me3 in the SKNBE2C. The analysis of gene expression microarrays showed that CDKN1C gene seems to function as a tumor suppressor; in fact, its expression is higher in 18 NB cell lines when compared to 6 healthy tissues (p = 7.15x10<sup>-15</sup>), in 46 relapsed NBs when compared to 56 non-relapsed NBs (p = 0.02). Moreover, Kaplan-Maier analysis with patients grouped by the optimal cut-off (calculated using R2 web tool) of CDKN1C expression for overall survival shows that higher CDKN1C expression is significantly associated with higher probability of survival in NB patients in different datasets.

### Summary/Conclusions

Using the genetic predisposition of BWS patients to develop NB, we identified rare and common variants in the CDKN1C tumor suppressor gene, located in DNA regulatory region and associated with the risk of developing NB. Future objectives include the functional study of these variants and the investigation of biological role of CDKN1C in NB development.

## **P040 Quantitative and semiquantitative assessment of 123I-MIBG uptake in prediction of unfavorable histopathology in patients with neuroblastic tumors**

*Chaurasiya, K.<sup>2</sup>, Kireeva, E.<sup>3</sup>, Shamanskaya, T.<sup>4</sup>, Kachanov, D.<sup>5</sup>, Likar, Y.<sup>6</sup>*

National Medical Research Center of Pediatric Hematology, Oncology & Immunology, Nuclear Medicine, Moscow, Russia <sup>2</sup>, National Medical Research Center of Pediatric Hematology, Oncology & Immunology, Nuclear Medicine, Moscow, Russia<sup>3</sup>, National Medical Research Center of Pediatric Hematology, Oncology & Immunology, Oncology, Moscow, Russia<sup>4</sup>, National Medical Research Center of Pediatric Hematology, Oncology & Immunology, Oncology, Moscow, National Medical Research Center of Pediatric Hematology, Oncology & Immunology<sup>5</sup>, National Medical Research Center of Pediatric Hematology, Oncology & Immunology, Nuclear Medicine, Moscow, Russia<sup>6</sup>

### **Background/Introduction**

123I-metaiodobenzylguanidine (123I-MIBG) scintigraphy is widely used for the detection and staging of neuroblastoma. The intensity of 123I-MIBG uptake in the primary neuroblastic tumors can vary significantly in same types of neuroblastic tumors as well as in different histological variants. Only visual assessment of 123I-MIBG uptake can be qualitative, but not quantitative. Fortunately, with the development of SPECT/CT and new software in recent years the semiquantitative (TLCRR – time to liver count rate ratio) and quantitative (SUVmax – maximum standardized uptake value) assessment of 123I-MIBG uptake in primary tumors is possible.

### **Aims**

Quantitative and semiquantitative assessment of 123I-MIBG uptake in prediction of unfavorable histopathology in patients with neuroblastic tumors.

### **Methods/Materials**

105 123I-MIBG-positive patients were included in this retrospective study. 123I-MIBGscintigraphy including whole body planar images and SPECT/CT were performed for all patients before any type of treatment. Semiquantitative (TLCRR) and quantitative (SUVmax) assessment of 123I-MIBG uptake were calculated and analyzed for all patients.

### **Results**

Out of 105 patients: 60 patients were with neuroblastoma (NB), 28 with ganglioneuroblastoma (GNB) and 17 with ganglioneuroma (GN). The values of SUVmax and TLCRR were significantly higher in neuroblastoma group than ganglioneuroma. The average values of SUVmax and TLCRR for NB were 7.5 and 5.6, for GNB - 3.1 and 2.58 and for GN - 1.7 and 1.3, respectively. Multiple statistical analysis were used including ROC curve for quality assessment of different models. AUC values for SUVmax, TLCRR and NSE were 0.923, 0.895, 0.858 in NB patients; 0.826, 0.735, 0.762 in GNB patients and 0.926, 0.898, 0.924 in GN patients respectively.

### **Summary/Conclusions**

SPECT/CT in combination with modern software allows quantitative assessment of 123I-MIBG uptake in neuroblastic tumor. The SUVmax and TLCRR can be used separately as well as in combination with NSE for prediction of histological variant. Our study demonstrates that quantitative assessment (SUVmax) of 123I-MIBG uptake alone as well as in combination with other parameters is more specific. Moreover, the quantitative assessment (SUVmax) of 123I-MIBG uptake is more simple method and it minimizes the possible error in patients with diffuse liver involvement.

## **P041 ScRNA-seq of *MYCN* and *LMO1* driven zebrafish neuroblastoma reveals intra- and inter-tumoral heterogeneity**

Fesmann, N.,<sup>1</sup> Heeren-Hagemann, A.<sup>2</sup>, Spanjaard, B.<sup>3</sup>, Gauert, A.<sup>4</sup>, Brame, L.<sup>5</sup>, Look, A.T.<sup>6</sup>, Eggert, A.<sup>7</sup>, Heeren-Hagemann, A.<sup>8</sup>, Junker, J.P.<sup>9</sup>

Max Delbrück Center for Molecular Medicine, Berlin Institute for Medical Systems Biology (BIMSB), Berlin, Germany<sup>1</sup>, Charité- University Hospital, Science Dept. of Paediatric Oncology/Haematology, Berlin, Germany<sup>2</sup>, Max Delbrück Center for Molecular Medicine, BIMSB, Berlin, Germany<sup>3</sup>, Charité- University Hospital, Science Dept. of Paediatric Oncology/Haematology, Berlin, Germany<sup>4</sup>, Charité- University Hospital, Science Dept. of Paediatric Oncology/Haematology, Berlin, Charité- University Hospital<sup>5</sup>, Harvard University, Dana-Farber Cancer Institute, Boston, USA<sup>6</sup>, Charité Universitätsmedizin, Pediatric Oncology and Hematology, Berlin, Germany<sup>7</sup>, Charité- University Hospital, Science Dept. of Paediatric Oncology/Haematology, Berlin, Germany<sup>8</sup>, Max Delbrück Center for Molecular Medicine, BIMSB, Berlin, Germany<sup>9</sup>

### Background/Introduction:

The cellular composition of primary and metastatic neuroblastoma (NB) tumours and their microenvironment has an essential impact on drug response and survival rate for young patients. Transgenic animal models such as zebrafish expressing human *MYCN*, which have been shown to resemble human NB tumours genetically and histologically, can help to understand the origin and consequence of inter- and intratumoral heterogeneity.

### Aims:

With single-cell RNA sequencing analysis of primary tumours from different transgenic zebrafish NB models, we are untangling the transcriptomic landscape of NB tumours and their microenvironment with the aim to understand inter- and intratumoral heterogeneity and the potentially resulting therapy resistance and disease relapse.

### Methods/Materials:

We sequenced 7k cells on average of four primary tumours from transgenic zebrafish expressing either *MYCN* alone or together with *LMO1* under the control of the dopamine  $\beta$ -hydroxylase (*dbh*) promoter and compared these to scRNA-seq data from two control fish expressing only a fluorescent reporter. The gene-expression pattern for different cell types of the prepared tumour-mass was locally resolved via fluorescent in situ hybridization (FISH) and antibody staining.

### Results:

We found an average of 20 cell subpopulations in each dissected tumour, including diverse cell types of the microenvironment. The tumours displayed heterogeneous degrees of immune infiltration as well as association of various fibroblast subtypes that were not present in control tissue. The gene expression profiles of neuroblastoma cells recapitulate their common sympathoadrenal origin, but also reveal transcriptionally distinct subgroups. As previously described by the group of Rogier Versteeg (Van Groningen et al., Nat. Gen., 2017), we find subpopulations of neuroblastoma cells that express mesenchymal marker genes. The *MYCN/LMO1* double transgenic fish develop tumours at two separate sites that we prepared and sequenced separately. ScRNA-seq data of both tumours from the same individual provides information on the differences in neuroblastoma transcriptional profiles arising from different cells of origin.

### Summary/Conclusions:

Our data provides an in-depth look into intra- and inter-tumour heterogeneity of transgenic neuroblastoma models on a single-cell level. The NB zebrafish with diverse patterns of immune infiltration and tumour cell heterogeneity make an excellent model system for studying the effects of chemotherapeutic treatment and knock-out of distinct cell populations. Moreover, we confirm that neuroblastoma cells show varying degrees of mesenchymal characteristics.



## **P042 Single-cell RNA-seq identifies neuroblastoma cell line subpopulations with enhanced invasive potential**

*Gont, A.*<sup>2</sup>, *Shan, A.*<sup>3</sup>, *Kaplan, D.*<sup>4</sup>, *Irwin, M.*<sup>5</sup>

Hospital for Sick Children, Neuroscience and mental health, Toronto, Canada <sup>2</sup>, University of Toronto, Institute of Medical Science, Toronto, Canada<sup>3</sup>, Hospital for Sick Children, Neuroscience and mental health, Toronto, Canada<sup>4</sup>, Hospital for Sick Children, HAEMATOLOGY/ONCOLOGY, Toronto, Hospital for Sick Children<sup>5</sup>

### Background/Introduction:

The major cause of death in neuroblastoma (NB) is metastatic relapse, and most therapies do not specifically target metastases. We previously developed a metastatic mouse model using intracardiac injection of human SK-N-AS NB cells followed by multiple rounds of *in vivo* selection from bone and brain metastatic sites. Gene expression profiles identified novel genes and pathways dysregulated in metastatic NB cells that when genetically or pharmacologically manipulated affected metastasis.

### Aims:

We now ask whether there is a transcriptionally-distinct subpopulation of cells within the parental population that has enhanced invasiveness and metastatic potential.

### Methods/Materials:

We used 10X Genomics single cell RNA sequencing (scRNA-seq) to transcriptionally profile between 2000 and 2500 cells from the parental cell line and two *in vivo*-selected cell lines that showed enhanced metastatic spread to bone/bone marrow (B5) and brain (BR2).

### Results:

Analysis of the merged dataset revealed that the B2 and BR5 cells were transcriptionally similar to each other occupying the same clusters. The parental cells show two predominant populations: population 1 (~40% of parental cells) resembled the parental gene bulk expression profiles, and population 2 (~60% of the parental cells) resembled the B5 and BR2 gene expression profile that we previously reported drove migration/invasion *in vitro* and/or metastasis *in vivo* including elevated expression of sphingosine kinase 1 (*SPHK1*).

These results suggest that subpopulations of the parental cells with enhanced metastatic gene expression are selected *in vivo* for honing to metastatic sites. To examine this, we identified a cell surface protein, TM4SF1, enriched in the parental populations transcriptionally resembling the B5 and BR2 cells. Using fluorescence activated cell sorting we generated both TM4SF1<sup>+</sup> and TM4SF1<sup>-</sup> lines from parental cells. TM4SF1<sup>+</sup> cells had increased *in vitro* migration and invasion and a gene expression profile enriched in cell motility and locomotion gene sets. Ongoing work involves measuring the *in vivo* metastatic potential of the TM4SF1<sup>+</sup> cell populations and investigating functional roles of TM4SF1 in promoting invasion.

Enrichment analysis of the increased metastatic populations and the TM4SF1<sup>+</sup> parental cells in the scRNA-seq data highlighted gene sets involved with 1) mesenchymal gene signatures, 2) migration and invasion, and 3) extracellular matrix and collagens. Using AUCell to compute gene set overlaps onto scRNA-seq data we showed that the increased metastatic B5, BR2 and TM4SF1<sup>+</sup> parental cells overlap with gene sets generated from the mesenchymal-subgroup of neuroblastoma. This analysis identifies potential gene programs and molecular pathways involved in enhancing invasion.

### Summary/Conclusions:

The use of scRNA-seq to dissect the heterogeneity of NB cells enabled the identification of enhanced metastatic subpopulations in NB and identified genes such as TM4SF1 that mark and/or drive invasive populations. This analysis contributes to our understanding of the molecular mechanisms governing

metastases and aims to identify novel targetable pathways that were not detected by profiling bulk tumor populations.

**P043 Evaluation of chromosomal aberrations and its influence on treatment results in patients with stage 3 neuroblastoma without *MYCN* amplification.**

Wieczorek, A.<sup>2</sup>, Szewczyk, K.<sup>3</sup>, Klekawka, T.<sup>4</sup>, Stefanowicz, J.<sup>5</sup>, Ussowicz, M.<sup>6</sup>, Drabik, G.<sup>7</sup>, Balwierz, W.<sup>8</sup>

Institute of Pediatrics, Jagiellonian University Medical College, Pediatric Oncology and Hematology, Kraków, Poland<sup>2</sup>, Institute of Pediatrics, Jagiellonian University, Medical College, Medical Genetics, Kraków, Poland<sup>3</sup>, Institute of Pediatrics, Jagiellonian University, Medical College, Pediatric Oncology and Hematology, Kraków, Poland<sup>4</sup>, Medical University of Gdansk, Pediatrics, Hematology and Oncology, Gdansk, Medical University of Gdansk<sup>5</sup>, Wrocław Medical University, Pediatric Bone Marrow Transplantation, Oncology, and Hematology, Wrocław, Poland<sup>6</sup>, University Children's Hospital of Krakow, Pathology Department, Kraków, Poland<sup>7</sup>, Institute of Pediatrics, Jagiellonian University, Medical College, Pediatric Oncology and Hematology, Kraków, Poland<sup>8</sup>

**Background/Introduction:**

Treatment strategies in children with localized unresectable neuroblastoma (NBL) are heterogeneous. Except of age and *MYCN* amplification there are no unequivocal risk factors predictive of outcome. Presence of segmental chromosomal aberration may also influence the prognosis. Better defining of risk factors at diagnosis is necessary to optimize the treatment strategies.

**Aims:**

The aim of the study is evaluation of influence of genetic aberrations on treatment results in patient with stage 3 NBL without *MYCN* amplification and its correlation with clinical and laboratory risk factors of treatment failure.

**Methods/Materials:**

Retrospective analysis of 40 stage 3 patients treated in 2000-2017 in 3 centers of Polish Pediatric Solid Tumors Group with complete follow-up data was performed. All children received standard chemotherapy, including 14 treated with radiotherapy as well. All potential known risk factor and laboratory markers and kind of treatment were evaluated. To evaluate genetic profile, in all children in which fresh or paraffin embedded (FFPE) tumor samples were available, comparative genomic hybridization (CGH) and fluorescence in situ hybridization (FISH) for *ALK* mutations were done. The Agilent SurePrint G3 CGH ISCA v2 Microarray Kit 8x60K array platform was used for genome evaluation. The resolution for aCGH evaluation was established on 150 kb. Frozen and FFPE aCGH profiles were matched for 7 patients to confirm and validate results. Two the most common mutations in the *ALK* gene (Phe1174Leu and Arg1275Gln) were performed.

**Results:**

We analyzed 40 children aged 0.2-127.8 months (mean 23.7, median 14.1), including 16 infants, 9 children aged 12-18 months and 15 over 18 months. Overall survival (OS) and progression free survival (PFS) at 5 years were 0.95 and 0.9, respectively. CGH results were available in 29 pts, including 14 NCA and 11 SCA profiles. *ALK* mutation was found only in 1 infant with SCA profile, who responded to standard therapy and is disease free for over 5 years. Complete remission was obtained in 60%, partial response in 32,5%. Three relapses (1 distant and 2 local) and 1 progression were diagnosed, all 3 with known profile had SCA. Two children died, including 1 death of toxicity. PFS was lower in SCA group (5-y 0.78 vs 1.0, p=0.05). Relapses occurred 3, 4 and 6 years after primary diagnosis. Three of 4 patients with therapy failure had no previous radiotherapy. Multivariate analyses showed the age >18 months (p=0.0) and SCA profile (p=0.03) at diagnosis were the risk factors of relapse.

**Summary/Conclusions:**

All observed treatment failures were found in patients over 18 months, and with SCA profile (3/4 known profiles), and mostly (3/4) without radiotherapy. Children with stage 3 NBL without *MYCN*



amplification over 18 months and with SCA profile should also have radiotherapy considered. As relapse may occur late after therapy, patients with SCA >18 months should be strictly followed after treatment.

## **P044 Fluorescence-guided tumor detection in Neuroblastoma using the organoid methodology: Preclinical validation**

Wellens, L.M.<sup>2</sup>, Deken, M.M.<sup>3</sup>, Jeremiasse, B.<sup>4</sup>, Llibre, G.<sup>5</sup>, De la Jara, F.<sup>6</sup>, Johnson, H.R.<sup>7</sup>, Vahrmeijer, A.L.<sup>8</sup>, Dekkers, J.F.<sup>9</sup>, Rios, A.C.<sup>10</sup>, Wijnen, M.H.W.A.<sup>11</sup>

Princess Máxima Center, Surgery, Utrecht, Netherlands<sup>2</sup>, LUMC, Surgery, Leiden, Netherlands<sup>3</sup>, Princess Máxima Center, Surgery, Utrecht, Netherlands<sup>4</sup>, Princess Máxima Center, Imaging, Utrecht, Princess Máxima Center<sup>5</sup>, Princess Máxima Center, Imaging, Utrecht, Netherlands<sup>6</sup>, Princess Máxima Center, Imaging, Utrecht, Netherlands<sup>7</sup>, LUMC, Surgery, Leiden, Netherlands<sup>8</sup>, Princess Máxima Center, Imaging, Utrecht, Netherlands<sup>9</sup>, Princess Máxima Center, Imaging, Utrecht, Netherlands<sup>10</sup>, Princess Máxima Center, Surgery, Utrecht, Netherlands<sup>11</sup>

### Background/Introduction:

Neuroblastoma surgery is considered a big challenge for the pediatric surgeon. Total resection remains the goal of surgery, however it is difficult to distinguish between tumor- and healthy- tissue. Tumor-specific fluorescent imaging agents for different targets are upcoming and recently introduced for clinical use.

### Aims:

To evaluate the use of fluorescence navigation during debulking surgery for neuroblastoma, multiple imaging targets were tested on a high throughput screening platform using the organoid methodology.

### Methods/Materials:

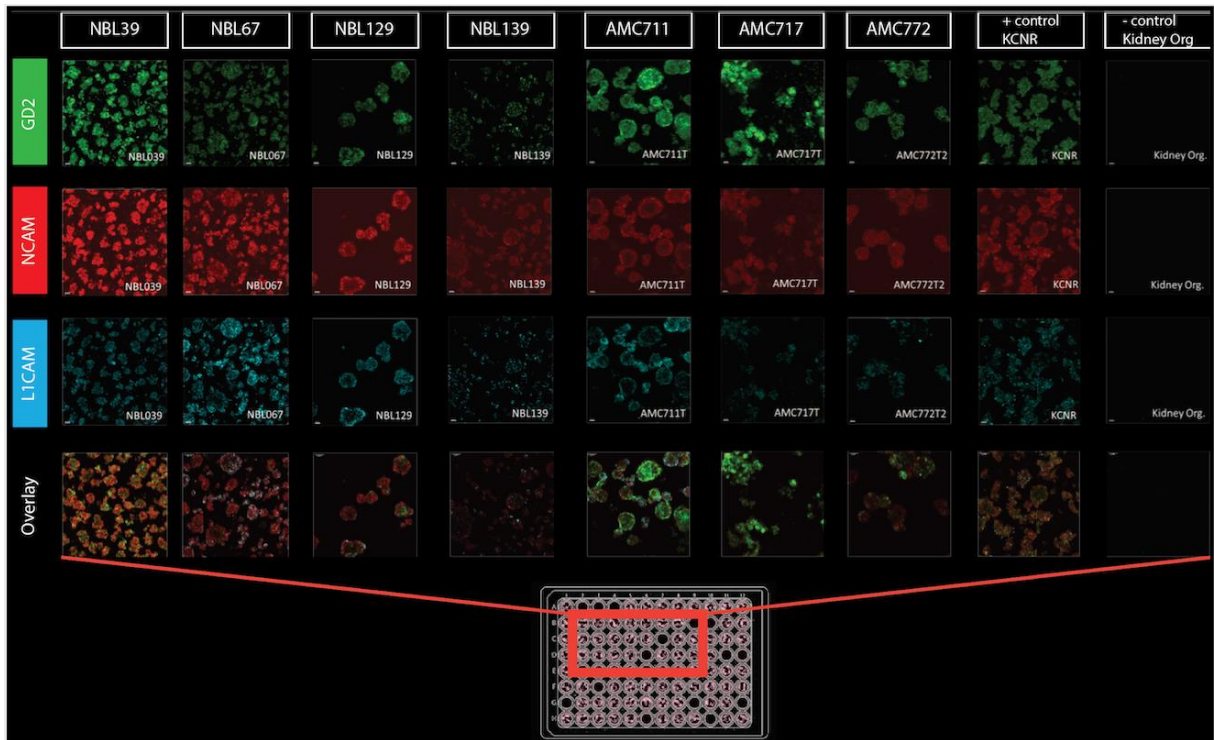
Comparing flowcytometry and 3D multiplex confocal imaging, differential expression between 9 different patient derived neuroblastoma organoid lines were compared. Results were validated *in vivo* in xenografts by conjugating the probes of interest to a near-infra red fluorophore and using a real time intraoperative detecting system. Dose escalation-, blocking-, pharmacokinetic-, and biodistribution studies were performed.

### Results:

The tested targets on our platform for this study were GD2, NCAM and L1CAM. Expression differed per line for FACS and 3D imaging. Target GD2 was validated *in vivo* and imaged between 24 - 128 hours. The optimal dose for fluorescence tumor detection by targeting GD2 was 1nmol with optimal imaging between 72 - 96 hours after administration. This dosage corresponds with a dose of 10mg/m<sup>2</sup> in children.

### Summary/Conclusions:

This study describes for the first time the organoid platform as a screening tool for fluorescence-guided surgery probes and the development and evaluation of anti-GD2-IRDye800CW for the use in a clinical trial.



## **P045 A comparison between 123I-MIBG imaging, trephine biopsy histology, GD2-immunocytology, and RT-qPCR to assess bone marrow involvement in high-risk neuroblastoma**

*Blom, A.J.<sup>2</sup>, Van Zogchel, L.M.J.<sup>3</sup>, Stutterheim, J.<sup>4</sup>, Zappeij-Kannegieter, L.<sup>5</sup>, De Keizer, B.<sup>6</sup>, De Krijger, R.R.<sup>7</sup>, Schumacher-Kuckelkorn, R.<sup>8</sup>, Hero, B.<sup>9</sup>, Van der Schoot, E.<sup>10</sup>, Tytgat, G.A.M.<sup>11</sup>*

Princess Máxima Center for Pediatric Oncology, Research, Utrecht, Netherlands<sup>2</sup>, Princess Máxima Center for Pediatric Oncology, Research, Utrecht, Netherlands<sup>3</sup>, Princess Máxima Center for Pediatric Oncology, Research, Utrecht, Netherlands<sup>4</sup>, Sanquin Research - Department of Experimental Immunohematology, Experimental Immunohematology, Amsterdam, Sanquin Research - Department of Experimental Immunohematology<sup>5</sup>, Princess Máxima Center for Pediatric Oncology, Radiology/Nuclear Medicine, Utrecht, Netherlands<sup>6</sup>, Princess Máxima Center for Pediatric Oncology, Pathology, Utrecht, Netherlands<sup>7</sup>, Children's Hospital University of Cologne, Pediatric Hematology and Oncology, Köln, Germany<sup>8</sup>, Children's Hospital University of Cologne, Pediatric Hematology and Oncology, Köln, Germany<sup>9</sup>, Sanquin Research - Department of Experimental Immunohematology, Experimental Immunohematology, Amsterdam, Netherlands<sup>10</sup>, Princess Máxima Center for Pediatric Oncology, Research, Utrecht, Netherlands<sup>11</sup>

### Background/Introduction:

Bone marrow (BM) is the most common site of dissemination in patients with metastatic neuroblastoma. Currently, histology of BM trephine biopsies, GD2-immunocytology, and 123I-MIBG (meta-iodobenzylguanidine) scintigraphy + SPECT-CT are used to assess BM infiltration. We have previously demonstrated that minimal residual disease detected by RT-qPCR (reverse transcriptase quantitative PCR) using neuroblastoma-specific target genes is a sensitive method to detect BM disease involvement. In this study, we compared the various diagnostic modalities to assess BM involvement.

### Aims:

To perform matched comparisons of RT-qPCR, trephine biopsy histology, GD2-immunocytology, and 123I-MIBG scintigraphy + SPECT-CT assessing BM infiltration in high-risk neuroblastoma patients. Moreover, the additional value of bilateral versus unilateral sampling was investigated.

### Methods/Materials:

After matching bilateral BM aspirates and biopsies to 123I-MIBG imaging (performed within one week), 73 BM assessments from 23 high-risk neuroblastoma patients were identified from a single center (22/23 INRG Stage M >1 year; 1/23 INRG Stage M <1 year with *MYCN* amplification). RT-qPCR was performed as previously described (Stutterheim 2009; van Wezel 2016). GD2-immunocytology was done as previously described using pooled bilateral samples (Swerts 2005). Imaging results from 123I-MIBG scintigraphy and SPECT-CT were quantitatively evaluated using Curie scores by two nuclear medicine physicians. Histology of bone marrow trephine biopsies was assessed by a pathologist using immunohistochemical markers (CD56, chromogranin A, synaptophysin).

### Results:

Of the 73 matched BM assessments, 17 samples were taken at diagnosis, 35 during induction chemotherapy, 10 at the end of induction chemotherapy, and 11 after consolidation therapy. All 17 diagnostic samples were RT-qPCR positive (15/17 bilateral, 2/17 unilateral). BM involvement could be confirmed by trephine biopsy and/or whole-body MIBG imaging in 14/17 cases. In the other 3/17 cases, stage M disease was diagnosed on the basis of soft tissue metastatic disease. In 2/3 cases, GD2-immunocytology was positive.

RT-qPCR was positive for pelvic BM infiltration in 79% compared to 41% for trephine biopsy histology, 43 % for GD2-immunocytology, 40% for MIBG scintigraphy, and 36% for SPECT-CT (Figure 1A). Overall, whole-body MIBG scintigraphy detected bone marrow dissemination in 64%.

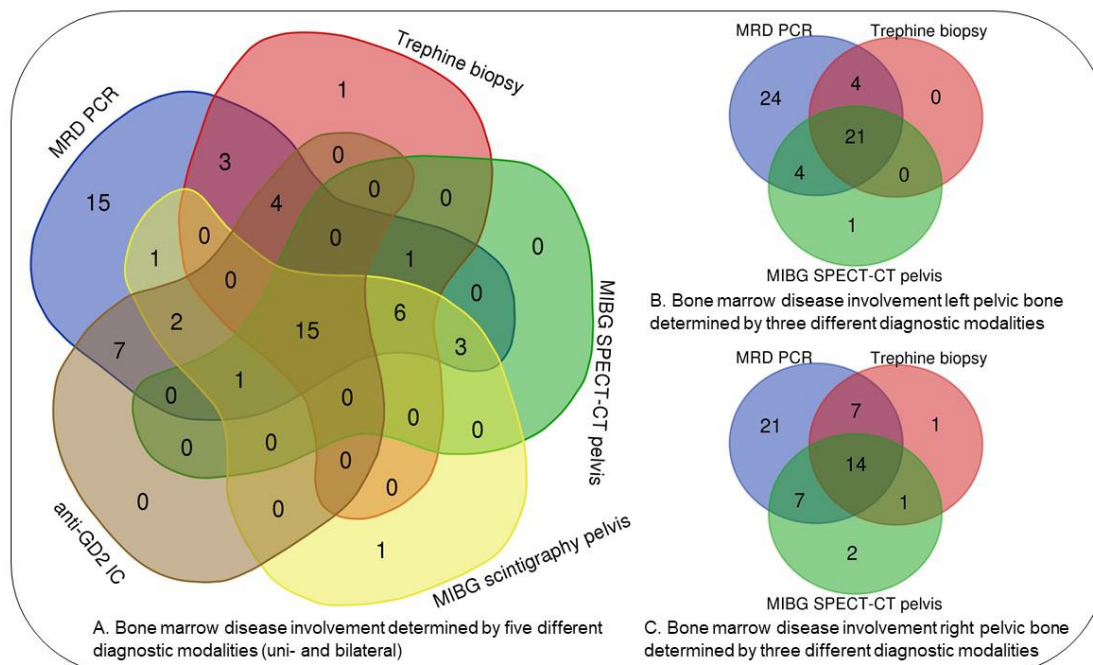
Of the 15/73 bilateral negative RT-qPCR samples, 5/15 showed BM involvement on whole-body MIBG imaging, and one sample had a unilateral positive trephine biopsy. These six samples were taken during and after induction chemotherapy and after consolidation therapy (respectively, n=4; 1; 1).

Eleven of 30 positive trephine-biopsies were unilaterally positive only. In 9/11 samples other diagnostic modalities (RT-qPCR and/or MIBG) identified BM disease dissemination. In 2/11 BM involvement could have been missed if unilateral sampling had prevailed.

Fourteen samples were unilaterally RT-qPCR-positive. In 4/14 samples, the other diagnostic modalities were unable to detect BM involvement, and thus are at the risk of being missed if unilateral samples would have been taken.

#### Summary/Conclusions:

For the detection of BM involvement, application of complementary diagnostic modalities is superior to one single technique. RT-qPCR seems to be the most sensitive technique to detect BM disease involvement. Bilateral pelvic BM sampling identified more BM involvement than unilateral sampling. These results need to be validated in a prospective analysis. The correlation of RT-qPCR positivity with outcome warrants further investigation in a prospective study.



Caption 1: Bone marrow disease involvement determined by different diagnostic modalities

## **P046 Single cell analysis for circulating neuroblastoma cells**

*Hiyama, E.*<sup>2</sup>, *Hiyama, Y.*<sup>3</sup>, *Kurihara, K.*<sup>4</sup>, *Masato, M.*<sup>5</sup>, *Ueda, Y.*<sup>6</sup>, *Sotomaru, Y.*<sup>7</sup>

Hiroshima University, Natural Science Center for Basic Research and Development, Hiroshima, Japan<sup>2</sup>, Hiroshima University, Natural Science Center for Basic Research and Development, Hiroshima, Japan<sup>3</sup>, Hiroshima University, Pediatric Surgery, Hiroshima, Japan<sup>4</sup>, Hiroshima University, Pediatric Surgery, Hiroshima, Hiroshima University<sup>5</sup>, Hiroshima University, Pediatric Surgery, Hiroshima, Japan<sup>6</sup>, Hiroshima University, Natural Science Center for Basic Research and Development, Hiroshima, Japan<sup>7</sup>

### **Background/Introduction:**

Circulating tumor cell (CTC) has improved the study of cancer disease as it represents a noninvasive biopsy that can be used as prognostic and prediction biomarkers as well as for future molecular diagnosis as an accurate selection of molecular targets and effective drugs.

### **Aims:**

As tumor heterogeneity and difference of malignant grade exist in neuroblastoma (NBL), we aimed to evaluate genetic and phenotypic profiles, as well as lipid-metabolomic molecular profile at the single cell level of CTC for better understanding the heterogeneity and progression of NBL.

### **Methods/Materials:**

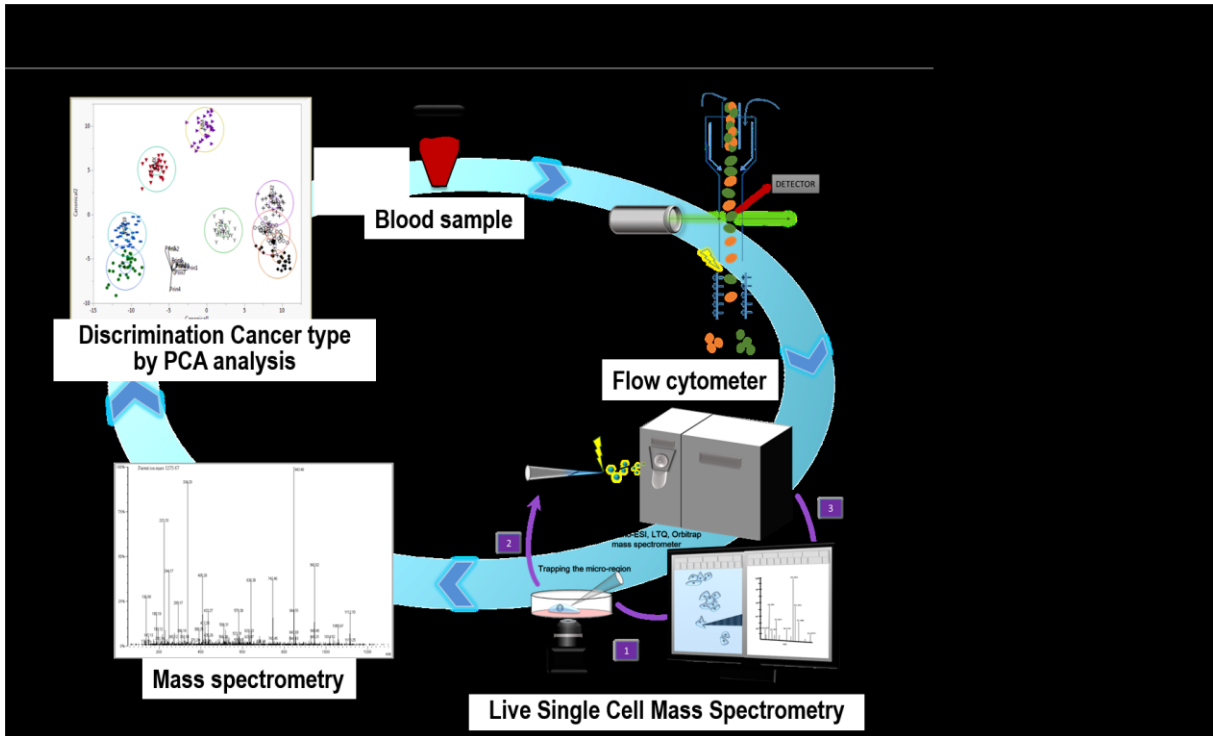
For attempt of CTC analysis in NBL patients, we have isolated single-CTCs and then analyzed genomic aberrations and expressions using next-generation sequencing after whole genome/transcriptome amplifications (WGA/WTA). And we have tried direct trapping of a single cell within a nanospray tip followed by super-sonication after the addition of ionization solvent for metabolomics analysis (Fig. 1 and 2).

### **Results:**

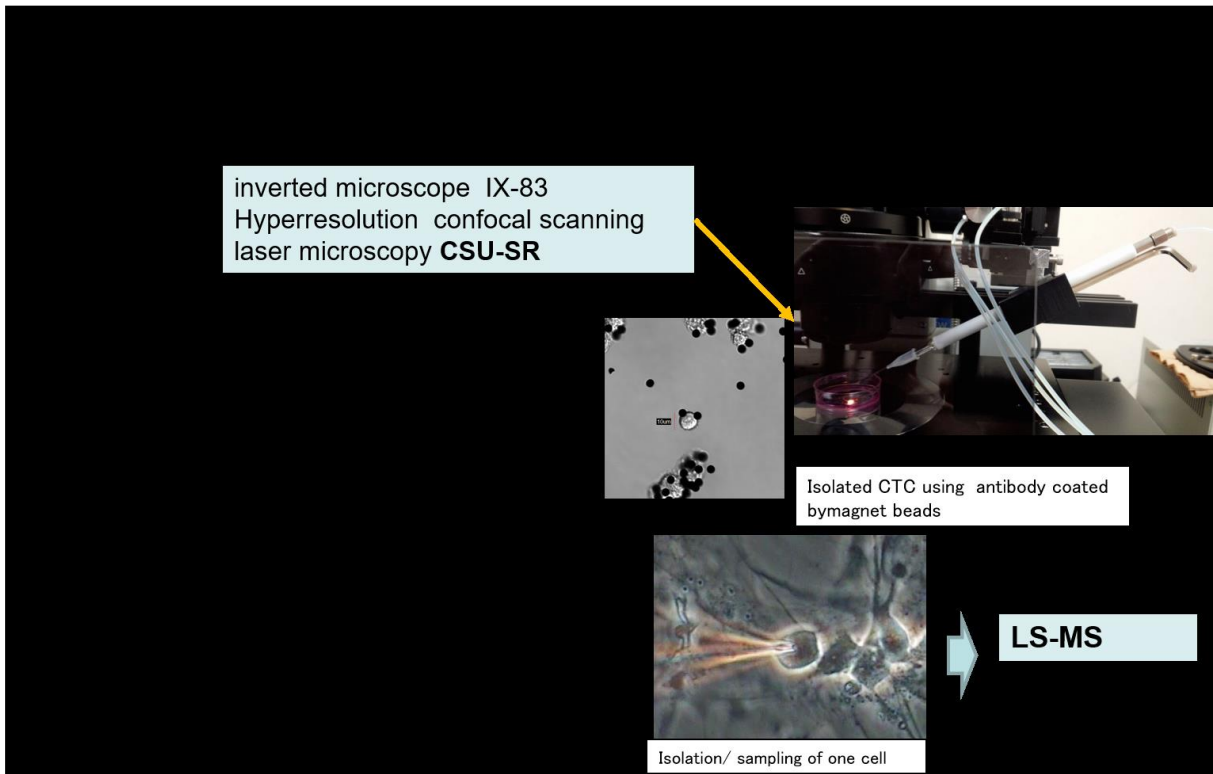
In the NBL-CTCs, the average amount of amplified DNA was 19.0 µg, and the percentage of reads mapped to any targeted region relative to all reads mapped to the reference was 66.5-98.9%. The concordance rates of CTC and tumor mutations were 49-80%. The genomic expression analysis in CTC showed the activation of cancer metastatic, neurogenic, stem cell signaling pathway genes. Metabolomic analysis detected catechol amine metabolites, which are specific to NBL, and drugs included in the patient's course of therapy in addition to vital molecules such as amino acids.

### **Summary/Conclusions:**

This "direct single-cell analysis method" seems to be useful for direct and wide range genomic and molecular marker detection in NBL-CTC, for evaluating heterogeneity of NBL tumor at diagnosis as well as during treatments. This method will be applicable for selection of effective drugs and evaluation of treatment efficacy in future.



Caption 1: Fig. 1. Single CTC analysis system



Caption 2: Fig. 2. Single CTC analysis platform





**P047 Quercetin alleviate cognitive decline in ovariectomised mice by targeting chromatin machinery and relieving transcriptional repression.**

*Aggarwal, A.<sup>2</sup>, Sandhir, R.<sup>3</sup>, Rishi, V.<sup>4</sup>*

National Agri-food Biotechnology Institute, Nutrition, Mohali, India <sup>2</sup>, Department of Biochemistry, Panjab University, Biochemistry, Chandigarh, India<sup>3</sup>, National Agri-food Biotechnology Institute, Nutrition, Mohali, India<sup>4</sup>

**Background/Introduction:**

The synergism between estrogen and histone tail acetylation-mediated memory formation is not clearly understood.

**Aims:**

Here, we attempt to study the altered histone acetylation homeostasis linked changes in cognition following ovariectomy and evaluate the protective effect of quercetin.

**Methods/Materials:**

Changes in spatial memory and learning functions assessed by Morris water maze, Novel object recognition test and Elevated plus maze. Different neuroplasticity markers, histone modifications and chromatin remodelers (HAT and HDAC) were measured through western blotting and real time PCR.

**Results:**

A significant reduction in estradiol levels with subsequent depletion in spatial memory and learning functions were observed in ovariectomised (OVX) mice. Correspondingly, a significant decline in neuroplasticity markers like Brain derived neurotrophic factor (BDNF), Synaptophysin (SYP), Post synaptic density (PSD)-95 were observed in cortex and hippocampus of OVX animals. Notably, histone acetyl-transferase (HAT)/histone deacetylase (HDAC) balance was significantly disrupted in cortex and hippocampus of OVX mice. Lowered extracellular signal regulated kinases (ERK) and cAMP response element binding protein (CREB) activation observed in OVX brain regions might account for diminished HAT activity. Altered HAT/HDAC homeostasis results in lowered histone (H)3 acetylation in OVX brain that may suppress transcriptional activation of neuroplasticity-related genes. Quercetin supplementation to OVX mice for 4 weeks was able to ameliorate cognitive impairment by restoring HAT/HDAC homeostasis through ERK activation and reversing alterations in neuroplasticity markers in cortex and hippocampus of OVX mice.

**Summary/Conclusions:**

Taken together, our results suggest that quercetin alleviate ovariectomy-induced cognitive decline by targeting chromatin assembly.

## **P048 Oncogenic transcription factors are over-represented in mutated active DNA binding sites in neuroblastoma**

*Lasorsa, V.A.<sup>2</sup>, Cimmino, F.<sup>3</sup>, Avitabile, M.<sup>4</sup>, Cantalupo, S.<sup>5</sup>, Montella, A.<sup>6</sup>, De Angelis, B.<sup>7</sup>, Morini, M.<sup>8</sup>, De Torres, C.<sup>9</sup>, Castellano, A.<sup>10</sup>, Locatelli, F.<sup>11</sup>, Iolascon, A.<sup>12</sup>, Capasso, M.<sup>13</sup>*

CEINGE - Biotecnologie Avanzate Scarl, Università degli Studi di Napoli Federico II, Naples, Italy <sup>2</sup>, CEINGE - Biotecnologie Avanzate Scarl, Università degli Studi di Napoli Federico II, Naples, Italy<sup>3</sup>, CEINGE - Biotecnologie Avanzate Scarl, Università degli Studi di Napoli Federico II, Naples, Italy<sup>4</sup>, CEINGE - Biotecnologie Avanzate Scarl, Università degli Studi di Napoli Federico II, Naples, CEINGE - Biotecnologie Avanzate Scarl<sup>5</sup>, CEINGE - Biotecnologie Avanzate Scarl, Università degli Studi di Napoli Federico II, Naples, Italy<sup>6</sup>, IRCCS Ospedale Pediatrico Bambino Gesù, Department of Pediatric Haematology and Oncology, Roma, Italy<sup>7</sup>, IRCCS Istituto Giannina Gaslini, Laboratory of Molecular Biology, Genova, Italy<sup>8</sup>, Hospital Sant Joan de Déu, Department of Oncology, Barcelona, Spain<sup>9</sup>, IRCCS Ospedale Pediatrico Bambino Gesù, Department of Pediatric Haematology and Oncology, Roma, Italy<sup>10</sup>, IRCCS Ospedale Pediatrico Bambino Gesù, Department of Pediatric Haematology and Oncology, Roma, Italy<sup>11</sup>, CEINGE - Biotecnologie Avanzate Scarl, Università degli Studi di Napoli Federico II, Naples, Italy, <sup>12</sup>, CEINGE - Biotecnologie Avanzate Scarl, Università degli Studi di Napoli Federico II, Naples, Italy <sup>13</sup>

### Background/Introduction:

The contribution of coding mutations to oncogenesis has been largely clarified whereas little is known about somatic mutations in noncoding DNA and their role in driving tumors remains controversial.

### Aims:

Here, we used an alternative approach to interpret the functional significance of noncoding somatic mutations in promoting tumorigenesis of neuroblastoma (NB).

### Methods/Materials:

Noncoding somatic mutations obtained from whole genome sequencing of 151 NBs were integrated with ENCODE v3 data of DNase Hypersensitive Sites and Transcription Factors Binding Sites (TFBSs). We located somatic mutations in regulatory elements specifically active in NB cells (tsa-TFBS), non-specifically active in NB cells (ntsa-TFBS), and non-active (na-TFBS). Within these types of DNA elements, enrichment analysis was used to identify transcription factors (TFs) whose binding sites harboured more or less mutations than the expected by using the ENCODE data as reference. A gene expression signature including the TFs enriched in mutated tsa-TFBS was built using RNAseq data from 498 NB tumors. We then integrated DNA and RNA sequencing data and assessed the effects of mutations in tsa-TFBS on mRNA levels.

### Results:

The number of somatic mutations in tsa-TFBSs was significantly lower as compared to ntsa-TFBS and na-TFBS ( $P < 0.05$ ) and altered, on average, more TFBSs compared to the others ( $P < 0.0001$ ). Surprisingly, even if the number of somatic mutations was lower in tsa-TFBS, their median of pathogenic scores, estimated by CADD and FunSeq2, was higher than those in ntsa-TFBS and na-TFBS ( $P < 0.0001$ ).

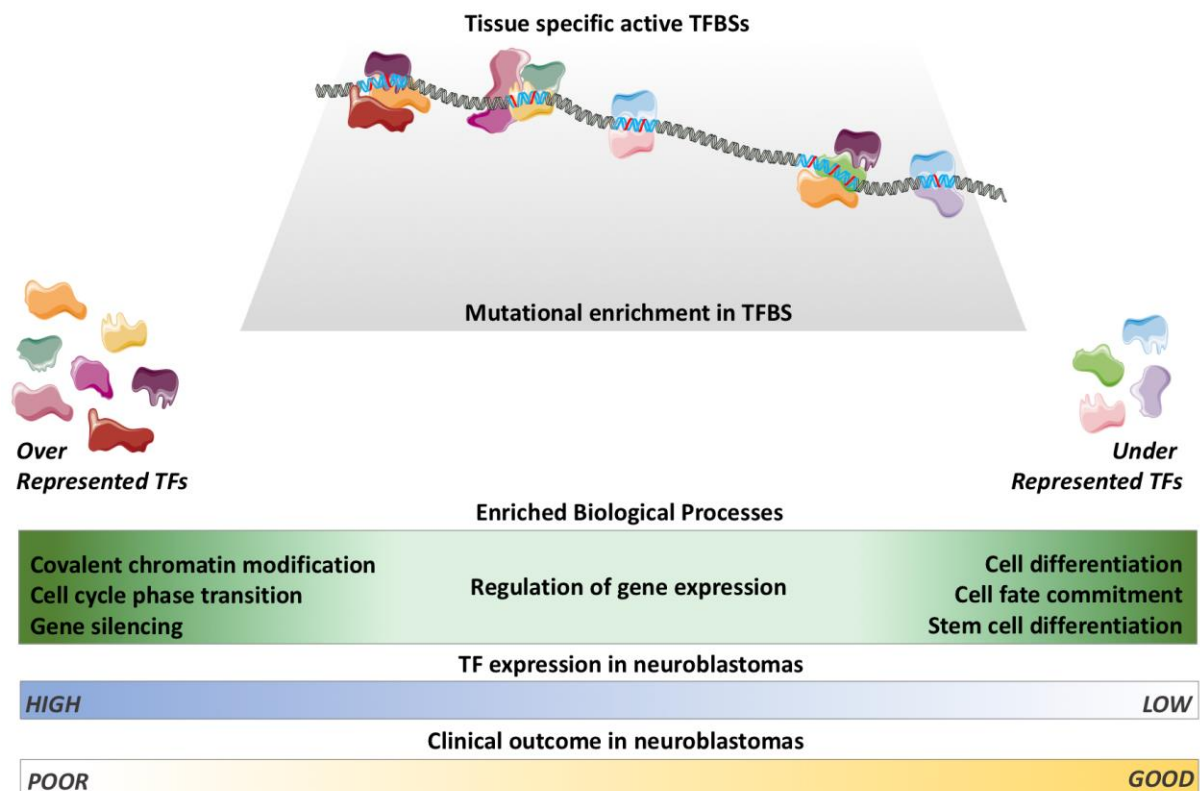
Within the mutated tsa-TFBSs, we found 18 over-represented TFs involved mainly in cell cycle phase transitions, and 15 under-represented TFs primarily regulating cell differentiation.

Among tumors positive for the over-represented TFs-based gene signature, a significantly higher frequency of *MYCN* amplified ( $P = 2.4 \times 10^{-29}$ ), INSS stage 4 ( $P = 1.27 \times 10^{29}$ ) and high-risk tumors ( $P = 1.5 \times 10^{-51}$ ) was observed. This signature was also able to predicted overall survival and event-free survival ( $P = 2.0 \times 10^{-16}$ ).

Moreover, we searched for recurrent mutations in tsa-TFBSs of over-represented TFs. We found a cluster of mutations altering EZH2 binding site and that significantly reduced *MCF2L* ( $P=3.97 \times 10^{-18}$ ) and *ADPRHL1* ( $P=3.35 \times 10^{-03}$ ) expression. Interestingly, the low expression of these genes correlated with unfavourable NB prognostic markers ( $P<0.001$ ) and poor survival ( $P<1.00 \times 10^{-07}$ ). A second cluster of mutations, nearby *CTTNBP2*, altered the binding sites of 6 over-represented TFs (RBBP5, EZH2, SIN3A, POLR2A, TAF1, E2F1). Low mRNA levels of *CTTNBP2* were found in the mutated samples ( $P=8.86 \times 10^{-07}$ ) and correlated with poor prognosis ( $P<0.05$ ). Similarly, a third cluster of mutations, within POLR2A binding site, reduced the expression of *LSP1*, involved in ERK/MAPK pathway ( $P=2.48 \times 10^{-03}$ ), whose low expression correlated with unfavourable NB prognostic markers ( $P<0.05$ ) and poor survival ( $P<0.01$ ).

**Summary/Conclusions:**

We propose a novel approach to study the involvement of regulatory mutations in NB that could be extended to other cancers and provide further evidence that alterations of gene expression may have relevant effects in NB development.



## **P049 Therapeutic targeting of ubiquitin-specific protease 5 in *MYCN*-driven neuroblastoma**

*Kleynhans, A.*<sup>2</sup>, *Mittra, R.*<sup>3</sup>, *Nagy, Z.*<sup>4</sup>, *Kim, P.*<sup>5</sup>, *Ciampa, O.*<sup>6</sup>, *Zhu, S.*<sup>7</sup>, *Carter, D.R.*<sup>8</sup>, *Cheung, B.B.*<sup>9</sup>, *Marshall, G.M.*<sup>10</sup>

Children Cancer Institute, Embryonal Cancer Therapy and Prevention, Sydney, Australia<sup>2</sup>, Children Cancer Institute, Embryonal Cancer Therapy and Prevention, Sydney, Australia<sup>3</sup>, Children Cancer Institute, Embryonal Cancer Therapy and Prevention, Sydney, Australia<sup>4</sup>, Children Cancer Institute, Embryonal Cancer Therapy and Prevention, Sydney, Children Cancer Institute<sup>5</sup>, Children Cancer Institute, Embryonal Cancer Therapy and Prevention, Sydney, Australia<sup>6</sup>, Department of Biochemistry and Molecular Biology, Mayo Clinic, Department of Biochemistry and Molecular Biology, Minnesota, USA<sup>7</sup>, Children Cancer Institute, Embryonal Cancer Therapy and Prevention, Sydney, Australia<sup>8</sup>, Children Cancer Institute, Embryonal Cancer Therapy and Prevention, Sydney, Australia<sup>9</sup>, Children Cancer Institute, Embryonal Cancer Therapy and Prevention, Sydney, Australia<sup>10</sup>

### Background/Introduction:

Histone deacetylase (HDAC) inhibitors are effective in preclinical models of the childhood cancer, neuroblastoma. HDAC inhibitor, suberanoyl hydroxamic acid (SAHA), has shown clinical success in the treatment of cutaneous T-cell lymphoma but lacks efficacy as a single agent in many solid tumours, including neuroblastoma.

### Aims:

To identify new compounds that potentiate SAHA activity and characterize their molecular mechanism.

To determine the oncogenic role of USP5 in neuroblastoma.

### Methods/Materials:

We first performed a cell-based, high-throughput drug screen using a MYC-expressing breast cancer cell line, treated with 1 µM of SAHA to identify novel compounds which act synergistically with SAHA. For mechanistic studies, we performed cell viability, colony formation assay, co-immunoprecipitation, ubiquitin assay, and chromatin immunoprecipitation (ChIP) assays. For the *in vivo* studies, we used neuroblastoma-bearing transgenic TH-*MYCN* homozygous mice and *MYCN*;GFP zebrafish.

### Results:

Previously we have shown that a small enhancer molecule, SE486-11, was able to synergistically enhance the cytopathic effects of SAHA in neuroblastoma cells. Treatment with the combination led to a decrease in cell viability, an increase in *MYCN* ubiquitination and completely inhibited tumour growth in TH-*MYCN* homozygous mice. A SILAC (Stable Isotope Labelling by Amino Acids in Cell Culture) analysis of protein from neuroblastoma cells treated with combination therapy, revealed that Ubiquitin-Specific Protease 5 (USP5) expression was significantly reduced by the combination.

More recently, *MYCN*;EGFP transgenic fish, with established neuroblastomas were treated with the combination which markedly inhibited neuroblastoma tumorigenesis in the zebrafish. A major role of USP5 is regulation of the relative cellular levels of unanchored mono- and polyubiquitins. We found that the combination treatment caused an increase in Lys48-linked polyubiquitination and that overexpression of USP5 reduced polyubiquitin levels following combination treatment. An online data base revealed that high USP5 expression in primary human neuroblastoma samples predicted poor prognosis in *MYCN* amplified patients. This implicates USP5 as a potential oncogenic driver in neuroblastoma. To determine the oncogenic role of USP5 in *MYCN*-driven neuroblastoma, USP5 was knock-downed by specific siRNAs in *MYCN*-amplified neuroblastoma cell lines, causing a significant decrease in *MYCN* protein expression and its half-life. Chromatin immunoprecipitation assays demonstrated an enrichment of *MYCN* protein near the USP5 transcription start site. Doxycycline-induced repression of *MYCN* expression in SHEP-tet21N correlated with decreased USP5 protein and mRNA levels. Co-immunoprecipitation assays showed that USP5 and *MYCN* formed a protein

complex. Genetic suppression of USP5 in *MYCN*-amplified neuroblastoma cell lines led to a significant reduction in cell viability, cell proliferation and colony formation.

Summary/Conclusions:

Taken together, our findings suggest that USP5 and *MYCN* participate in a forward feedback expression regulatory loop and that inhibition of USP5 function in neuroblastoma cells using small molecule inhibitors could be a novel therapeutic approach for *MYCN*-amplified tumours.

## **P050 An oncogenic role for the splicing associated protein, SNRPD3 in MYCN-driven neuroblastoma**

*Salib, A.*<sup>2</sup>, *Nagy, Z.*<sup>3</sup>, *Jayatilke, N.*<sup>4</sup>, *Mayoh, C.*<sup>5</sup>, *Carter, D.R.*<sup>6</sup>, *Cheung, B.B.*<sup>7</sup>, *Marshall, G.M.*<sup>8</sup>

Childrens cancer institute, Embryonal Cancer Therapy and Prevention, Randwick, Australia <sup>2</sup>,  
Childrens cancer institute, Embryonal Cancer Therapy and Prevention, Randwick, Australia<sup>3</sup>,  
Childrens cancer institute, Bioinformatics, Randwick, Australia<sup>4</sup>, Childrens cancer institute,  
Bioinformatics/computational biology, Randwick, Childrens cancer institute<sup>5</sup>, Childrens cancer institute,  
Embryonal Cancer Therapy and Prevention, Randwick, Australia<sup>6</sup>, Childrens cancer institute,  
Embryonal Cancer Therapy and Prevention, Randwick, Australia<sup>7</sup>, Childrens cancer institute,  
Embryonal Cancer Therapy and Prevention, Randwick, Australia<sup>8</sup>

### Background/Introduction:

Neuroblastoma is a rare solid tumour in early childhood with less than 50% survival rate for high-risk patients. Amplification of the *MYCN* oncogene remains the single most important genetic predictor of poor prognosis. Many of the pro-tumorigenic functions of *MYCN* are attributed to its ability to regulate global gene expression programs. Alternative splicing is another important component of gene expression regulation. Alternative splicing allows for diverse transcriptional variants that can lead to diverse cellular phenotypes and characterising a wide variety of biological functions. Dysregulation of alternative splicing has been found to effect pathways that make up the hallmarks of cancer. We hypothesise that dysregulation of alternative splicing may be associated with an aggressive *MYCN*-driven neuroblastoma phenotype.

### Aims:

In this study, we aim to identify the molecular mechanism by which *MYCN* regulates alternative splicing programs in neuroblastoma.

### Methods/Materials:

To address the above hypothesis and aims siRNA knockdown of SNRPD3 in BE2C, Kelly and SHEP.tet21N cells were performed to assess cell viability, proliferation and colony formation. Protein and mRNA levels were measured using western blotting and RT-qPCR. RNA-sequencing was performed to assess differentially spliced genes using the leafcutter program.

### Results:

Microarray analysis on ganglia and tumour tissue collected during tumour initiation in transgenic TH-*MYCN*<sup>+/+</sup> and wildtype mice revealed a significant increase in the mRNA expression of SNRPD3, a core component of the spliceosome. Analysis of a 649 neuroblastoma patient cohort (Kocak dataset) revealed high SNRPD3 expression is correlated with poor prognosis. Using co-immunoprecipitation experiments, we identified SNRPD3 as a novel *MYCN* binding protein. siRNA knockdown of SNRPD3 reduced cell viability, proliferation and colony number in *MYCN*-amplified neuroblastoma cell lines. Further investigation using the *MYCN*-inducible, SHEP.tet21N cells, showed that SNRPD3 knockdown exhibited a more pronounced lethality in the presence of *MYCN*.

RNA-sequencing was performed in SHEP.tet21N cells with suppression of SNRPD3 by siRNAs to identify aberrantly spliced genes and signalling pathways involved in the regulation of alternative splicing in neuroblastoma. Our analysis revealed that there is an overall increase in the number of genes being spliced when *MYCN* is expressed. Interestingly, SNRPD3 knockdown in the presence of *MYCN* resulted in a further 25-fold increase in the number of genes being differentially spliced. This pronounced effect on splicing was *MYCN*-specific, with SNRPD3 knockdown alone showing little effect on differential splicing. Gene ontology and KEGG pathway analysis of *MYCN*+ve/SNRPD3-ve dependent splicing identified enrichment in cell cycle pathways.

### Summary/Conclusions:

Based on these results, we conclude that *MYCN*-driven transcriptional up-regulation and physical interaction with SNRPD3 contributes to changes in alternative splicing that perpetuate *MYCN*-driven oncogenesis. Disruption of SNRPD3 in the presence of *MYCN* was sufficient to induce widespread mis-splicing. That impacts cell cycle machinery and results in cell cycle inhibition/cell death. The selective relationship exhibited between *MYCN* and SNRPD3 indicates that SNRPD3 could serve as a novel therapeutic target in *MYCN*-amplified neuroblastoma.

## P051 MicroRNA-based therapeutics for the treatment of high-risk neuroblastoma

*Holliday, H.*<sup>2</sup>, *Dodson, E.*<sup>3</sup>, *Nikolic, I.*<sup>4</sup>, *Deng, N.*<sup>5</sup>, *Simpson, K.*<sup>6</sup>, *Kamili, A.*<sup>7</sup>, *Wheatley, M.*<sup>8</sup>, *McCarroll, J.*<sup>9</sup>, *Mestdagh, P.*<sup>10</sup>, *Marshall, G.*<sup>11</sup>, *Fletcher, J.*<sup>12</sup>, *Swarbrick, A.*<sup>13</sup>

Garvan Institute of Medical Research, Cancer, Sydney, Australia <sup>2</sup>, Garvan Institute of Medical Research, Cancer, Sydney, Australia<sup>3</sup>, Peter MacCallum Cancer Centre, Victorian Centre for Functional Genomics, Melbourne, Australia<sup>4</sup>, Garvan Institute of Medical Research, Cancer, Sydney, Garvan Institute of Medical Research<sup>5</sup>, Peter MacCallum Cancer Centre, Victorian Centre for Functional Genomics, Melbourne, Australia<sup>6</sup>, Children's Cancer Institute, Molecular Targets and Cancer Therapeutics, Sydney, Australia<sup>7</sup>, Children's Cancer Institute, Molecular Targets and Cancer Therapeutics, Sydney, Australia<sup>8</sup>, Children's Cancer Institute, Translational Cancer Nanomedicine, Sydney, Australia<sup>9</sup>, Ghent University, Center for Medical Genetics, Ghent, Belgium<sup>10</sup>, Children's Cancer Institute, Gene Regulation in Cancer, Sydney, Australia<sup>11</sup>, Children's Cancer Institute, Molecular Targets and Cancer Therapeutics, Sydney, Australia, <sup>12</sup>, Garvan Institute of Medical Research, Cancer, Sydney, Australia <sup>13</sup>

### Background/Introduction:

Neuroblastoma is a deadly childhood cancer arising in the developing sympathetic nervous system. High-risk patients are currently treated with intensive multi-layered chemotherapy which is not always curative and leaves surviving patients with life-long side effects, underscoring an urgent need for safer treatments. miRNA-based drugs represent an exciting new class of cancer therapeutic. However, our understanding of miRNA function in neuroblastoma is fragmented, restricting our ability to capitalise on their therapeutic potential.

### Aims:

We aim to screen and validate miRNAs that sensitise neuroblastoma cells to chemotherapy *in vitro* and test candidate therapeutic miRNAs in pre-clinical models of high-risk neuroblastoma.

### Methods/Materials:

An extensive functional genomic screen of >1200 miRNA mimics was conducted in neuroblastoma and normal-like cell lines to discover miRNAs with therapeutic promise. This was performed in combination with low doses (IC30) of doxorubicin and vincristine chemotherapies. Selected candidate miRNAs are currently being tested in the pre-clinical COG-N-519 patient-derived xenograft (PDX) model, originating from a patient with high-risk disease after several lines of treatment. Star-POEGMA nanoparticles are used to deliver candidate miRNAs either as a single agent, or in combination with topotecan chemotherapy. Ongoing work includes mechanistic experiments to assess miRNA uptake and their impact on gene expression, and therapeutic experiments to monitor tumour growth and survival.

### Results:

Three miRNAs (miR-99b-5p, miR-380-3p and miR-485-3p) had potent synthetic lethal interaction with doxorubicin *in vitro* specifically in high-risk cell lines (Kelly and SK-N-DZ), but not low-risk neuroblastoma (SHEP) or normal (IMR90 and MCF-10A) cell lines. Analysis of a clinical cohort revealed that these miRNAs are possible tumour suppressors as they undergo recurrent copy number loss, and low expression predicts poor outcome.

Encouragingly, miR-99b-5p could be effectively delivered (40-fold overexpression) to PDX tumours. RNA-Seq analysis revealed downregulation of key neuroblastoma dependency genes *MYCN*, *PHOX2B*, *HAND1/2*, *GATA3*, *ISL1* and *EZH2* in miR-99b-5p-treated tumours. Importantly, many of these genes encode core regulatory lineage-defining transcription factors in neuroblastoma (1,2), and are considered to be 'undruggable'. Additionally, miRNA treatment resulted in upregulation of neuronal differentiation genes, suggesting that miR-99b-5p can either differentiate the tumour cells, or



selectively kill undifferentiated tumour cells. Future single cell RNA-Seq experiments will be used to address these possibilities.

#### Summary/Conclusions:

We have identified novel chemosensitising miRNAs specific to high-risk neuroblastoma that can be administered *in vivo* to drive favourable gene expression changes. If effective in longer term preclinical studies, we predict that restoring the function of tumour suppressive miRNAs represents an attractive new therapeutic strategy for the treatment of neuroblastoma patients.

#### References

1. Van Groningen et al. Nat Genet. 2017

## **P052 COX2 expression is increased in relapsed Neuroblastoma samples with 11q deletion and non-amplified MYCN**

Gradowski Nascimento, T.<sup>2</sup>, França Junior, N.<sup>3</sup>, Herai, R.<sup>4</sup>, Noronha, L.<sup>5</sup>, Cavalli, L.R.<sup>6</sup>, Esposito, S.E.<sup>7</sup>

Pontifícia Universidade Católica do Paraná, Programa de Pós-Graduação em Ciências da Saúde, Curitiba, Brazil <sup>2</sup>, Pontifícia Universidade Católica do Paraná, Programa de Pós-Graduação em Ciências da Saúde, Curitiba, Brazil<sup>3</sup>, Pontifícia Universidade Católica do Paraná, Programa de Pós-Graduação em Ciências da Saúde, Curitiba, Brazil<sup>4</sup>, Pontifícia Universidade Católica do Paraná, Laboratório de Patologia, Curitiba, Pontifícia Universidade Católica do Paraná<sup>5</sup>, Instituto de Pesquisa Pelé Pequeno Príncipe, Laboratório de genoma, Curitiba, Brazil<sup>6</sup>, Pontifícia Universidade Católica do Paraná, Programa de Pós-Graduação em Ciências da Saúde, Curitiba, Brazil<sup>7</sup>,

### Background/Introduction:

Despite still challenging, important prognostic genetic markers have been associated with the low survival rates in high-risk neuroblastoma. Additionally to the genetic background, the tumor microenvironment inflammatory state plays a role in the modulation of tumor progression. The presence of pro-inflammatory markers was related to the metastatic phenotypes, and more recently, it was suggested that losses in the 11q chromosome could define a subset of therapy-resistant neuroblastoma. At cellular and molecular level, this subset was characterized to be inflammatory driven and with high activity of the COX/mPGES-1/PGE2 pathway.

### Aims:

This study aimed to evaluate the expression of COX-2 in a set of tumor samples collected in diagnosis and post chemotherapeutic treatment.

### Methods/Materials:

Neuroblastic specimens were obtained retrospectively for the years 2001-2014 from the archives of the Division of Anatomical Pathology, Pequeno Príncipe Pediatric Hospital (Brazil) as formalin-fixed paraffin-embedded (FFPE) blocks. A subgroup of 82 specimens were collected at diagnosis prior to any cancer treatment (Pre-CT) and 20 were collected after chemotherapy (Post-CT), out of which 11 cases were paired with pre and post-CT samples. Immunohistochemical expression of COX-2 was evaluated through quantitative analyses of cytoplasmic staining. Copy number alterations (CNA) were analyzed by array-CGH in paired samples of patients (Pre-CT and post-CT). *MYCN* oncogene content was assayed by fluorescence in situ hybridization (FISH). The genes from 11q23 deletion chromosomal locus were extracted using RefSeq gene model as provided by the a-CGH analysis. Next, the genes were subjected to protein-protein interaction analysis using String database.

### Results

COX-2 expression was positive in all NB tumorigenic subgroups. Spearman multivariate analysis showed an anti-correlation between COX-2 expression and *MYCN* amplification ( $\rho = -0.693$ ,  $p < 0.05$ ). A significant increase in COX-2 expression was verified in post-CT samples when compared with pre-CT unpaired samples ( $n = 77$ ,  $p < 0.05$ ) in non-amplified *MYCN*. A-CGH results showed no significant differences in the median of copy number alterations in Pre-CT ( $15.36 \pm 3.2$ ) and Post-CT ( $12.18 \pm 2.4$ ) samples. COX-2 expression in pre-CT samples correlates with 11q23 loss ( $\rho = 0.631$ ,  $p = 0.045$ ), 7q gain ( $\rho = 0.853$ ;  $p = 0.002$ ), and 7p gain ( $\rho = 0.798$ ;  $p = 0.006$ ). Other significant correlations were found for 11q loss with 1q gain ( $\rho = 0.694$ ;  $p = 0.038$ ), 7p gain ( $\rho = 0.908$ ;  $p < 0.001$ ), 7q gain ( $\rho = 0.771$ ;  $p = 0.005$ ), and 9q gain ( $\rho = 0.654$ ;  $p = 0.029$ ). Analysis of the genes lost in the 11q23 chromosomal locus demonstrated a strong and positive involvement of MMPs-related genes that interact or are involved in the collagen formation, including MMP7, MMP10, MMP13, MMP1 and MMP3.

### Summary/Conclusions:

COX-2 expression is increased in post-treated samples in NB tumors without *MYCN* amplification. It also correlates with chromosome 11q deletions, with strong and positive involvement of MMPs-related genes involved in the extracellular matrix remodeling.

## **P053 Genome methylation defines EZH2 inhibitor sensitivity in neuroblastoma**

*Endo, Y.*<sup>2</sup>, *Kamijo, T.*<sup>3</sup>, *Endo, Y.*<sup>4</sup>, *Takenobu, H.*<sup>5</sup>, *Shinno, Y.*<sup>6</sup>, *Okada, R.*<sup>7</sup>, *Satoh, S.*<sup>8</sup>, *Mukae, K.*<sup>9</sup>, *Sugino, R.P.*<sup>10</sup>, *Haruta, M.*<sup>11</sup>, *Hattori, N.*<sup>12</sup>, *Nakazawa, A.*<sup>13</sup>, *Ohira, M.*<sup>14</sup>, *Ushijima, T.*<sup>15</sup>, *Nio, M.*<sup>16</sup>

Saitama Cancer Center, Research Institute for Clinical Oncology, Ina, Saitama, Japan <sup>2</sup>, Saitama Cancer Center, Research Institute for Clinical Oncology, Ina, Saitama, Japan<sup>3</sup>, Saitama Cancer Center, Research Institute for Clinical Oncology, Ina, Saitama, Japan<sup>4</sup>, Saitama Cancer Center, Research Institute for Clinical Oncology, Ina, Saitama, Saitama Cancer Center<sup>5</sup>, Saitama Cancer Center, Research Institute for Clinical Oncology, Ina, Saitama, Japan<sup>6</sup>, Saitama Cancer Center, Research Institute for Clinical Oncology, Ina, Saitama, Japan<sup>7</sup>, Saitama Cancer Center, Research Institute for Clinical Oncology, Ina, Saitama, Japan<sup>8</sup>, Saitama Cancer Center, Research Institute for Clinical Oncology, Ina, Saitama, Japan<sup>9</sup>, Saitama Cancer Center, Research Institute for Clinical Oncology, Ina, Saitama, Japan<sup>10</sup>, Saitama Cancer Center, Research Institute for Clinical Oncology, Ina, Saitama, Japan<sup>11</sup>, National Cancer Center Research Institute, Department of Epigenetics, Division of Epigenomics, Tokyo, Japan, <sup>12</sup>, Saitama Children's Medical Center, Department of Clinical Research/Pathology, Saitama, Japan <sup>13</sup>, Saitama Cancer Center, Research Institute for Clinical Oncology, Ina, Saitama, Japan <sup>14</sup>, National Cancer Center Research Institute, Department of Epigenetics, Division of Epigenomics, Tokyo, Japan <sup>15</sup>, Tohoku University School of Medicine, Department of Pediatric Surgery,, Sendai, Japan <sup>16</sup>

### Background/Introduction:

Polycomb repressive complex 2 (PRC2) is a multiprotein chromatin-modifying complex that is essential for vertebrate development and differentiation. PRC2 is composed of a trimeric core of SUZ12, EED, and EZH1/2, which catalyze the methylation of histone H3 at lysine 27 (H3K27me1/2/3). EZH2 is a H3K27 methylase and a target of cancer epigenetic treatments. We recently reported the oncogenic roles of EZH2 in NB and NTRK1 (TRKA) epigenetic regulation by EZH2 in the *MYCN*-amplified aggressive neuroblastomas (NB) (Li et al., *ONCOGENE*, 2018). Several EZH2 inhibitors (EZH2is) have been developing and the effects of them on NB cells have studied. These studies indicated that the combination of multiple drugs with EZH2 inhibitors will be required for curative NB therapy (Chen L et al., *J Clin Invest.* 2018; Henrich et al., *Cancer Res* 2018; Li et al., *ONCOGENE*, 2018).

### Aims:

To improve EZH2i treatments for curative NB therapy, we investigated EZH2i-sensitivity biomarkers by using aggressive NB model cell lines.

### Methods/Materials:

We performed transcriptome and methylome analysis by using EZH2i-sensitive and -resistant NB cells. A microarray analysis was performed using the Agilent platform of 8 x; 60 K design ID G4851B (Agilent Technologies, USA). A DNA methylation analysis of clinical data was performed using the Infinium HumanMethylation450 BeadChip Kit (Illumina, USA). The Combination Index (CI) was calculated by the Chou-Talalay (2010) method using CompuSyn (<http://www.combosyn.com/>).

### Results:

Here, we investigated the effects and function of small molecule EZH2i on aggressive NB model cell lines. We examined the antitumor effect of EZH2i using WST assay and colony formation assay. By EZH2i EPZ-6438, suppression was observed in SK-N-SH and SK-N-BE cells (sensitive cells) dose-dependently, whereas it was not observed in IMR32 and NGP cells (resistant cells). FACS analysis showed apoptosis and G0/G1 cell cycle arrest in sensitive cells. Transcriptome analysis and GSEA indicated significant changes were observed in the gene set related to cell cycle arrest and differentiation in the sensitive cells. We selected genes induced at mRNA level by EPZ-6438 only in the sensitive cells and confirmed tumor suppressor function in NB cells. Almost of the EPZ-6438-induced gene promoters were marked by H3K27me3 in *MYCN*-amplified NB cell lines. Interestingly, a

part of the EPZ-6438-induced gene promoters have CpG islands methylated in NB tumor samples (Henrich et al., Cancer Res 2018; Li et al., ONCOGENE, 2018). Further, combination of EPZ-6438 and 5-aza-deoxycytidine, suppressed proliferation in the EPZ-6438-resistant 3 NB cell lines effectively. These inhibitors suppressed the resistant NB cell proliferation synergistically as CI was less than 1.0. Transcriptome/methylome analysis of the EPZ-6438 and 5-aza-deoxycytidine-treated IMR32 cells revealed the combinational epigenetic regulation of the tumor suppressors and oncogene expression.

Summary/Conclusions:

Finally, methylome analysis of the promoters can be a biomarker of EPZ-6438-included epigenomic treatments for unfavorable NB patients. We will present the responsible CpG island methylation as biomarkers for the application of EZH2i/DNMTi combination therapy.

## **P054 Polycomb EZH1 regulates cell cycle/drug sensitivity of neuroblastoma cells in concert with MYCN**

*Kamijo, T.,<sup>1</sup> Shinno, S.<sup>2</sup>, Shinno, S.<sup>3</sup>, Takenobu, T.<sup>4</sup>, Endo, E.<sup>5</sup>, Okada, O.<sup>6</sup>, Sugino, R.P.<sup>7</sup>, Satoh, S.<sup>8</sup>, Mukae, M.<sup>9</sup>, Wada, W.<sup>10</sup>, Akter, A.<sup>11</sup>, Haruta, H.<sup>12</sup>, Nakazawa, N.<sup>13</sup>, Yoshida, Y.<sup>14</sup>, Ohira, O.<sup>15</sup>, Hishiki, H.<sup>16</sup>*

Saitama Cancer Center, Research Institute for Clinical Oncology, Ina, Saitama, Japan<sup>1</sup>, Saitama Cancer Center, Research Institute for Clinical Oncology, Ina, Saitama, Japan<sup>2</sup>, Saitama Cancer Center, Research Institute for Clinical Oncology, Ina, Saitama, Japan<sup>3</sup>, Saitama Cancer Center, Research Institute for Clinical Oncology, Ina, Saitama, Japan<sup>4</sup>, Saitama Cancer Center, Research Institute for Clinical Oncology, Ina, Saitama, Saitama Cancer Center<sup>5</sup>, Saitama Cancer Center, Research Institute for Clinical Oncology, Ina, Saitama, Japan<sup>6</sup>, Saitama Cancer Center, Research Institute for Clinical Oncology, Ina, Saitama, Japan<sup>7</sup>, Saitama Cancer Center, Research Institute for Clinical Oncology, Ina, Saitama, Japan<sup>8</sup>, Saitama Cancer Center, Research Institute for Clinical Oncology, Ina, Saitama, Japan<sup>9</sup>, Saitama Cancer Center, Research Institute for Clinical Oncology, Ina, Saitama, Japan<sup>10</sup>, Saitama Cancer Center, Research Institute for Clinical Oncology, Ina, Saitama, Japan<sup>11</sup>, Saitama Cancer Center, Research Institute for Clinical Oncology, Ina, Saitama, Japan<sup>12</sup>, Saitama Children's Medical Center, Department of Clinical Research/Pathology, Saitama, Japan<sup>13</sup>, Chiba University School of Medicine, Department of Pediatric Surgery, Chiba, Japan<sup>14</sup>, Saitama Cancer Center, Research Institute for Clinical Oncology, Ina, Saitama, Japan<sup>15</sup>, Chiba University School of Medicine, Department of Pediatric Surgery, Chiba, Japan<sup>16</sup>

### **Background/Introduction:**

Polycomb repressive complex 2 (PRC2) is a multiprotein chromatin-modifying complex that is essential for vertebrate development and differentiation. PRC2 is composed of a trimeric core of SUZ12, EED, and EZH1/2, which catalyses the methylation of histone H3 at lysine 27 (H3K27me1/2/3). Recently, the role of PRC2, especially EZH2-related functions has been received a lot of attentions in NB tumorigenesis (Chen L et al., J Clin Invest. 2018; Laurel T. B-E et al., EJC. 2017; Tsubota S et al., Cancer Res. 2018; Li Z et al., ONCOGENE. 2018). Enhancer of zeste 1 polycomb repressive complex 2 subunit (EZH1), a homolog of EZH2, has different biochemical activities and effects on chromatin modification compared to EZH2.

### **Aims:**

We studied the EZH1 roles in NB biology by EZH1 knockdown and inhibitor treatments by using transcriptome and comprehensive chromatin modification analyses for development of a new epigenetic NB therapy.

### **Methods/Materials:**

Transcriptome analysis were performed using the Agilent platform of 8 x 60 K design ID G4851B. qChIP and ChIPseq experiments were done according to the previous paper (Li Z et al., ONCOGENE. 2018). To evaluate synergistic efficiency of UNC1999 and 5-FU to suppress NB cells, the Combination Index (CI) was calculated by the Chou-Talalay (2010) method using CompuSyn (<http://www.combosyn.com/>).

### **Results:**

resulted in significant cell death as well as xenograft inhibition. EZH1 depletion decreased the level of H3K27me1; the interaction and protein stabilization of MYCN and EZH1 indicated the cooperative roles in epigenetic transcriptional regulation. Transcriptome analysis of EZH1-depleted cells resulted in up-regulation of neural differentiation-related pathways, such as "Nervous system development (GO:0007399)", and "Neuron projection development (GO:0031175)"; down-regulation of the cell cycle progression-related pathways, such as "DNA recombination (GO:0006310)", "cell cycle (GO:0007049)", and "mitotic cell cycle (GO:0000278)". In particular, GSEA revealed down-regulation of reactome E2F-mediated regulation of DNA

replication along with key genes of this process, TYMS, POLA2, and CCNA1. TYMS and POLA2 were transcriptionally activated by *MYCN* and EZH1-related epigenetic modification. ChIPseq/qChIP analysis showed the *MYCN*/EZH1 co-localization, which indicated that EZH1 and *MYCN* bind to the TYMS promoter regions in *MYCN*-amplified NB cell lines. Further, luciferase experiments using the TYMS promoter region confirmed *MYCN*-related TYMS induction. Treatment with the EZH1/2 inhibitor UNC1999 also induced cell death, decreased H3K27 methylation, and reduced the levels of TYMS and POLA2 in NB cells. Previous reports indicated neuroblastoma cells are resistant to 5-fluorouracil (5-FU) and TYMS (encoding 5-FU-target thymidylate synthetase) has been considered the primary site of action for folate analogues. Intriguingly, UNC1999 treatment significantly sensitized *MYCN*-amplified neuroblastoma cells to 5-FU treatment.

#### Summary/Conclusions:

Taken together, EZH1 plays roles as an inhibitor of neuronal differentiation and as a transcriptional activator for thymidylate synthetase (TYMS), in cooperation with *MYCN* in NB. EZH1/2 inhibition may be an effective strategy for development of a new epigenetic treatment for NB and combination therapy with 5-FU seems to be promising for *MYCN*-amplified NBs.

## **P055 The functional annotation and exploration the SOX11 enhanceosome**

*Louwagie, A.*<sup>2</sup>, *Decaestecker, B.*<sup>3</sup>, *Delhaye, L.*<sup>4</sup>, *D'haene, E.*<sup>5</sup>, *James de Bony, E.*<sup>6</sup>, *Olexiouk, V.*<sup>7</sup>, *Vergult, S.*<sup>8</sup>, *Versteeg, R.*<sup>9</sup>, *Koster, J.*<sup>10</sup>, *Van Nes, J.*<sup>11</sup>, *Ek, S.*<sup>12</sup>, *Roberts, S.*<sup>13</sup>, *Eyckerman, S.*<sup>14</sup>, *Mestdagh, P.*<sup>15</sup>, *De Preter, K.*<sup>16</sup>, *Speleman, F.*<sup>17</sup>

Ghent University, Department of Biomolecular Medicine, Ghent, Belgium<sup>2</sup>, Ghent University, Department of Biomolecular Medicine, Ghent, Belgium<sup>3</sup>, VIB-UGent, Center of Medical Biotechnology, Ghent, Belgium<sup>4</sup>, Ghent University, Department of Biomolecular Medicine, Ghent, Ghent University<sup>5</sup>, Ghent University, Department of Biomolecular Medicine, Ghent, Belgium<sup>6</sup>, Ghent University, Department of Biomolecular Medicine, Ghent, Belgium<sup>7</sup>, Ghent University, Department of Biomolecular Medicine, Ghent, Belgium<sup>8</sup>, Academic Medical Center, Department of Oncogenomics, Amsterdam, Netherlands<sup>9</sup>, Academic Medical Center, Department of Oncogenomics, Amsterdam, Netherlands<sup>10</sup>, Academic Medical Center, Department of Oncogenomics, Amsterdam, Netherlands<sup>11</sup>, Lund University, Department of Immunotechnology, Lund, Sweden,<sup>12</sup> Memorial Sloan Kettering Cancer Center, Department of Pediatrics, New York, USA<sup>13</sup>, VIB-UGent, Center for Medical Biotechnology, Ghent, Belgium<sup>14</sup>, Ghent University, Department of Biomolecular Medicine, Ghent, Belgium<sup>15</sup>, Ghent University, Department of Biomolecular Medicine, Ghent, Belgium<sup>16</sup>, Ghent University, Department of Biomolecular Medicine, Ghent, Belgium<sup>17</sup>

### Background/Introduction:

Neuroblastoma (NB) is the most common extra cranial solid tumor during early childhood, arising from progenitors of the sympathetic nervous system. We identified the adrenergic specific transcription factor *SOX11* as a lineage dependency factor and a putative master regulator of epigenetic plasticity in NB. The expression of lineage identity genes is typically regulated by cell type specific enhancers thereby acting as putative cell-specific vulnerabilities that may be targeted while minimizing harm to the normal development of tissues.

### Aims:

We aimed to gain insight into the long-range control of *SOX11* expression in NB cells as a first step towards cell type specific therapeutic targeting of *SOX11*-driven neuroblastoma.

### Methods/Materials:

We analyzed H3K27ac data to map noncoding regulatory enhancer regions in the vicinity of the *SOX11* locus. Chromatin conformation analysis (4C-seq) additionally shows evidence for looping contacts between the *SOX11* promotor region and the *SILC1* locus, a lncRNA downstream of *SOX11*. For further exploration of the function of *SILC1*, chromatin isolation by RNA purification (ChIRP) is performed to identify lncRNA bound DNA sequences. Functional inhibition of the *SILC1* locus is explored using dCas9-KRAB silencing in NB cells.

### Results:

We identified a super-enhancer in a large gene poor region, 1.1 Mb distal to the *SOX11* locus. This region coincides with the previously annotated lncRNA coined *SILC1* which was shown to be implicated in neurite outgrowth and neuron regeneration. *SILC1* was found to be strongly expressed in adrenergic NB cells while absent in mesenchymal NB cells and highly correlated to *SOX11* expression levels in both NB cell lines and tumors. Also, in the normal developing sympathetic lineage, *SILC1* expression pattern closely resembled that of *SOX11*. Using 4C-seq, we observed looping in this highly active region between the *SOX11* and *SILC1* promoter in adrenergic NB cells while absent in the mesenchymal NB cell line SH-EP. We are currently unraveling the role of *SILC1* using ChIRP sequencing and a dCas9-KRAB silencing system.

### Summary/Conclusions:



In conclusion, our data suggests that *SILC1* plays a crucial role in assisting the function of the transcription factor *SOX11* thus offering an entry point for targeted therapy for *SOX11*-driven neuroblastoma.

## **P056 Combination therapy with the CDK7 inhibitor and the tyrosine kinase inhibitor exerts synergistic anticancer effects against *MYCN*-amplified neuroblastoma**

*Tee, A.E.L.*<sup>2</sup>, *Ciampa, O.*<sup>3</sup>, *Wong, M.*<sup>4</sup>, *Fletcher, J.*<sup>5</sup>, *Kamili, A.*<sup>6</sup>, *Chen, J.*<sup>7</sup>, *Carter, D.*<sup>8</sup>, *Cheung, B.*<sup>9</sup>, *Marshall, G.M.*<sup>10</sup>, *Liu, P.Y.*<sup>11</sup>, *Liu, T.*<sup>12</sup>

CHILDRENS CANCER INSITUTE AUSTRALIA, Histone Modification, Sydney, Australia <sup>2</sup>,  
CHILDRENS CANCER INSITUTE AUSTRALIA, Molecular Carcinogenesis, Sydney, Australia<sup>3</sup>,  
CHILDRENS CANCER INSITUTE AUSTRALIA, Histone Modification, Sydney, Australia<sup>4</sup>,  
CHILDRENS CANCER INSITUTE AUSTRALIA, Experimental Therapeutics, Sydney, CHILDRENS  
CANCER INSITUTE AUSTRALIA<sup>5</sup>, CHILDRENS CANCER INSITUTE AUSTRALIA, Experimental  
Therapeutics, Sydney, Australia<sup>6</sup>, CHILDRENS CANCER INSITUTE AUSTRALIA, Histone  
Modification, Sydney, Australia<sup>7</sup>, CHILDRENS CANCER INSITUTE AUSTRALIA, Molecular  
Carcinogenesis, Sydney, Australia<sup>8</sup>, CHILDRENS CANCER INSITUTE AUSTRALIA, Molecular  
Carcinogenesis, Sydney, Australia<sup>9</sup>, CHILDRENS CANCER INSITUTE AUSTRALIA, Molecular  
Carcinogenesis, Sydney, Australia<sup>10</sup>, CHILDRENS CANCER INSITUTE AUSTRALIA, Histone  
Modification, Sydney, Australia<sup>11</sup>, CHILDRENS CANCER INSITUTE AUSTRALIA, Histone  
Modification, Sydney, Australia, <sup>12</sup>

### Background/Introduction:

The most common cause of death from diseases in children is cancer. Neuroblastoma is accountable for approximately 8% of all paediatric cancers but 15% of all paediatric cancer mortality.(Maris et al., 2007; Matthay et al., 2016) Neuroblastoma arises from embryonic sympathoadrenal lineage of neural crest cells and is commonly situated within the adrenal medulla, head, chest, neck and pelvis.(Brodeur, 2003). *MYCN* oncogene amplification occurs in approximately 25% of human neuroblastoma tissues and correlates with poor patient prognosis.(Maris et al., 2007; Matthay et al., 2016)

*MYCN* oncogene encodes N-Myc oncoprotein, a Myc family transcription factor important for cell cycle progression, cell proliferation, resistance to apoptosis *in vitro* as well as neuroblastoma formation and progression *in vivo*.(Gustafson and Weiss, 2010; Huang and Weiss, 2013) While N-Myc presents as a therapeutic target, Myc oncoproteins are considered “undruggable” as they have no active site for ligand binding by small molecule compounds.(Beltran, 2014; Dang, 2013) As such, targeting N-Myc down-stream targets, N-Myc protein stability or *MYCN* gene transcription provides an attractive approach for inhibiting the N-Myc oncogenic pathway.

*MYCN* oncogene transcription is regulated by super-enhancers and cyclin dependent kinase 7 (CDK7).(Chipumuro et al., 2014) CDK7 binds to Transcription Factor II Human and regulates transcriptional initiation, pausing and elongation by phosphorylating the serine 5 and serine 7 residues of the C-terminal domain of RNA polymerase II at super-enhancer-associated oncogene loci.(Chipumuro et al., 2014; Christensen et al., 2014; Kwiatkowski et al., 2014). The covalent CDK7 inhibitor THZ1 binds to the cysteine 312 residue adjacent to the ATP cleft in the canonical kinase domain of CDK7 protein, suppresses CDK7 function, RNA polymerase II phosphorylation and the transcription of super-enhancer-associated oncogenes including *MYCN*, *MYC* and *BLC2*, and induces cancer cell growth inhibition.(Chipumuro et al., 2014; Christensen et al., 2014; Kwiatkowski et al., 2014; Nagaraja et al., 2017; Rusan et al., 2018; Wong et al., 2017b)

Receptor tyrosine kinases are a family of enzymes which are key regulators of cell proliferation, differentiation, metabolism, survival and apoptosis.(Lemmon and Schlessinger, 2010) Tyrosine kinases are activated due to ligand binding or constitutively activating mutations and induces phosphatidylinositol 3-kinase (PI3K) activation, leading to glycogen synthase kinase 3b inactivation, c-Myc and N-Myc protein dephosphorylation at threonine 58, and c-Myc and N-Myc protein stabilization.(Gustafson and Weiss, 2010)

In this study, we have performed drug screening and identified TKIs, including ponatinib and lapatinib, as the approved oncology drugs which exerted the best synergistic anticancer effects with the CDK7

inhibitor THZ1 against *MYCN*-amplified neuroblastoma cells. Combination therapy with THZ1 and ponatinib or lapatinib synergistically reduced gene expression of the protein kinase *PNUTS* and thereby reduced N-Myc protein but not mRNA expression, and synergistically induced *MYCN*-amplified neuroblastoma cell apoptosis.

#### Aims:

We aimed to screen the US Food and Drug Administration-Approved Oncology Drugs Set V from the National Cancer Institute, and identified tyrosine kinase inhibitors (TKIs), including ponatinib and lapatinib, as the Approved Oncology Drugs exerting the best synergistic anticancer effects with THZ1 in *MYCN*-amplified neuroblastoma cells. We then validated the synergistic anti-cancer effects and to identify the underlying mechanisms of action of THZ1 and RTK inhibitor combination therapy against *MYCN*-amplified neuroblastoma.

#### Methods/Materials:

##### Cell culture

BE(2)-C cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and Kelly and CHP134 cells were cultured in Roswell Park Memorial Institute Medium (RPMI) 1640, all supplemented with 10% fetal calf serum. BE(2)-C cells were obtained from Barbara Spengler (Fordham University, New York, NY) 20 years ago, and Kelly and CHP134 cells were purchased from the European Collection of Cell Cultures in 2010 (Sigma, Sydney, Australia). All cell lines were maintained in a humidified incubator at 37°C and 5% CO<sub>2</sub> in air. The identity of cell lines was confirmed in 2016, 2017 and 2018 by small tandem repeat profiling conducted at the Garvan Institute of Medical Research or Cellbank Australia.

##### Alamar blue assays

Alamar blue assays were performed as we described.(Sun et al., 2017; Sun et al., 2015) Briefly, cells were transfected with control or *PNUTS* siRNAs for 96 hours, or treated with vehicle control or anticancer agents for 72 hours. The cells were incubated with Alamar blue (Invitrogen) for the last five hours, and cell culture plates were read on a microplate reader at 570/595 nm. Results were quantified according to the optical density absorbance units and expressed as percentage changes in the number of cells, relative to control siRNA-transfected or vehicle control-treated samples.

##### Drug screening

For primary screening, *MYCN* oncogene-amplified BE(2)-C neuroblastoma cells were seeded into 96-well plates and treated in triplicates with vehicle control, the CDK7 inhibitor THZ1 at 16nM which reduced the number of BE(2)-C cells by approximately 30% on its own, the US Food and Drug Administration (FDA)-Approved Oncology Drugs (AODs) Set V from National Cancer Institute at 1 μM, or combination for 72 hours. Cell viability was determined by Alamar blue assays. Synergistic/additive interaction between THZ1 and the AODs was examined by fractional product method and expressed as R values.(Greco et al., 1995)

AODs which reduced the number of viable BE(2)-C cells by ≥ 90% on their own and AODs which synergized with THZ1 in reducing the number of viable BE(2)-C cells with a R value of less than 0.7 were shortlisted for secondary drug screening. BE(2)-C cells were treated with a range of doses of THZ1, the shortlisted AODs, or combination of THZ1 and the AODs for 72 hours, followed by Alamar blue assays. To determine if the effect of THZ1 and the AOD were synergistic or additive, Bliss-additivity was calculated with Bliss-additive formula.(Bliss and Bartels, 1946) If the actual combination effect was greater than that of the predicted additivity, THZ1 and the AOD exerted synergistic anticancer effect. Synergy was further validated by calculating combination indexes (CIs) for effective doses for 75% and 90% cell number reduction with the Chou-Talalay method(Chou and Talalay, 1984) and CompuSyn software (Combosyn Inc, <http://www.combosyn.com/>), where CI <1, 1 or >1 indicates synergistic, additive and antagonistic effect, respectively.

##### Apoptosis analysis

Neuroblastoma cells were transfected with control siRNA, PNUTS siRNA-1 or PNUTS siRNA-2 for 72 hours, or treated with vehicle control, 32nM THZ1, 250nM ponatinib, 1000nM lapatinib, THZ1 plus ponatinib, or THZ1 plus lapatinib for 72 hours. Cells were then stained with Annexin V-FITC and propidium iodide, followed by flow cytometry analysis of cells positively stained by Annexin V-FITC and/or propidium iodide, using FACS Canto Flow Cytometer (BD Biosciences, Franklin Lakes, NJ). The percentage of Annexin V and/or propidium iodide positively stained cells was analyzed with FlowJo Version 10 (TreeStar Inc., Ashland, OR) as we described.(Sun et al., 2015)

#### siRNA transfection

Neuroblastoma cells were plated into 6 well plates or T25 flasks and transfected with siRNAs using Lipofectamine 2000 (Life Technologies, Grand Island, NY) according to the manufacturer's instructions and as we described previously.(Liu et al., 2014; Wong et al., 2017a) RNA or protein were harvested for RT-PCR and immunoblot analyses.

#### Plasmid transfection

pCMV6-entry empty vector and pCMV6-entry PNUTS expression constructs were purchased from Origene (Origene, Rockville, MD). Neuroblastoma cells were transfected with the constructs using Lipofectamine 2000 reagent (Life Technologies) according to the manufacturer's protocol and as we described.(Sun et al., 2017; Sun et al., 2015)

#### Real time reverse transcription PCR (RT-PCR)

RNA was extracted from cells with RNeasy Plus Mini kit (Qiagen, Hamburg, Germany) and quantified with a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's instructions. RT-PCR was performed using Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA) for cDNA synthesis and gene specific primers and Power SYBR Green Master Mix (Invitrogen) as the fluorescent dye for PCR in Applied Biosystems 7900 (Applied Biosystems, Grand Island, NY). All primers were synthesized by Sigma (Sigma). The comparative threshold cycle ( $\Delta\Delta\text{Ct}$ ) method(Schmittgen and Livak, 2008) was used to evaluate fold changes in target genes, relative to the housekeeping gene actin.

#### Immunoblot

Neuroblastoma cells were lysed in RIPA buffer (150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-Cl pH 7.5) containing protease inhibitors (Sigma, St Louis, MO) and phosphatase inhibitors (Roche, <http://en.wikipedia.org/wiki/Penzberg>, <http://en.wikipedia.org/wiki/Germany>). Protein was extracted, quantified with the Bicinchoninic Acid Assay kit (Pierce, Rockford, IL), and loaded onto sodium dodecyl sulphate-polyacrylamide gels, followed by electrophoresis and transfer to nitrocellulose membranes. After blocking, membranes were probed with rabbit anti-PNUTS (1:1,000) (Bethyl Laboratories, Montgomery, TX) and mouse anti-N-Myc (1:1000) (Santa Cruz Biotechnology) antibodies. The membranes were then incubated with a goat anti-rabbit or goat anti-mouse antibody conjugated to horseradish peroxidase (1:10000) (both from Santa Cruz Biotechnology), and protein bands were visualized with SuperSignal (Pierce). The membranes were finally probed with an anti-actin antibody (1:30000) (Sigma) as loading controls.

#### Affymetrix microarray differential gene expression study

BE(2)-C neuroblastoma cells were treated with vehicle control, THZ1, ponatinib, lapatinib, THZ1 plus ponatinib, or THZ1 plus lapatinib. Six hours later, RNA was extracted from the cells with RNeasy Mini kit (Qiagen), and differential gene expression was investigated using Affymetrix Arrays (Affymetrix, Santa Clara, CA), as we described.(Liu et al., 2014; Sun et al., 2017; Sun et al., 2015) Microarray data were analyzed in R (<http://www.r-project.org/>) with bioconductor package (<http://www.bioconductor.org/>), and normalized with GenePattern software (Version 3.9.0 Broad Institute) and LimmaGP modules (Version 20.0) available at (<https://pwbc.garvan.org.au/gp/>), and deposited at Gene Expression Omnibus website (Series GSEXXXXX).

#### Statistical analysis

Experiments for statistical analysis were performed at least 3 times. Data were examined with Graphpad Prism 6 program and expressed as mean  $\pm$  standard error. Differences were analyzed for statistical significance with one-way ANOVA among groups or two-sided unpaired *t* test for two groups.

#### Results:

We screened the Approved Oncology Drugs Set and identified 17 AODs which reduced cell viability by  $\geq 90\%$  on their own and the 5 AODs which synergized with THZ1 were then subjected to secondary screen. BE(2)-C cells were treated with vehicle control, a range of dosages of THZ1, the AODs, or combination, followed by Alamar blue assays. Synergy or additivity of the THZ1 and AOD combination therapies was calculated using the Bliss-additivity model.(Bliss and Bartels, 1946) The synergy/additivity analysis showed that vincristine and doxorubicin, two first line chemotherapy drugs for treating neuroblastoma patients, did not synergize with THZ1; that the other DNA topoisomerase II inhibitors, the other microtubule inhibitors, the MEK inhibitor, the gene transcription/histone deacetylase/RNA synthesis inhibitors as well as the proteasome and protein translation inhibitors did not considerably synergize with THZ1; and that the TKIs ponatinib, lapatinib and nilotinib synergized with THZ1. The primary and secondary screens therefore identify the TKIs as the AODs exerting the best synergistic anticancer effects with THZ1 against *MYCN*-amplified neuroblastoma.

We next examined whether TKIs and THZ1 exerted synergistic anticancer effects in a range of *MYCN*-amplified neuroblastoma cell lines with little toxicity to normal cells. *MYCN*-amplified KELLY and CHP134 neuroblastoma cells were treated with vehicle control, a range of doses of THZ1, ponatinib, lapatinib, nilotinib, THZ1 plus ponatinib, lapatinib or nilotinib for 72 hours, followed by Alamar blue assays. Combination effect analysis with the Bliss-additivity model demonstrated that combination therapy with THZ1 and ponatinib, lapatinib or nilotinib synergistically reduced the number of viable Kelly and CHP134 cells. In addition, combination index (CI) values for reducing BE(2)-C, Kelly and CHP134 neuroblastoma cell viability by 75% and 90% by the combination therapies were generated by Compusyn(Bijnsdorp et al., 2011), and the CI values further confirmed that combination therapy with THZ1 and ponatinib, lapatinib or nilotinib synergistically reduced the number of viable BE(2)-C, Kelly and CHP134 cells with CI values  $< 0.5$  in all cases.

Ponatinib and nilotinib both target the tyrosine kinase Bcr-ABL(Rosti et al., 2017) while lapatinib targets the tyrosine kinase ERBB2 and EGFR.(Rimawi et al., 2015) As the concentrations for achieving considerable synergistic anticancer effects with THZ1 was higher for nilotinib (up to 8 $\mu$ M) than ponatinib (up to 1 $\mu$ M) and lapatinib (up to 2 $\mu$ M), we used ponatinib and lapatinib for further studies. BE(2)-C and Kelly neuroblastoma and WI-38 fibroblast cells were treated with vehicle control, THZ1, ponatinib, lapatinib, or combination of THZ1 and ponatinib or lapatinib for 72 hours, followed by staining with the apoptosis marker Annexin V. Flow cytometry analysis showed that THZ1 and ponatinib, as well as THZ1 and lapatinib, synergistically induced apoptosis in 72% and 70% BE(2)-C and 66% and 58% Kelly neuroblastoma cells but not in WI38 fibroblasts. Taken together, the data suggest that THZ1 and the TKIs ponatinib and lapatinib synergistically induce apoptosis in neuroblastoma but not normal non-malignant cells.

THZ1 suppresses *MYCN* gene transcription by blocking CDK7 binding to the *MYCN* gene promoter.(Chipumuro et al., 2014) We examined whether THZ1 and the TKI ponatinib and lapatinib synergistically regulate N-Myc mRNA and protein expression. BE(2)-C and Kelly cells were treated with vehicle control, THZ1, ponatinib, lapatinib, THZ1 plus ponatinib, or THZ1 plus lapatinib for 48 hours. RT-PCR and immunoblot analyses showed that THZ1 alone reduced N-Myc mRNA and protein expression, and that combination therapy with THZ1 and the TKIs did not co-operatively reduce N-Myc mRNA expression, but synergistically blocked the expression of N-Myc protein. The data suggest that THZ1 and TKI combination therapy synergistically blocks N-Myc protein expression through a post-transcriptional mechanism.

To examine the mechanism through which THZ1 and the TKI ponatinib and lapatinib synergistically reduce N-Myc protein expression and neuroblastoma cell proliferation and survival, we performed Affymetrix microarray gene expression experiments in BE(2)-C cells after treatment with vehicle control, THZ1, ponatinib, lapatinib, THZ1 plus ponatinib or THZ1 plus lapatinib for 6 hours. Using two

fold change as the cut-off point, 13 genes were found to be commonly down-regulated and one gene commonly up-regulated by both THZ1 and ponatinib as well as THZ1 and lapatinib combinations. Lysine rich coiled-coil 1 (KRCC1) and phosphatase 1 nuclear targeting subunit (PNUTS, also known as PPP1R10), the genes most synergistically downregulated by THZ1 and TKI combination therapies were selected for validation.

BE(2)-C and Kelly cells were treated with vehicle control, THZ1, ponatinib, lapatinib, THZ1 plus ponatinib, or THZ1 plus lapatinib for 48 hours. RT-PCR analysis showed that monotherapy with THZ1 reduced KRCC1 and PNUTS mRNA expression and monotherapy with ponatinib or lapatinib showed no effect, and that combination therapy with THZ1 and ponatinib or lapatinib synergistically and dramatically reduced KRCC1 and PNUTS mRNA expression. Consistent with the RT-PCR data, immunoblot analysis showed that combination therapy with THZ1 and ponatinib or lapatinib synergistically blocked PNUTS protein expression. Taken together, the data demonstrate that the THZ1 and TKI combination therapy synergistically reduces KRCC1 and PNUTS expression in *MYCN*-amplified neuroblastoma cells.

PNUTS has recently been shown to directly binds to Aurora kinase A and c-Myc proteins and blocks c-Myc protein degradation.(Dingar et al., 2018; Wang et al., 2019) We therefore examined whether PNUTS regulates N-Myc protein expression and neuroblastoma cell proliferation and survival. Real-time RT-PCR and immunoblot analyses showed that transfection of BE(2)-C and Kelly cells with two independent PNUTS siRNAs, PNUTS siRNA-1 or PNUTS siRNA-2, efficiently knocked down PNUTS mRNA and protein expression, and considerably reduced N-Myc protein but not N-Myc mRNA expression. BE(2)-C and Kelly cells were next transfected with an empty vector or PNUTS expression construct. Immunoblot analysis demonstrated that PNUTS over-expression up-regulated N-Myc protein expression.

BE(2)-C and Kelly cells were then transfected with control siRNA or PNUTS siRNAs for 72 hours, followed by Alamar blue assays or staining with Annexin V and flow cytometry analysis of Annexin V positively stained cells. Alamar blue assays showed that knocking down PNUTS expression significantly reduced the number of viable BE(2)-C and Kelly neuroblastoma cells, and flow cytometry analysis of Annexin V stained cells showed that knocking down PNUTS expression led to BE(2)-C and Kelly cell apoptosis. Taken together, the data demonstrate that PNUTS is important for N-Myc protein expression and neuroblastoma cell proliferation and survival.

#### Summary/Conclusions:

In summary, our screening of AODs identifies TKIs ponatinib, lapatinib and nilotinib as the AODs exerting the best synergistic anticancer effects with THZ1 against *MYCN* gene-amplified neuroblastoma cells. Combination therapy with THZ1 and the TKIs synergistically blocks *PNUTS* gene expression and N-Myc protein expression, and synergistically induces apoptosis in *MYCN*-amplified neuroblastoma but not normal non-malignant cells. PNUTS up-regulates N-Myc protein expression, and PNUTS knockdown induces *MYCN*-amplified neuroblastoma cell growth inhibition and apoptosis. As CDK7 inhibitors are currently under clinical evaluation in patients, our data suggest the addition of the TKIs in patients in clinical trials.

## **P057 CASZ1 represses the core transcriptional regulatory circuitry in neuroblastoma via an interconnected feedback loop**

*Liu, Z.<sup>2</sup>, Zhang, Z.<sup>3</sup>, Xu, M.<sup>4</sup>, Lei, H.<sup>5</sup>, Shern, J.<sup>6</sup>, Thiele, C.<sup>7</sup>*

National Cancer Institute, Pediatric Oncology Branch, Bethesda, USA <sup>2</sup>, National Cancer Institute, Pediatric Oncology Branch, Bethesda, USA<sup>3</sup>, National Cancer Institute, Pediatric Oncology Branch, Bethesda, USA<sup>4</sup>, National Cancer Institute, Pediatric Oncology Branch, Bethesda, National Cancer Institute<sup>5</sup>, National Cancer Institute, Pediatric Oncology Branch, Bethesda, USA<sup>6</sup>, National Cancer Institute, Pediatric Oncology Branch, Bethesda, USA<sup>7</sup>

### Background/Introduction:

Neuroblastoma (NB) is neural crest-derived and commonly arises in the adrenal gland. NB core transcriptional regulatory circuitry (CRC) contains essential transcription factors (TFs) ISL1, HAND2, GATA3, PHOX2B and TBX2, and these TFs form an interconnected autoregulatory feed-forward loop to determine an adrenergic phenotype of NB. What TFs directly repress the NB CRC are unknown.

### Aims:

The zinc-finger TF CASZ1 orchestrates cell commitment in normal neuroblasts and functions as a Chr1p36 tumor suppressor in NB. Here we investigate whether CASZ1 regulates the CRC to affect NB cell identity.

### Methods/Materials:

CASZ1b overexpressing NB cell lines are established. Single-cell RNA-seq (scRNA-seq), bulk RNA-seq and ChIP-seq are performed to investigate the role of CASZ1 in controlling CRC.

### Results:

Interrogation of published scRNA-seq data of the developing adrenomedullary cells within the adrenals of murine embryos reveals that CASZ1 is heterogeneously expressed with low levels in the schwann cell precursors (SCPs), median levels in the bridge state cells and sympathoblasts, and high levels in more differentiated chromaffin cells. NB CRC components are highly expressed in sympathoblasts, but other types of adrenomedullary cells have variable mRNA levels. This suggests that together with CRC TFs, CASZ1 is involved in the regulation of SCPs stemness and chromaffin cell differentiation. Published microarray data analyses shows that levels of CASZ1 is 2-fold lower while levels of NB CRC components is 2-100x higher in NB compared to levels in the adrenals ( $p < 0.0001$ ), suggesting that CASZ1 is silenced while CRC components are aberrantly upregulated in NB. Published ChIP-seq data analyses show that NB CRC components bind to CASZ1 gene locus. Knock-down of HAND2 in NB cells decreases HAND2 binding to CASZ1 gene locus resulting in a >2-fold increase of CASZ1 mRNA levels, indicating that the CRC represses CASZ1 expression. Consistently, scRNA-seq of NB cells reveals that CASZ1 is expressed in less than 5% of cells and CASZ1 expressing cells have lower levels of CRC components. Restoration of CASZ1b in SY5Y cells results in growth inhibition and neurite extension. Consistently, retinoids up-regulates CASZ1 and CASZ1 knock-down attenuates retinoids induced neuronal differentiation. RNA-seq results show that restoration of CASZ1b in NB results in a positive enrichment of neurotransmitter transporter, catecholamine secretion and neuron projection gene signatures ( $p < 0.002$ ), indicating a chromaffin cell differentiation. Importantly, CASZ1b restoration in NB cells down-regulates the expression of CRC components ( $p < 0.05$ ) and results in a negative enrichment of the NB adrenergic gene signature that determined by CRC ( $p < 0.001$ ). ChIP-seq results show that restoration of CASZ1b in SY5Y cells leads to an enrichment of CASZ1b binding within super enhancers (SEs) and typical enhancers (TEs) of NB CRC components which is accompanied by decreased H3K27ac signal mainly in TEs. Thus, CASZ1b directly represses CRC components through decreasing TEs activity.

### Summary/Conclusions:

Our study identifies that CASZ1 forms a negative feedback regulatory circuit with established NB CRC to switch the NB adrenergic phenotype to a more differentiated chromaffin cell phenotype. This leads to a novel model in which the CRC forms an interconnected feedback loop with a TF to control cell identity in health and disease.



## **P058 Novel strategies for delivering small RNAs to treat neuroblastoma**

Boloix, A.<sup>2</sup>

Vall Hebron Research Institute, Pediatric Oncology, Barcelona, Spain <sup>2</sup>

### Background/Introduction:

MicroRNAs are a class of small non-coding RNAs that are crucial regulators of gene expression and found to be deregulated in multiple human diseases such as cancer, both in adult and pediatric tumours. In particular, an overall reduction of miRNA expression has been shown to contribute to the progression and outcome of patients with neuroblastoma, one of the most aggressive pediatric solid tumours that could benefit from microRNA-restoration therapies. Nevertheless, miRNA-based therapies have not reached the market due, in part, to suboptimal biodistribution and short half-life in bloodstream. Nanomedicine promises the precise delivery of nucleic acids into tumours with reduced off-target toxicities. Hence, conjugation of miRNA to nanoparticles could be a good strategy to test anti-tumour effects of miRNAs *in vivo*. However, there is still a lack of a standard formulation for their clinical administration.

### Aims:

The aim of this project is to develop a novel nanocarrier able to deliver tumour suppressive miRNAs to treat high-risk-neuroblastoma tumours.

### Methods/Materials:

Novel unilamellar lipid-based nanoparticles were prepared using a compressed fluid-based technology named DELOS-SUSP process (Depressurization of an Expanded Liquid Organic Solution-Suspension) and further purified by diafiltration. The tumour suppressor miR-323a-5p was conjugated with nanovesicles through electrostatic interactions. The delivery of miR-323a-5p to target cells was measured by quantitative RT-qPCR. MiRNA target genes levels were monitored by RT-qPCR and western blot. Finally, the phenotypic effects of miR-323a-5p overexpression on cell proliferation were measured by crystal violet staining.

### Results:

We have synthesized a potential clinical formulation based on novel nanoparticles. Compared to other nanoparticles, such as liposomes, our nanovesicles are small (80-100nm), unilamellar, with high homogeneity and positive charge. These properties grant an excellent long-term colloidal stability and an efficient entrapment of small RNA (sRNA) in their surface. miR-323a-5p nanocomplexes showed efficient miRNA loading, resistance to nucleases, high transfection efficiency and intracellular accumulation of mature miRNA forms. From the functional point of view, treatment of neuroblastoma cells with miR-323a-5p nanoconjugates induced downregulation of *bona fide* miR-323a-5p targets, such as CCND1 or CHAF1A, at mRNA and protein level. Finally, miR-323a-5p nanoconjugates were capable of halting the proliferation of neuroblastoma cells.

### Summary/Conclusions:

In summary, we developed a new nanomedicine composed by a tumour-suppressive microRNA and an innovative nanocarrier which together could be a potential tool for the treatment of high-risk neuroblastoma.

## **P059 *MYCN* mRNA as a post-transcriptional regulator of RNA transcripts via microRNAs in High-Risk Neuroblastoma**

*Gómez-González, S.*<sup>2</sup>, *García, M.*<sup>3</sup>, *Garrido-García, A.*<sup>4</sup>, *García-Gerique, L.*<sup>5</sup>, *Lemos, I.*<sup>6</sup>, *Rodríguez Núñez, E.*<sup>7</sup>, *Suñol, M.*<sup>8</sup>, *Carcaboso, A.M.*<sup>9</sup>, *Mora, J.*<sup>10</sup>, *Lavarino, C.*<sup>11</sup>

Fundació Sant Joan de Déu, Institut de Recerca Sant Joan de Déu, Developmental Tumor Biology Laboratory, Barcelona, Spain<sup>2</sup>, Fundació Sant Joan de Déu, Institut de Recerca Sant Joan de Déu, Developmental Tumor Biology Laboratory, Barcelona, Spain<sup>3</sup>, Fundació Sant Joan de Déu, Institut de Recerca Sant Joan de Déu, Developmental Tumor Biology Laboratory, Barcelona, Spain<sup>4</sup>, Fundació Sant Joan de Déu, Institut de Recerca Sant Joan de Déu, Developmental Tumor Biology Laboratory, Barcelona, Spain<sup>5</sup>, Fundació Sant Joan de Déu, Institut de Recerca Sant Joan de Déu, Developmental Tumor Biology Laboratory, Barcelona, Spain<sup>6</sup>, Hospital Sant Joan de Deu, Pathology, Barcelona, Spain<sup>7</sup>, Hospital Sant Joan de Deu, Pathology, Barcelona, Spain<sup>8</sup>, Fundació Sant Joan de Déu, Institut de Recerca Sant Joan de Déu, Developmental Tumor Biology Laboratory, Barcelona, Spain<sup>9</sup>, Hospital Sant Joan de Deu, Pediatric Oncology, Barcelona, Spain<sup>10</sup>, Fundació Sant Joan de Déu, Institut de Recerca Sant Joan de Déu, Developmental Tumor Biology Laboratory, Barcelona, Spain<sup>11</sup>

### Background/Introduction

MicroRNAs (miRNAs) are small RNA sequences which downregulate gene expression through targeting complementary sequences in mRNA 3'-untranslated region (UTR), known as miRNA response elements (MRE). miRNAs can regulate multiple mRNAs; whereas a single mRNA transcript can be regulated by multiple miRNAs. Competition for shared miRNAs between protein-coding and noncoding transcripts, known as "competing endogenous RNA" (ceRNA), represents a mechanism of gene regulation. ceRNAs act as a miRNA sponge or decoy for common miRNAs. By altering the pool of miRNAs, ceRNAs affect expression levels of other mRNAs. Aberrant expression of specific miRNAs have been reported to actively contribute to neuroblastoma (NB) pathogenesis.

In NB, the amplification of the oncogene *MYCN* is associated with high-risk disease. Interestingly, *MYCN* mRNA 3'-UTR contains MRE highly conserved among vertebrates, suggesting strong functional relevance. *MYCN* mRNA can thus function as a sponge that regulates other mRNAs via shared miRNAs, acting as ceRNA. In *MYCN* amplified tumors, the increase of mRNA abundance may affect ceRNA networks and consequently alter the transcriptional output of key genes. The effect of concomitant deregulated levels of multiple miRNAs on gene regulatory processes in NB has not been studied.

### Aims

This study aimed to explore *MYCN* mRNA as a post-transcriptional regulator of distinct RNA transcripts via shared pool of miRNAs, and characterize the ceRNA network associated with *MYCN* amplification.

### Methods/Materials

We generated and analyzed genome-wide miRNA and gene expression microarray data (Affymetrix GeneChip miRNA4.0 and Affymetrix U219; respectively) from twenty primary NB tumors. *MYCN* status was characterized using qPCR or/and SNP arrays (Affymetrix CytoscanHD). Clinical and biological data was available for all NB included in the study. Bioinformatic tools and publicly available databases such as TargetScan were used to examine mRNA/miRNA interactions. NB cell lines, patient-derived xenografts (PDXs) and primary NB tumors were used to validate the ceRNA network by real-time qPCR.

### Results

Unsupervised analysis of the miRNA microarray data revealed differential profiles among NB clinical risk groups. These miRNA expression profiles were found to be significantly associated with *MYCN* amplification status. We developed and applied an R-based bioinformatic tool that performs correlation expression analyses between miRNA and RNA transcripts. The analysis identified a potential ceRNA network associated with *MYCN* amplification. After data filtering, the

most promising *MYCN* targets and their associated miRNAs were validated in NB cell lines, in PDX and in a set of independent primary NB tumors. The results enabled us to identify a network of ceRNAs that affect biological pathways that contribute to the aggressive phenotype of the disease.

#### Summary/Conclusions

High levels of *MYCN* mRNA act as a miRNA sponge deregulating the expression of several ceRNAs that compete for common miRNAs. These findings could provide a potential functional explanation of how genes with no previously known direct interactions are potentially interlinked and identify interactions between relevant signaling pathways.

## **P060 Integrative analysis of genome-wide DNA methylation at single-base resolution identifies epigenetic subtypes of high-risk neuroblastoma**

Henrich, K.O.<sup>2</sup>, Okonechnikov, K.<sup>3</sup>, Wehrmann, L.<sup>4</sup>, Gartlgruber, M.<sup>5</sup>, Dreidax, D.<sup>6</sup>, Chavez, L.<sup>7</sup>, Pfister, S.M.<sup>8</sup>, Westermann, F.<sup>9</sup>

DKFZ, German Cancer Research Center, Neuroblastoma Genomics, Heidelberg, Germany <sup>2</sup>, DKFZ, German Cancer Research Center, Division of Pediatric Neurooncology, Heidelberg, Germany<sup>3</sup>, DKFZ, German Cancer Research Center, Neuroblastoma Genomics, Heidelberg, Germany<sup>4</sup>, DKFZ, German Cancer Research Center, Neuroblastoma Genomics, Heidelberg, DKFZ, German Cancer Research Center<sup>5</sup>, DKFZ, German Cancer Research Center, Neuroblastoma Genomics, Heidelberg, Germany<sup>6</sup>, DKFZ, German Cancer Research Center, Division of Pediatric Neurooncology, Heidelberg, Germany<sup>7</sup>, DKFZ, German Cancer Research Center, Division of Pediatric Neurooncology, Heidelberg, Germany<sup>8</sup>, DKFZ, German Cancer Research Center, Neuroblastoma Genomics, Heidelberg, Germany<sup>9</sup>

### Background/Introduction:

Neuroblastoma is a pediatric tumor derived from precursors of the sympathetic nervous system. Half of the children diagnosed have high-risk disease, disproportionately contributing to overall childhood cancer-related deaths. Known genetic aberrations only partially explain this aggressive tumor behavior, and evidence is accumulating that epigenetic deregulation plays a prominent role in neuroblastoma pathogenesis.

### Aims:

Our aim is to analyze the regulatory and developmental role of DNA methylation on a genome-wide scale by an integrated approach covering genomes, transcriptomes and chromatin landscapes of primary neuroblastomas as well as transcriptional trajectories of the developing adrenal medulla.

### Methods/Materials:

Whole-genome bisulfite sequencing (WGBS) and RNAseq were performed in 51 primary neuroblastomas including *MYCN*-amplified, *TERT*-rearranged, ALT-positive and low-risk tumors. For 35 of these, H3k27ac ChIPseq was performed. Array-based DNA methylation estimation complemented by RNAseq was done in an additional cohort of 180 neuroblastomas. Single cell RNAseq data from the developing mouse adrenal medulla were used for projection of epigenetically defined subtype-specific profiles. Targeted *in vitro* demethylation of candidate regions was done using a CRISPR-dCas9-TET1 construct.

### Results:

Dimension reduction approaches and unsupervised clustering on WGBS data identified three epigenetically defined groups being characterized by low-risk disease, amplified *MYCN* or presence of telomere maintenance mechanisms (TMMs, including *TERT* and ALT), respectively. Differentially methylated regions, partially methylated regions and DNA methylation valleys specific for these subgroups were identified and integration of associated transcriptional programs revealed subgroup-specific regulatory networks. Targeted demethylation of candidate regulatory sites revealed direct transcriptional impact. H3K27ac integration identified enhancer and super enhancer methylation patterns as highly characteristic of subtype identity, and array-based enhancer methylation analysis confirmed the relevance of the three epigenetic subtypes in a large tumor cohort. WGBS and RNAseq-based tracing of enhancer networks found subgroup-specific transcription factor/enhancer networks converging on a limited number of nodes. Projecting methylation-defined transcriptional profiles of the three subgroups onto developmental trajectories of mouse adrenal medulla precursors revealed divergent candidate cells of origin.

### Summary/Conclusions:

Our integrative investigation of DNA methylation at single nucleotide level identifies neuroblastoma subtypes, suggests their differential developmental origin and sheds light on their epigenetically manifested regulatory networks, which will aid identification of therapeutic intervention targets.

## **P061 The oncogenic transcriptional regulators CtBP-1/2 are novel potential therapeutic targets in neuroblastoma.**

*Rozen, E.J.<sup>2</sup>, Wigglesworth, K.<sup>3</sup>, Huang, T.<sup>4</sup>, Ali, A.<sup>5</sup>, Desaboini, N.R.<sup>6</sup>, Royer, W.<sup>7</sup>, Shohet, J.M.<sup>8</sup>*

University of Massachusetts Medical School, Pediatrics, Worcester, USA <sup>2</sup>, University of Massachusetts Medical School, Pediatrics, Worcester, USA<sup>3</sup>, University of Massachusetts Medical School, Pediatrics, Worcester, USA<sup>4</sup>, University of Massachusetts Medical School, Biochemistry and Molecular Pharmacology, Worcester, University of Massachusetts Medical School<sup>5</sup>, University of Massachusetts Medical School, Biochemistry and Molecular Pharmacology, Worcester, USA<sup>6</sup>, University of Massachusetts Medical School, Biochemistry and Molecular Pharmacology, Worcester, USA<sup>7</sup>, University of Massachusetts Medical School, Pediatrics, Worcester, USA<sup>8</sup>

### Background/Introduction:

Neuroblastoma (NB) is a relatively rare disease, representing 6-10% of all childhood tumors, but a significant ~15% of childhood cancer deaths. NB is a solid tumor that arises from the developing sympathetic precursors of the neural crest. CtBP-1 and -2 constitute an evolutionarily conserved family of redox-sensing transcriptional regulators with pleiotropic roles during development and adult lifespan. A very unique feature of CtBPs is the presence of a catalytic dehydrogenase domain, requiring NAD(P)H as a cofactor. CtBP-2 has been shown to modulate neural crest development by repressing melanocyte lineage differentiation and promoting commitment towards sympathoadrenal fates. More recently, accumulating evidence support a role for CtBPs as oncogenic factors that are over-expressed in many cancer types, and whose expression levels significantly correlate to worse prognosis and more aggressive phenotypes. CtBPs can simultaneously repress the transcription of several tumor-suppressive genes (e.g. p53) while inducing pro-oncogenic functions (e.g. c-Myc upregulation), such as cell proliferation, EMT and migration. In the NB cell line SH-SY5Y, RNAi-mediated CtBP2 downregulation resulted in decreased expression of c-Myc, MMP9 and MMP2 proteins, with concomitant reductions of *in vitro* cell growth, proliferation, migration and invasion. Thus, development of highly selective and specific inhibitors targeting CtBPs transcriptional functions could lead to improved therapeutic strategies in NB and other cancers. In this context, inhibition of CtBPs' catalytic domain has proven beneficial in murine *in vivo* models of colon cancer, pancreatic ductal adenocarcinoma and intestinal adenomatous polyposis. The most promising inhibitory molecules targeting this domain are derivatives of the substrate competitor 2-hydroxy-imino phenylpyruvate (HIPP), but their current potency and specificity require further improvement.

### Aims:

Several evidences suggest a role for CtBPs in cancer, and more specifically in NB, but many details of the molecular mechanisms remain to be elucidated. Thus, we sought to assess the effect of CtBPs downregulation and/or pharmacological inhibition on different NB cellular processes. Moreover, we have established a collaboration with the Department of Biochemistry and Molecular Pharmacology (UMass Medical School) whereby novel CtBP inhibitor (HIPP) derivatives are being designed, synthesized and tested on NB cells.

### Methods/Materials:

We use inducible shRNA-expression systems and pharmacological inhibitors together with high throughput imaging cytometry to assess the effect of CtBP downregulation/inhibition on several NB cell biology. Luciferase reporter assays and RT-qPCR are being used to assess CtBP transcriptional output in response to the different treatments to test. Bioinformatic analysis of available NB patient databases (R2 Platform). RNA-seq analysis will be performed to further characterize the transcriptional landscape regulated by CtBPs in NB cells.

### Results:

We observe significant decreases in NB cell proliferation and viability upon shRNA downregulation of either CtBP-1 or CtBP-2. As shown for other solid tumors, we demonstrate that CtBPs expression

negatively correlate with NB patient survival probability in multiple annotated clinical cohorts. We have recently started screening a novel set of HIPP chemical derivatives by means of biophysical studies (isothermal titration calorimetry, multi-angle light scattering and protein crystallography) as well as cell-based approaches to optimize performance as inhibitors of CtBPs function. CtBPs-dependent transcriptional activity and oncogenic functions in response to most promising compounds will be assessed in a panel of NB cell lines.

Summary/Conclusions:

Our preliminary analysis confirm a role for CtBPs as regulators of NB cell homeostasis and warrant for further characterization. Current and future efforts will contribute to clarify the mechanisms by which these transcriptional modulators mediate NB initiation, progression and/or metastasis, as well as to uncover improved inhibitory molecules with enhanced therapeutic value.

## **P062 DYRK family kinases are critical modulators of Neuroblastoma cell homeostasis and tumorigenicity.**

*Rozen, E.J.<sup>2</sup>, Wigglesworth, K.<sup>3</sup>, Huang, T.<sup>4</sup>, Wu, J.<sup>5</sup>, Shohet, J.M.<sup>6</sup>*

University of Massachusetts Medical School, Pediatrics, Worcester, USA <sup>2</sup>, University of Massachusetts Medical School, Pediatrics, Worcester, USA<sup>3</sup>, University of Massachusetts Medical School, Pediatrics, Worcester, USA<sup>4</sup>, University of Massachusetts Medical School, Pediatrics, Worcester, University of Massachusetts Medical School<sup>5</sup>, University of Massachusetts Medical School, Pediatrics, Worcester, USA<sup>6</sup>

### Background/Introduction:

Neuroblastoma (NB) is the most common solid malignancy in early childhood and high-risk disease accounts for a disproportionately high mortality when compared to other childhood cancer types. The *MYCN* oncogene is amplified and/or overexpressed in 25% of patients with this childhood cancer, making it the most powerful prognostic marker to date. *MYCN* drives the initiation and progression of neuroblastoma through a transcriptional program regulated by multiple protein-protein and protein-DNA interactions in malignant cells. Our group recently described a novel MYC(N) interaction with the tumor suppressor p53, leading to alterations in its transcriptional behavior. In search for potential modulators of this interaction, we focused on the 'Dual specificity tyrosine phosphorylation-regulated kinase' (DYRK) family, with particular attention to members DYRK1A and DYRK2. Interestingly, DYRK kinases have been shown to phosphorylate and modulate both p53 and MYC, constituting potential molecular regulators of the MYC(N)-p53 interaction. DYRK1A has been shown to play a critical role for neural development from flies to human. Altered expression has been linked to Down Syndrome and Autism Spectrum disorders as well as some types of cancers. An increasing body of literature suggests several roles for DYRK2 in the regulation of cancer cell homeostasis and tumor progression. In particular, treatment of NB cell lines with the DYRK family inhibitor Harmine, resulted in caspase activation and subsequent cell death in a *MYCN* amplification-sensitive fashion.

### Aims:

We sought to characterize the function of the DYRK family of kinases (DYRK1A and DYRK2 members in particular) as modulators of different aspects of NB cell biology and tumorigenicity.

### Methods/Materials:

We used lentiviral-mediated shRNA expression systems to constitutively or inducibly downregulate DYRKs expression in NB cell lines *in vitro* and in mouse xenograft tumor models. Pharmacological inhibition or shRNA knockdown were used to evaluate effects on cell viability, proliferation and apoptosis *in vitro*. Bioinformatic analysis of NB cell/patient databases was used for expression-survival correlation plots.

### Results:

Here, we demonstrate that expression and function of either DYRK1A or DYRK2 is essential for proper NB cell survival and proliferation *in vitro*. Importantly, DYRK2 expression shows a significant negative correlation to survival probability of NB patients. This is in agreement with the fact that NB cell lines show the highest *DYRK2* mRNA expression among all other cancer cell lines analyzed (Cancer Cell Line Encyclopedia, The Broad Institute). Ongoing experiments will evaluate the impact of conditional DYRK1A/2 knockdown in orthotopic xenograft models. Current efforts also aim at elucidating the transcriptional mechanisms regulated by these kinases in NB cells by means of RNA-seq analysis.

### Summary/Conclusions:

Altogether, our findings demonstrate that DYRK kinases indeed are critical modulators of NB cell pathophysiology. We anticipate that additional preclinical studies will support the potential translation of these findings into clinical therapeutic tools.





## **P063 Regulation of *MYCN*/MAX interaction and transcriptional function by the PAK2 stress kinase.**

Rozen, E.J.<sup>2</sup>, Royer, W.<sup>3</sup>, Wigglesworth, K.<sup>4</sup>, Huang, T.<sup>5</sup>, Shohet, J.M.<sup>6</sup>

University of Massachusetts Medical School, Pediatrics, Worcester, USA <sup>2</sup>, University of Massachusetts Medical School, Biochemistry and Molecular Pharmacology, Worcester, USA<sup>3</sup>, University of Massachusetts Medical School, Pediatrics, Worcester, USA<sup>4</sup>, University of Massachusetts Medical School, Pediatrics, Worcester, University of Massachusetts Medical School<sup>5</sup>, University of Massachusetts Medical School, Pediatrics, Worcester, USA<sup>6</sup>

### Background/Introduction:

The MYC oncoprotein family (*MYCN*, c-MYC, and MYCL) are short-lived transcription factors (TF) that are collectively overexpressed or deregulated in more than half of all human cancers, thus constituting potential therapeutic targets of crucial relevance. In neuroblastoma (NB), *MYCN* drives tumor initiation and progression through a transcriptional program regulated by multiple protein-protein and protein-DNA interactions. MYC family TFs require hetero-dimerization with its transcriptional partner MAX to exert their functions. c-MYC/MAX dimerization is regulated in part via phosphorylation of 3 residues within the C-terminal basic Helix-Loop-Helix domain (bHLH) of c-MYC (S358, S373 and T400) by the stress-induced 'p21-activated kinase 2' (PAK2). Such post-translational modifications disrupt c-MYC/MAX interaction (S373 and T400) and DNA binding (S358), resulting in inhibition of c-MYC-dependent transcription, proliferation and transformation. Furthermore, PAK2-mediated disruption of c-MYC/MAX interaction was shown to trigger a switch on Retinoic Acid Receptor  $\alpha$  (RAR $\alpha$ ) from repression to induction of differentiation on leukemic cells, constituting a novel and unexpected MAX-independent c-MYC tumor-suppressor function. We observed that two of such c-MYC residues (S373 and T400, which mediate interaction with MAX) are conserved in both *MYCN* and MAX bHLH domains, suggesting that they may also be targets of PAK2 kinase activity and thus mediate the functional behavior of the *MYCN*/MAX transcriptional complex.

### Aims:

We aimed to test whether PAK2 has the ability to interact with and phosphorylate *MYCN* and/or MAX on these and/or other residues, and ultimately modulate their function.

### Methods/Materials:

Bioinformatic analysis of sequence alignments and NB cell/patient databases (Cancer Cell Line Encyclopedia, The Broad Institute and R2 Platform), co-immunoprecipitation assays, *in vitro* kinase assays from endogenously and exogenously expressed proteins, site-directed mutagenesis and pharmacological inhibition of kinase activity.

### Results:

We have confirmed PAK2/*MYCN*/MAX interaction by co-immunoprecipitation. Furthermore, we show that a constitutively active PAK2 mutant can specifically phosphorylate *MYCN* in *in vitro* kinase assays. Moreover, we have generated *MYCN* point-mutant versions of the two PAK2 target residues to phosphorylation-mimetic Aspartic Acid residues (S400D, T427D or both) and confirmed their role in the modulation of *MYCN*/MAX interaction and function. Finally, we propose a potential role for the related PAK family member PAK3, given its remarkably high expression level in NB cell lines (CCLE) and its very significant correlation to NB patient survival probability (R2 platform).

### Summary/Conclusions:

Present and future efforts will contribute to fully elucidate the role of PAK kinases on *MYCN* and MAX phosphorylation and function in *in vivo* NB mouse models. Our preliminary results highlight the PAK family kinases as novel regulators of oncogenic MYC(N)/MAX transcription and open new roads to the design of improved translational strategies to fight NB.



## **P064 The role of PCP4 and its potential gene regulation network in neuroblastoma**

Zhan, Y.<sup>2</sup>, Dong, R.<sup>3</sup>, Yang, R.<sup>4</sup>

Children's Hospital of Fudan University, Pediatric surgery, Shanghai, China <sup>2</sup>, Children's Hospital of Fudan University, Pediatric surgery, Shanghai, China<sup>3</sup>, Children's Hospital of Fudan University, Pediatric surgery, Shanghai, China<sup>4</sup>

### Background/Introduction:

Neuroblastoma (NB), the most common malignant solid tumor in children's abdomen, accounts for 8%-10% of childhood malignant tumors. But the molecular mechanism of NB tumorigenesis is still unclear. Our research shows that the *PCP4* and its gene network may play an important role in NB. *PCP4* (Purkinje cell protein 4), also named Calmodulin regulator protein, stands on the top of Calmodulin (CaM) signaling cascade, implying it may have a complex downstream regulation network in NB.

### Aims:

To investigate the functions of potential *PCP4* gene network in NB, and to promote the understanding of the molecular mechanism of the tumorigenesis in NB.

### Methods/Materials:

Immunofluorescence and Western blot were used to detect the expression of *PCP4* in the NB pathological sections and tissues. And the *PCP4* overexpression or knockdown and other genes overexpression or knockdown in NB cell lines, SK-N-BE(2)C and the SK-N-SH, were achieved by lentiviral transduction or plasmid transfection. Functions of cell lines were analyzed by the abilities of proliferation, migration and other abilities. Next, we performed a differential proteomics analysis to figure out the potential gene network of *PCP4*, and then verify the expression by the qPCR, Western Blot and immunohistochemical staining.

### Results:

According to the immunofluorescence results, *PCP4* expression was down-regulated in NB tumor cells. And compared with the control, *PCP4* overexpressed NB cell lines showed a poor proliferative and migration abilities, while *PCP4* knockdown cell lines obtained a more invasive phenotype. We performed the differential proteomics analysis to investigate the downstream gene network of *PCP4*. Then we got 289 different expressed genes as potential targets. After the target gene expression detection and bioinformatics analysis, we may construct a *PCP4* gene network in NB.

### Summary/Conclusions:

We found that *PCP4* plays a tumor suppressive role in NB with its potential gene network. This would help us further understand of the mechanism of NB.

## **P065 MYCN cooperates with HAND2 to control neuroblastoma growth and differentiation**

*Xu, M.*<sup>2</sup>, *Liu, Z.*<sup>3</sup>, *Thiele, C.*<sup>4</sup>

National Institutes of Health, National Cancer Institute, Bethesda, USA <sup>2</sup>, National Institutes of Health, National Cancer Institute, Bethesda, USA<sup>3</sup>, National Institutes of Health, National Cancer Institute, Bethesda, USA<sup>4</sup>

### Background/Introduction:

Neuroblastoma (NB) is a neural crest derived tumor of the developing sympathetic nervous system. Amplification of *MYCN* correlates with high-risk disease. How *MYCN* alters the transcriptional landscape of presumptive sympathoadrenal progenitors to cause NB remains ill-defined.

### Aims:

To identify critical nodes regulating *MYCN*'s diverse effects on growth, tumorigenicity and differentiation in NB.

### Methods/Materials:

The *MYCN* interactome was defined by co-immunoprecipitation(co-IP) and protein mass spectrometric analyses in IMR32 cells. RNAi, RNA-seq and ChIP-seq were performed to investigate how the novel protein partners affect *MYCN* oncogenic activity.

### Results:

Co-IP and protein mass-spectrometry results showed that *MYCN* interacts with 8 neural crest lineage-transcription factors (TFs) that are essential for sympathoadrenal development. Among these *HAND2*, *PHOX2B*, *ISL1*, *TBX2* and *GATA3* are components of a core transcriptional regulatory circuitry that maintains NB proliferative state and cell identity. SiRNA silencing of these 8 TFs led to a 30-80% decrease of NB cell proliferation. Realtime PCR showed that most of these TFs regulate the expression of selected *MYCN* target genes, but only *HAND2* completely phenocopies for this set of *MYCN* target genes. Proximity ligation assays support the interaction between *HAND2* and *MYCN*. Structure/function studies showed a critical region of *MYCN* that is required for *HAND2* interaction shares only ~30% homology with c-Myc. Consistently, antibodies to *HAND2* immunoprecipitate *MYCN* but not c-Myc. Silencing *MYCN* and *HAND2* together inhibited the growth of NB cells to the same extent as silencing either gene alone. However, only the combined decrease of *HAND2* and *MYCN* significantly induced neurite-like extensions. RNA-seq after silencing either *MYCN* or *HAND2* revealed that ~45% genes regulated by *MYCN* (811 out of 1822 genes) were also regulated by *HAND2*. Gene set enrichment analysis after si *HAND2* showed a significant negative enrichment for MYC signature genes ( $p=0.05$ ,  $FDR=0.05$ ). For genes commonly regulated by both *MYCN* and *HAND2*, the combined silencing of *MYCN* and *HAND2* resulted in significantly increased changes of mRNA levels compared to silencing of either alone. Ingenuity pathway analysis (IPA) showed that the commonly regulated genes after silencing of both *MYCN* and *HAND2* are positively enriched in NGF and TRKA pathway (neural differentiation,  $p=0.05$ ) and negatively enriched in STAT3 pathway (proliferation,  $p=0.05$ ). Chromatin immunoprecipitation sequencing (ChIP-seq) using an anti-*HAND2* and *MYCN* antibodies identified that genes bound by both *HAND2* and *MYCN* are positively enriched in noradrenergic neuron differentiation ( $p=0.05$ ). Importantly, *HAND2* silenced cells resulted in decreased binding not only of *HAND2* but also *MYCN* on the same genomic locus and decreases in RNA Pol II on the associated gene bodies consistent with decreased transcription elongation.

### Summary/Conclusions:

Our results suggest a mechanism for NB tumorigenesis in which *MYCN* interacts with *HAND2* and forms a module on genomic DNA to facilitate the upregulation of *MYCN* signature genes. The *HAND2/MYCN* interaction may function to raise the threshold which sympathoadrenal progenitors must overcome to implement a differentiation program.



## **P066 Identification of core genes involved in high risk neuroblastoma without *MYCN* amplification by bioinformatic analysis**

Yuan, X.<sup>2</sup>, Du, S.<sup>3</sup>, Jin, Q.<sup>4</sup>

Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Department of Pediatric Hematology and Oncology, Shanghai, China <sup>2</sup>, Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Department of Pediatric Hematology and Oncology, Shanghai, China<sup>3</sup>, Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Department of Pediatric Hematology and Oncology, Shanghai, China<sup>4</sup>

### Background/Introduction:

Neuroblastoma (NB) is the most common extracranial malignancy in childhood and with great heterogeneity, high-risk NB usually has dismal long-term survival.

### Aims:

The aim of this study was to explore the differentially expressed genes (DEGs) of high-risk patients and to provide bioinformatics basis for the occurrence and treatment of neuroblastoma.

### Methods/Materials:

GSE49710 dataset from the Gene Expression Omnibus (GEO) database was downloaded and differentially expressed genes were analyzed by GEO2R tool. The gene ontology (GO) function analysis and pathway enrichment analysis of DEGs were carried out using the DAVID database and Metascape database<sup>3</sup> respectively. Then, the protein-protein interaction (PPI) network was constructed using the STRING database. Hub genes were screened using Cytoscape software and verified using the R2 Genomics Analysis and Visualization Platform. Finally, we obtained the transcription factor (TF) of hub genes using the Network Analyst online tool.

### Results:

A total of 209 DEGs were identified, which were mainly enriched in cell cycle, DNA replication, and p53 signaling pathway. Six genes (KIF20A, RRM2, CDK1, TOP2A, DLGAP5 and CCNB2) were selected as hub gene based on the degree of centrality. The overall survival reflected that high expression of these six crucial genes significantly predicted poor prognosis. Importantly, using the median of DLGAP5 mRNA expression as the cut-off points, multivariable Cox regression analysis showed that high levels of DLGAP5 mRNA expression was associated with poor patient overall survival and event-free survival, independent of disease stage, age at diagnosis and *MYCN* amplification status. TF analysis showed that MYBL2, SOX2 and FOXM1 were regulators of these six hub genes.

### Summary/Conclusions:

The identification of the crucial genes and pathways would contribute to the development of novel molecular targets and biomarker-driven treatments for high-risk neuroblastoma.

## **P067 A polycomb group protein RING1A preferentially mediates H2AK119 ubiquitination with supporting neuroblastoma cell proliferation.**

*Sato, S.<sup>2</sup>, Mariko, M.<sup>3</sup>, Ohira, M.<sup>4</sup>, Kamijo, T.<sup>5</sup>*

Saitama Cancer Center, Research Institute for Clinical Oncology, Saitama, Japan<sup>2</sup>, Dokkyo Medical University Koshigaya Hospital, Department of Pediatric Surgery, Saitama, Japan<sup>3</sup>, Saitama Cancer Center, Research Institute for Clinical Oncology, Saitama, Japan<sup>4</sup>, Saitama Cancer Center, Research Institute for Clinical Oncology, Saitama, Saitama Cancer Center<sup>5</sup>

### Background/Introduction:

Polycomb repressive complex (PRC) mediates histone modification and subsequent target gene silencing in various types of cancers as well as in early development. PRC2 encompasses EZH1/2 histone methyltransferases and involves H3K27 methylation. Recent studies have revealed that EZH2 contributes to malignant status of neuroblastoma (NB), dampening expression of tumor suppressor genes. On the other hand, the core complex of PRC1 is composed by RING1A/B E3 ligases and PCGF family proteins, which mono-ubiquitinates H2AK119. PRC1 is classified into several subtypes according to the PCGF family proteins (ex. with PCGF1 → PRC1.1), and they potentially control distinct target genes. Since PCGF4, also referred as BMI1, accelerates NB cell proliferation, PRC1 is expected to be oncogenic and druggable in NB. However, further investigations are required to understand behavior of the divergent PRC1 subtypes and to clarify the druggability.

### Aims:

In this study we have perturbed RING1A/B expression and challenged to uncover PRC1-mediated gene regulatory network as well as phenotypes in NB.

### Methods/Materials:

We employed shRNAs to suppress the expression of RING1A/B in NGP and SK-N-BE cells. The cell viability was quantified by WST-8 and flat colony formation assays. The RNA samples were subjected to SurePrint G3 Human GE microarray ver.3. Raw signals were normalized in GeneSpring GX. Differentially expressed genes were defined by one-way AVOVA test's q-value < 0.1 and fold change > 1.5.

### Results:

The knockdown of RING1A or RING1B impaired NB cell growth. Intriguingly, the RING1A knockdown remarkably attenuated H2AK119ub, whereas the RING1B knockdown did not. The double knockdown of RING1A/B resulted in H2AK119ub reduction, which was comparable to the RING1A single knockdown. These data indicate that RING1A preferentially mediates H2AK119ub in NB cells with a distinct mechanism from RING1B. To reveal the specific target genes of RING1A/B, we performed microarray analysis and then selected genes up-regulated in the double knockdown, specifically in the RING1A and RING1B knockdown (RING1A/B\_DKD\_up, RING1A\_KD\_up and RING1B\_KD\_up genes). Gene ontology analysis revealed that the RING1A/B\_DKD\_up genes were exclusively associated with organ morphogenesis and development. Moreover, ChIP-X enrichment analysis (ChEA) showed that the promoter regions of the RING1A/B\_DKD\_up genes were exclusively occupied by polycomb group proteins. These results suggest that physiological PRC1 function is remained in NB. Besides, we identified potential tumor suppressors in the RING1A\_KD\_up genes. Of note, ChEA displayed that KDM2B binding was exclusively enriched in the RING1A\_KD\_up genes promoter but not in that of the RING1B\_KD\_up genes. Since KDM2B is a component of PRC1.1, this result implies preferential association of RING1A to PRC1.1 to repress unique target gene expression in NB.

### Summary/Conclusions:

In our study, RING1A involves cell proliferation and preferentially mediated H2AK119ub with unique target gene silencing in NB. Moreover, according to ChEA, RING1A\_KD\_up genes were expected to



be regulated by PRC1.1, that encompasses RING1A and KDM2B. Although no compound is available for its inhibition currently, PRC1.1 is to be a potential therapeutic target in NB.

## **P068 Functional connection between neuroblastoma hotspot locus 6p22.3 lncRNAs and oncogenic/tumor suppressor pathways: Exploring molecular mechanisms to therapeutic approaches**

*Kanduri, C.<sup>2</sup>, Mitra, S.<sup>3</sup>, Juvvuna, P.K.<sup>4</sup>, Marco, M.D.<sup>5</sup>, Kosalai, S.T.<sup>6</sup>, Muralidharan, S.V.<sup>7</sup>, Raimondi, I.<sup>8</sup>, Ménard, M.<sup>9</sup>, Huarte, M.<sup>10</sup>, Weiss, W.A.<sup>11</sup>, Fischer, M.<sup>12</sup>, Kogner, P.<sup>13</sup>, Johnsen, J.I.<sup>14</sup>, Mondal, T.<sup>15</sup>*

Institute of Biomedicine, Gothenburg University, Department of Medical Biochemistry and Cell Biology, Gothenburg, Sweden <sup>2</sup>, Institute of Biomedicine, Gothenburg University, Department of Medical Biochemistry and Cell Biology, Gothenburg, Sweden<sup>3</sup>, Institute of Biomedicine, Gothenburg University, Department of Medical Biochemistry and Cell Biology, Gothenburg, Sweden<sup>4</sup>, Institute of Biomedicine, Gothenburg University, Department of Medical Biochemistry and Cell Biology, Gothenburg, Sweden<sup>5</sup>, Institute of Biomedicine, Gothenburg University, Department of Medical Biochemistry and Cell Biology, Gothenburg, Sweden<sup>6</sup>, Institute of Biomedicine, Gothenburg University, Department of Medical Biochemistry and Cell Biology, Gothenburg, Sweden<sup>7</sup>, University of Navarra, Cima, Pamplona, Spain<sup>8</sup>, University of California San Francisco, Departments of Neurology, Pediatrics, and Neurological Surgery, San Francisco, USA<sup>9</sup>, University of Navarra, Cima, Pamplona, Spain<sup>10</sup>, University of California San Francisco, Departments of Neurology, Pediatrics, and Neurological Surgery, San Francisco, USA<sup>11</sup>, Center for Molecular Medicine Cologne, University of Cologne, Institute for Neurophysiology, The Cologne Cluster of Excellence in Cellular Str, Cologne, Germany, <sup>12</sup>, Department of Women's and Children's Health, Karolinska Institutet, Childhood Cancer Research Unit, Stockholm, Sweden <sup>13</sup>, Department of Women's and Children's Health, Karolinska Institutet, Childhood Cancer Research Unit, Stockholm, Sweden <sup>14</sup>, Institute of Biomedicine, Gothenburg University, Department of Medical Biochemistry and Cell Biology, Gothenburg, Sweden <sup>15</sup>

### Background/Introduction:

Long noncoding RNAs (lncRNAs) have emerged as important players in cancer development and progression but mechanistically how they contribute to the disease remain less understood. Genome wide association studies (GWAS) have identified several loci which are associated with the aggressive neuroblastoma (NB) and till date the most significant locus associated with aggressive NB is 6p22.3, which harbors three lncRNAs *CASC15-003*, *CASC15-004* and *NBAT1* (6p22 lncRNAs). 6p22 lncRNAs show decreased expression in high-risk NB and their lower expression correlates with poor disease outcome. Previously, we demonstrated that 6p22 lncRNAs regulate differentiation of the NB cells by regulating REST/NRSF and SOX9/CHD7/USP36 pathways, and that their interplay determines undifferentiated state which is a major hall mark of NB.

### Aims:

6p22 lncRNAs in the regulation of oncogenic/tumor pathways that drive NB tumor aggressiveness

### Methods/Materials:

Tissues from NB patients and NB cell lines were used in the study.

Methods used in this study: Chromatin immunoprecipitation (ChIP), Luciferase assay, Immunocytochemistry (ICC), NB cell line derived xenografts, immunoblot, RNA extraction, sequencing and transcriptomic analysis, Proximity ligation assay (PLA), Immunoprecipitation and Ubiquitination assays.

### Results:

In the current study we have explored how the lower expression of 6p22.3 locus encoded lncRNAs influences p53 and *MYCN* dependent pathways that may contribute to tumor aggressiveness.

NB has low mutation rate for the p53 gene. Alternative ways of p53 inactivation have been proposed in NB via an abnormal cytoplasmic accumulation of wild-type p53. However, mechanisms leading to p53 inactivation via cytoplasmic accumulation are not well investigated. We show that NB risk-

associated 6p22.3 locus derived tumor suppressor lncRNA *NBAT1* is a p53 responsive lncRNA and regulates p53 sub-cellular localization. Low *NBAT1* expression provides resistance to genotoxic drugs by promoting cytoplasmic p53 accumulation. Altered CRM1 function in the *NBAT1* depleted cells contributes to p53 cytoplasmic accumulation and the loss of p53 dependent nuclear gene expression during genotoxic drug treatment. CRM1 inhibition rescues p53 dependent nuclear functions in the *NBAT1* depleted cells and sensitizes the cells to genotoxic drugs. Combined inhibition of CRM1 and MDM2 was even more effective in sensitizing aggressive NB cells with p53 cytoplasmic localization.

Interestingly, we found that the levels of 6p22 lncRNAs also correlate with *MYCN* protein levels. Overexpression of 6p22 lncRNAs in *MYCN* amplified cell lines and non-amplified cell lines leads to decreased *MYCN* protein levels whereas their KD resulted in stabilization of *MYCN*. We show that 6p22 lncRNA and its common interacting protein USP36, a ubiquitin-specific protease, determine *MYCN* levels. ICC and Co-IP studies reveal *MYCN* interaction with USP36 and their colocalization at the nucleolus, respectively. Importantly, we have characterized 6p22 lncRNAs/USP36/*MYCN* pathway regulated genes that can contribute to *MYCN* dependent NB disease progression.

#### Summary/Conclusions:

This study, using NB as a disease model system, has uncovered how lncRNA mediated oncogenic and tumor suppressor pathways converge to regulate cancer progression. Of note, our mechanistic studies of 6p22 lncRNA/p53/CRM1 propose a novel combination therapy for high-risk NB.

## **P069 ADRA1B as a novel pharmacological target for neuroblastoma**

*Broso, F.*<sup>2</sup>, *Gatto, P.*<sup>3</sup>, *Sidarovich, V.*<sup>4</sup>, *Adami, V.*<sup>5</sup>, *Tebaldi, T.*<sup>6</sup>, *Tonini, G.P.*<sup>7</sup>, *Quattrone, A.*<sup>8</sup>

University of Trento, Cellular, computational and integrative biology, Trento, Italy <sup>2</sup>, University of Trento, Cellular, computational and integrative biology, Trento, Italy<sup>3</sup>, University of Trento, Cellular, computational and integrative biology, Trento, Italy<sup>4</sup>, University of Trento, Cellular, computational and integrative biology, Trento, Italy<sup>5</sup>, University of Trento, Cellular, computational and integrative biology, Trento, Italy<sup>6</sup>, Città della Speranza, Pediatric Research Institute, Padova, Italy<sup>7</sup>, University of Trento, Cellular, computational and integrative biology, Trento, Italy<sup>8</sup>

### Background/Introduction:

High-risk neuroblastoma (NB) is an aggressive tumour with a survival rate below 50%. A drug combination strategy can provide new answers to this unmet therapeutic need. 13-cis retinoic acid (13-cisRA) is an anti-proliferative and pro-differentiative agent used in the post-consolidation phase of NB therapy, while naturally occurring polyphenols have been shown to exert anti-proliferative effects in several cancer cell lines. To identify molecules able to potentiate the activity of 13-cisRA, we screened a library of 169 natural compounds for their effects on the viability of the NB cells CHP134 challenged with a sub-lethal dose of 13-cisRA. The *in vitro* screening identified *isorhamnetin* as a synergistic molecule leading to 80% reduction in cell viability.

### Aims:

The project aims to identify the molecular basis of the synergism 13cis-RA and isorhamnetin, in order to explore its possible role as a novel therapeutic target for neuroblastoma treatment.

### Methods/Materials:

We generated NB cell lines knocked out for ADRA1B. We used two NB cell lines: CHP134, sensitive to 13cis-RA, and SK-N-AS, which are known to be unresponsive to the retinoid. Cells were treated with 13-cisRA alone or in combination with isorhamnetin. The cell response was evaluated via metabolic assay, apoptosis assay, differentiation assay, and gene expression analysis.

### Results:

We found that the synergistic effect of 13cis-RA and isorhamnetin is accompanied by a marked increase in the expression of the catecholamine receptor adrenergic receptor alpha-1B (ADRA1B). The exposure of CHP134 ADRA1B KO cells to 13-cisRA provokes a reduction of cell viability, accompanied by an activation of Caspase3/7, comparable with what occurs in WT cells treated with both 13cis-RA and isorhamnetin. Furthermore, CHP134 KO cells challenged with 13-cisRA show an increase in the percentage of neurite protrusion, hallmark of neural differentiation, confirmed at the molecular level by the rise of the expression of neural differentiation markers  $\beta$ 3-Tubulin and tyrosine hydroxylase. Nonetheless, we detected a dramatic overexpression of the transcription factor SOX9 both at mRNA and protein level, followed by a coherent promotion of HES1 mRNA transcription. None of these markers resulted to be induced in SK-N-AS NB cells exposed to 13-cisRA, neither if we KO ADRA1B. Consistently, SK-N-AS cells do not show neurite protrusion. Testing the effect of combining the alpha-adrenergic antagonist, L765,314, with 13-cisRA, we observed a decrease of cell viability in CHP134 WT cells in line with that of KO cells exposed to the retinoid.

### Summary/Conclusions:

We can conclude that the deletion of ADRA1B sensitizes NB cells to 13-CisRA in terms of induction of apoptosis and neural differentiation. Since NB is a catecholamine-rich tumor, we propose that the interruption by KO of ADRA1B of the pro-proliferative circuit generated by catecholamines in NB restores in cells the ability to undergo the pro-differentiative and pro-apoptotic programs promoted by 13-CisRA and mediated by activation of SOX9, which has already been reported to be necessary to 13-cisRA to exert its effects in human breast cancer cell lines. Considering the druggable nature of the

ADRA1B receptors, we indicate ADRA1B as a novel pharmacological target for treating neuroblastoma.

## **P070 Exploration of PHF6-RRM2 interaction as functional 'replitase' protein complex and its role in replicative stress control in neuroblastoma**

*Depestele, L.*<sup>2</sup>, *Sanders, E.*<sup>3</sup>, *Loontjens, S.*<sup>4</sup>, *Eggermont, A.*<sup>5</sup>, *Nunes, C.*<sup>6</sup>, *Lake, M.*<sup>7</sup>, *Delhaye, L.*<sup>8</sup>, *Bollen, M.*<sup>9</sup>, *Impens, F.*<sup>10</sup>, *Eyckerman, S.*<sup>11</sup>, *Durinck, K.*<sup>12</sup>, *Speleman, F.*<sup>13</sup>

University of Ghent, Biomolecular Medicine, Ghent, Belgium<sup>2</sup>, University of Ghent, Biomolecular Medicine, Ghent, Belgium<sup>3</sup>, University of Ghent, Biomolecular Medicine, Ghent, Belgium<sup>4</sup>, University of Ghent, Biomolecular Medicine, Ghent, University of Ghent<sup>5</sup>, University of Ghent, Biomolecular Medicine, Ghent, Belgium<sup>6</sup>, KU Leuven, Laboratory of Biosignaling & Therapeutics, Leuven, Belgium<sup>7</sup>, University of Ghent, Biomolecular Medicine, Ghent, Belgium<sup>8</sup>, KU Leuven, Laboratory of Biosignaling & Therapeutics, Leuven, Belgium<sup>9</sup>, VIB Center for Medical Biotechnology, VIB Center for Medical Biotechnology, Ghent, Belgium<sup>10</sup>, University of Ghent, Biomolecular Medicine, Ghent, Belgium<sup>11</sup>, University of Ghent, Biomolecular Medicine, Ghent, Belgium,<sup>12</sup> University of Ghent, Biomolecular Medicine, Ghent, Belgium<sup>13</sup>

### Background/Introduction:

*PHF6* encodes a plant homeodomain (PHD) factor containing two imperfect PHD zinc-finger domains. Insights into its multiple functions recently started to emerge. *PHF6* germline mutations causes cognitive disabilities while somatic mutations are frequent in T-cell acute leukemia (T-ALL). We and others found evidence for a role in self-renewal and lineage commitment during differentiation. Recent work revealed a role in non-homologous end joining, G2 checkpoint recovery and fork restart. We identified the ribonucleotide reductase subunit M2 (RRM2) as top *PHF6* interacting protein in T-ALL by IP-MS. RRM2, involved in dNTP production, plays a crucial role in DNA replication and repair and has been proposed to be part of a so-called 'replitase' that shuttles to the nucleus to secure dNTP concentrations during fork repair. In view of our discovery of RRM2 as a crucial dependency gene in neuroblastoma (NB), further insights into its regulation in NB cells could be critically important.

### Aims:

We aimed to expand our IP-MS analyses of *PHF6* to NB cell lines and to further explore the role of the presumed *PHF6*/RRM2 replitase in NB.

### Methods/Materials:

IP-MS analysis was done on four NB cell lines. Interaction for top ranked proteins is investigated by proximity ligation assays and co-localization experiments using immunofluorescence. The auxin-inducible degron technology was used to conditionally degrade *PHF6* in the colon cancer cell line HCT116 as a model and further tested in NB. Transcriptome (RNA-seq), genome-wide open chromatin (ATAC-seq) and protein-DNA interaction mapping (CUT&RUN) is being performed upon *PHF6* knock down.

### Results:

Additional IP-MS analysis confirmed RRM2 as top *PHF6* interactor in NB and also identified topoisomerase 2B (TOP2B, implicated in transcription and chromatin architecture), two components of the nuclear pore complex (RANBP2 and ELYS, implicated in nuclear transport, DNA replication and response to replicative stress), as additional high ranked interactors. These findings suggest that *PHF6* may act at the nuclear pore complex, possibly to control transcription and transcription-coupled DNA repair of highly transcribed cell identity genes. Interestingly, differential gene expression analysis after *PHF6* depletion revealed an enriched gene set related to binding of *PHF6* to the histone modification H3K56ac. H3K56ac is predominantly present at newly transcribed regions and is required for cell cycle re-entry after DNA repair. Based on these findings, we hypothesize that *PHF6* chromatin binding and association with H3K56ac is required to shuttle RRM2 towards sites of DNA repair and to subsequently release cells from checkpoint inhibition, in line with the role of *PHF6* in fork restart. In addition to the H3K56ac set, we also found enrichment for ribosome biogenesis gene sets, in keeping

with previously attributed functions of PHF6. Finally, also a strong correlation with MYC and TP53 targets was observed.

#### Summary/Conclusions:

We confirm PHF6-RRM2 interaction in NB cells and propose that PHF6 is a component of the 'replitase', implicated in active regulation of RRM2 function during replication and DNA repair. In addition, we propose a role for PHF6 in the gene gating process at the nuclear pore facilitating high level transcription and nuclear export for transcripts of cell identity genes.

## **P071 Alternative splicing in neuroblastoma generates RNA-fusion transcripts and is associated with vulnerability to spliceosome inhibitors**

Holmberg, J.<sup>2</sup>, Shi, Y.<sup>3</sup>, Rraklli, V.<sup>4</sup>, Maxymovitz, E.<sup>5</sup>, Li, S.<sup>6</sup>, Westerlund, I.<sup>7</sup>, Bedoya Reina, O.<sup>8</sup>, Yuan, J.<sup>9</sup>, Bullova, P.<sup>10</sup>, Juhlin, C.C<sup>11</sup>, Stenman, A.<sup>12</sup>, Larsson, C.<sup>13</sup>, Kogner, P.<sup>14</sup>, O'Sullivan, M.J.<sup>15</sup>, Susanne, S.<sup>16</sup>, Holmberg, J.<sup>17</sup>

Karolinska Institutet, Department of Cell and Molecular biology, Stockholm, Sweden <sup>2</sup>, Karolinska Institutet, Department of Cell and Molecular Biology, Stockholm, Sweden<sup>3</sup>, Karolinska Institutet, Department of Cell and Molecular Biology, Stockholm, Sweden<sup>4</sup>, Karolinska Institutet, Department of Cell and Molecular Biology, Stockholm, Karolinska Institutet<sup>5</sup>, Karolinska Institutet, Department of Microbiology, Tumor- and Cellbiology, Stockholm, Sweden<sup>6</sup>, Karolinska Institutet, Department of Cell and Molecular Biology, Stockholm, Sweden<sup>7</sup>, Karolinska Institutet, Department of Microbiology, Tumor- and Cellbiology, Stockholm, Sweden<sup>8</sup>, Karolinska Institutet, Department of Cell and Molecular Biology, Stockholm, Sweden<sup>9</sup>, Karolinska Institutet, Department of Microbiology, Stockholm, Sweden<sup>10</sup>, Karolinska Institutet, Department of Oncology-Pathology, Stockholm, Sweden<sup>11</sup>, Karolinska Institutet, Department of Oncology-Pathology, Stockholm, Sweden, <sup>12</sup>, Karolinska Institutet, Department of Oncology-Pathology, Stockholm, Sweden <sup>13</sup>, Karolinska Institutet, Department of Women's and Children's Health, Stockholm, Sweden <sup>14</sup>, Trinity College, Trinity Translational Medicine Institute, Dublin, Ireland <sup>15</sup>, Karolinska Institutet, Department of Microbiology, Tumor- and Cellbiology, Stockholm, Sweden <sup>16</sup>, Karolinska Institutet, Department of Cell and Molecular Biology, Stockholm, Sweden <sup>17</sup>

### Background/Introduction:

The paucity of recurrent mutations has hampered efforts to understand and treat neuroblastoma. Aberrant splicing and splicing dependent RNA-fusion transcripts have been implicated in other types of cancer, however their role in neuroblastoma remains largely unexplored.

### Aims:

We aim to investigate the presence and possible role of aberrant splicing and splicing dependent RNA-fusion transcripts in neuroblastoma. In addition, we attend to establish whether the spliceosome can be targeted to treat neuroblastoma.

### Methods/Materials:

Bioinformatic analysis of RNA-sequenced neuroblastoma datasets was performed to elucidate risk-associated changes in expression levels of spliceosome factors and to identify novel RNA-fusion transcripts. Comparative analysis with other types of tumors and non-transformed tissue was executed to confirm neuroblastoma specificity of identified fusion candidates. One candidate was selected for functional characterization in an *in vitro* assay of neuronal differentiation. Splicing activity was attenuated in neuroblastoma cells by the spliceosome inhibitor, Pladienolide B. PCR with primers spanning fusion junctions was performed to elucidate the impact of attenuated splicing on the expression of fusion transcripts compared to their wild type cognates. To understand if targeting the spliceosome can reduce tumor growth in a preclinical model, we treated mice that had been xenografted with neuroblastoma cells with the Pladienolide B spliceosome inhibitor.

### Results:

Elevated expression of splicing associated factors was a strong predictor of high-risk neuroblastoma and reduced patient survival. We identified >900 primarily intrachromosomal RNA-fusion transcripts generated by genes in close proximity. Fusions were enriched in chromosomal regions gained or lost in neuroblastoma and included well-known neuroblastoma oncogenes. As a proof-of-principle that a gene product with altered properties can be produced by these fusions, we characterized the *ZNF451-BAG2* fusion which generates a truncated BAG2-protein capable of inhibiting retinoic acid-induced differentiation. A majority of fusions contained canonical splicing sites and a subset exhibited increased sensitivity to spliceosome inhibition resulting in loss of expression after treatment. This



effect was accompanied by selectively induced apoptosis in neuroblastoma cells *in vitro* as well as reduced growth of neuroblastoma xenografts upon treatment with the spliceosome inhibitor Pladienolide B.

Summary/Conclusions:

Our findings elucidate a splicing dependent mechanism able to generate altered gene products, relevant for neuroblastoma pathogenesis and representing possible novel drug targets. In addition, we show that targeting the spliceosome is a potential strategy to treat neuroblastoma.

**P072 Low DLG2 gene expression, a link between 11q-deleted and MYCN-amplified neuroblastoma, causes forced cell cycle progression, and predicts poor patient survival.**

Ejeskär, K.,<sup>1</sup> Keane, S.<sup>2</sup>, Keane, S.<sup>3</sup>

University of Skövde, School of Health Sciences, Skövde, Sweden<sup>1</sup>, University of Skövde, School of Health Sciences, Skövde, Sweden<sup>2</sup>, University of Skövde, School of Health Sciences, Skövde, Sweden<sup>3</sup>

Background/Introduction:

There are two groups of aggressive neuroblastomas, one with *MYCN* amplification, and another with 11q chromosomal deletion; these chromosomal aberrations are generally mutually exclusive. The *DLG2* gene resides in the 11q-deleted region, thus makes it an interesting neuroblastoma candidate tumor suppressor gene.

Aims:

The aim of this study was to evaluate the possibility of *DLG2* to be a tumor suppressor gene, important for the biology of aggressive neuroblastoma.

Methods/Materials:

We evaluated the association of *DLG2* gene expression in neuroblastoma with patient outcomes, stage and *MYCN* status, using online microarray data combining independent neuroblastoma patient data sets. Functional studies were also conducted using neuroblastoma cell models and the fruit fly.

Results:

Using the array data we concluded that higher *DLG2* expression was positively correlated to patient survival. We could also see that expression of *DLG2* was inversely correlated with *MYCN* status and tumor stage. Cell proliferation was lowered in both 11q-normal and 11q-deleted neuroblastoma cells after *DLG2* over expression, and increased in 11q-normal neuroblastoma cells after *DLG2* silencing. Higher level of *DLG2* increased the percentage of cells in the G2/M phase and decreased the percentage of cells in the G1 phase. We detected increased protein levels of Cyclin A and Cyclin B in a fruit fly model when over expressing *dMyc* or silencing *dmDLG*, indicating that both events results in enhanced cell cycling. Induced *MYCN* expression in neuroblastoma cells caused lowered *DLG2* gene expression, this was confirmed in the fly, when *dMyc* was over expressed, the *dmDLG* protein level was lowered, indicating a link between *Myc* over expression and low *dmDLG* level.

Summary/Conclusions:

We conclude that low *DLG2* expression level forces cell cycle progression, and that it predicts poor neuroblastoma patient survival. The low *DLG2* expression level could be caused by either *MYCN*-amplification or 11q-deletion.

## **P073 Predicting induction chemotherapy using dynamic modelling of the p53 network**

Fey, D.<sup>2</sup>

University College Dublin, Systems Biology Ireland, Dublin, Ireland <sup>2</sup>

### Background/Introduction:

Neuroblastoma has a diverse clinical presentation and course. Risk classification of Neuroblastoma has refined treatments with reduced therapy for low- and intermediate-risk disease and intensive therapy in high-risk cases. Advances in therapy for these high-risk patients increased 5-year overall survival to 50% and includes intensive induction chemotherapy comprising high dosages of DNA-damaging agents. These agents are highly toxic and have severe short- and long-term side effects such as neutropenia, secondary tumours, infertility, bone necrosis, and irreversible hearing loss. Unfortunately, patient responses vary and about 20% of patients do not respond. Thus, predictive and prognostic markers for refining risk-stratified Neuroblastoma therapies present an urgent clinical need. The p53 signal transduction network (STN) is critical for mediating the cellular DNA damage response. In response to chemotherapeutics, the activation of p53 regulates critical cell fate decisions such as cell-cycle arrest and apoptosis. Hereby, the specific time- and dose-dependent p53 activation patterns are critical for deciding which cell fate is triggered. Because these dynamic activation patterns are difficult to understand intuitively, systems biology utilises mathematical models. Recently, mathematical models have also begun to emerge as a new concept in patient-stratification. In contrast to classic biomarkers that usually consist of snapshot measurements mathematical models describe drug response mechanisms using a set of dynamic mathematical rules. Inherently, these models can predict time-dependent changes using precise computer simulations.

### Aims:

Our aim was to understand how the p53 STN links genotype to phenotype, and how specific expression profiles of the p53 network components control the dynamic p53 response and influence patient survival.

### Methods/Materials:

We built a mathematical model of the p53 network and calibrated it on dynamic perturbation data from cell lines. The model contains patient-specific parameters that can be adjusted to measurements from patient tumour samples. Retrospective tumour profiling data from several neuroblastoma cohorts (n=688, n=249, DNA array; n=143 RNA-seq) were used to simulate the patient-specific responses and test whether the p53 response patterns associate with tumour aggressiveness and patient survival. Both, sub-stratification and multivariate Cox regression was used to correct for potential confounding factors such as *MYCN* amplification and risk (Children's Oncology Group, COG). Sensitivity analysis was used to identify optimal combination drug-targets for non-responding patients.

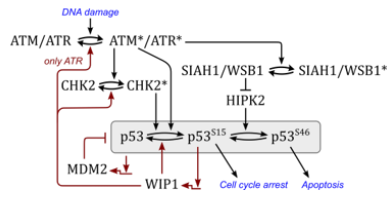
### Results:

We found several associations that link specific p53 STN response patterns to tumour aggressiveness and patient survival. In particular, the simulated p53 phosphorylation response of S15 (pS15) emerged as a robust prognostic marker in neuroblastoma, with increased pS15 responses indicating poor event-free and overall survival. In about 10% of the high-risk patients, the model predicted a highly activated pS15 cell-cycle-arrest signal and extremely poor event-free survival of less than 20%. The optimal drug-combination targets to re-instate the normal p53 activation pattern in these patients differed between patients. CHEK2 was the in optimal target in 69% of patients, SIAH1 in 28% of patients.

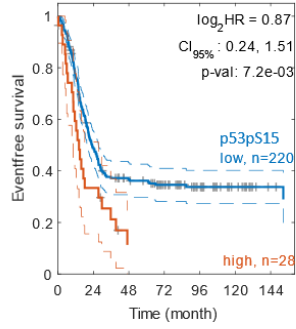
### Summary/Conclusions:

The results show that personalised mathematical modelling can help us to understand the p53 DNA responses of individual patient. Patient-specific simulations might be useful to predict therapy responses, stratify patients, and refine treatments.

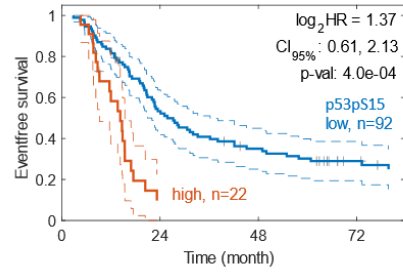
(A) p53 DNA-damage response model



(B) Training cohort: High risk patients



(C) 1<sup>st</sup> Validation cohort: High-risk patients



Caption 1: Patient stratification based on mathematical modeling of the p53 DNA damage response

## **P074 BET inhibition as monotherapy and combination therapy in MYC-driven neuroblastoma models**

*Firle, K.A.<sup>2</sup>, Szymansky, A.<sup>3</sup>, Witthauer, M.J.<sup>4</sup>, Dorado Garcia, H.<sup>5</sup>, Ahrens, K.<sup>6</sup>, Deubzer, H.<sup>7</sup>, Kuenkele, A.<sup>8</sup>, Henssen, A.G.<sup>9</sup>, Toedling, J.<sup>10</sup>, Hertwig, F.<sup>11</sup>, Eggert, A.<sup>12</sup>, Schulte, J.H.<sup>13</sup>*

Charité - Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin, Germany <sup>2</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin, Germany<sup>3</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin, Germany<sup>4</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin, Germany<sup>5</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin, Germany<sup>6</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin, Germany<sup>7</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin, Germany<sup>8</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin, Germany<sup>9</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin, Germany<sup>10</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin, Germany<sup>11</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin, Germany, <sup>12</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin, Germany <sup>13</sup>

### Background/Introduction:

MYC signaling is a predominant driver of high-risk neuroblastoma, caused either by amplification of *MYCN* or by activation of cMYC. The Bromodomain and Extra-terminal (BET) domain-containing protein BRD4 was reported to cooperate with *MYCN* in the epigenetic regulation of super-enhancer driven genes in neuroblastoma. Inhibition of BRD4 by small molecules such as JQ1 and OTX015 was shown to repress BET/*MYCN*-mediated transcriptional control and to exert antitumoural efficacy in *MYCN*-driven neuroblastoma. The BET inhibitor TEN-010, a derivative of JQ1, is currently in clinical trials for various MYC-driven adult tumours but its efficacy against neuroblastoma is yet to be established.

### Aims:

Our objective was to compare the antitumoural efficacy of TEN-010 to the previously published BET inhibitors and to evaluate its potential to be implemented in the clinical treatment regimen of high-risk neuroblastoma.

### Methods/Materials:

In a preclinical setup, we investigated the antitumoural activity of TEN-010 (n=18), OTX015 (n=12) and JQ1 (n=12) in a panel of neuroblastoma cell lines and fibroblasts. We employed an ATP detection assay to assess cell viability 72h after treatment. Western Blot and qPCR were used to measure changes in mRNA and protein expression. Effects on proliferation and cell death were investigated using colorimetric BrdU ELISA and Annexin V/7-AAD FACS analysis. To mimic the simultaneous administration of TEN-010 with conventional drugs of neuroblastoma study treatment, we subsequently turned towards combination therapies. We assessed combinations of TEN-010 with conventional chemotherapeutics (cisplatin, doxorubicin, etoposide, vincristine) in neuroblastoma cell lines IMR-5/75, CHP-134, NBL-S and SK-N-AS and fibroblast cell line BJ hTERT. Combinatorial effects were analysed using the Bliss Independence Model.

### Results:

In general, cell lines with high *MYCN*/*MYC* expression, according to RNA sequencing data, showed higher sensitivity to TEN-010 monotherapy than cell lines with low or moderate expression of these genes. Nine cell lines were highly sensitive to TEN-010 with IC50 values ranging 79nM to 632nM. Overall the efficacy of TEN-010 was comparable to the documented efficacies of JQ1 and OTX015. Treatment with TEN-010 exerted inhibition of proliferation and induction of cell death, which was

confirmed by BrdU ELISA and Annexin V/7-AAD FACS analysis. The efficacy of TEN-010 monotherapy was further enhanced when cells were treated for longer durations up to seven days. Western Blot and qPCR confirmed on-target activity of TEN-010 by upregulation of the bona-fide response marker HEXIM1. Investigating combinatorial treatment approaches, we observed synergistic interactions of TEN-010 and cisplatin or vincristine in *MYCN*-driven neuroblastoma cell lines, while combinations with etoposide or doxorubicin indicate additive effects. Conversely, all combinations except for etoposide showed antagonistic or additive interactions in fibroblasts, suggesting a potential benefit on toxic side effects.

#### Summary/Conclusions:

Our results support the notion that BET proteins have crucial functions in *MYC/MYCN*-driven neuroblastoma and promote BET inhibitors, such as TEN-010, as an effective, beneficial addition to current standard therapy regimen for high-risk neuroblastoma.

## **P075 Multi-omics network inference identifies novel drug targets in high risk neuroblastoma**

Vermeirssen, V.<sup>2</sup>, Bartenhagen, C.<sup>3</sup>, Fischer, M.<sup>4</sup>, Speleman, F.<sup>5</sup>, De Preter, K.<sup>6</sup>

Ghent University, Biomedical Molecular Biology & Biomolecular Medicine, Ghent, Belgium <sup>2</sup>, University of Cologne, Experimental Pediatric Oncology, Cologne, Germany<sup>3</sup>, University of Cologne, Experimental Pediatric Oncology & Center for Molecular Medicine Cologne (CMMC), Cologne, Germany<sup>4</sup>, Ghent University, Biomolecular Medicine, Ghent, Ghent University<sup>5</sup>, Ghent University, Biomolecular Medicine, Ghent, Belgium<sup>6</sup>

### Background/Introduction:

Neuroblastoma, an aggressive embryonal tumor of the sympathetic nervous system, is dominated by DNA copy number alterations. Tumors with segmental chromosomal aberrations are associated with poor survival. Although some oncogenic driver genes e.g. *MYCN* have been identified in neuroblastoma, many drivers remain to be found on the major recurrent DNA copy number alterations such as 2p and 17q.

Regulatory networks provide an in depth, systems-level understanding of complex biological processes and their perturbed regulation upon disease. Network inference, the prediction of regulatory networks from omics data and a set of potential regulators (e.g. driver genes), is known to benefit from different algorithms and data types.

Since cancer driving DNA copy number alterations are thought to target genes in a relatively small number of signaling and regulatory pathways, regulatory networks can provide a framework to study the convergence of these alterations (i.e. driver genes) on specific network modules (i.e. driver pathways).

### Aims:

We hypothesized that one or more dosage sensitive genes on the gained copy number regions act as cooperative drivers during neuroblastoma development. Through integrative computational analysis and network inference, we aim to identify these causal drivers and the regulatory networks in which they operate, since these may open up new avenues for targeted therapy.

### Methods/Materials:

On available matching transcriptome, copy number alteration and clinical data for > 200 primary tumor samples, we applied several multi-omics data integration strategies, including the multi-omics module network inference algorithm Lemon-Tree and different data in the regulator assignment such as expression data for genes on the recurrently amplified 2p and 17q regions and for all transcription factors. Using ensemble methodologies, we clustered genes in consensus co-expression modules and predicted a prioritized list of drivers and regulators for each of them. We characterized modules and drivers in the regulatory network through network analysis, functional enrichment analysis, clinical data correspondence and further integration with other data.

### Results:

Module network inference resulted in over 150 tight co-expression modules containing over 8000 genes. Over 90% of the modules showed significant functional enrichment, often related to the hallmarks of cancer. Retaining the 1% top predictions, we identified novel drivers on chromosomes 2p and 17q as well as additional transcriptional regulators. Several predicted drivers are known to participate in biological pathways overrepresented in the co-expression modules. Amongst others, we detected *BRIP1* and *RRM2* as neuroblastoma driver genes, for which we have gathered preclinical evidence already (see abstracts Vanhauwaert et al., Nunes et al.). Correlation to neuroblastoma specific transcriptomic signatures and survival data provided further validation of the predicted modules and drivers. We discuss the obtained hypotheses in light of known experimental findings in neuroblastoma. We are currently integrating patient single cell transcriptome data with this reference regulatory network for patient-specific drug target identification.

### Summary/Conclusions:

In conclusion, we report on the relevance of multi-omics network inference for identifying novel key driver genes and regulators of neuroblastoma. We provide a neuroblastoma reference regulatory network, from which novel drug targets and perturbed biological pathways for specific patient subtypes are identified.



## **P076 The neuroblastoma surfaceome is regulated by adrenergic or mesenchymal subtype-specific transcriptional regulatory networks.**

*Kendsersky, N.<sup>2</sup>, Yarmarkovich, M.<sup>3</sup>, Rokita, J.L.<sup>4</sup>, Maris, J.<sup>5</sup>*

University of Pennsylvania, Departments of Medicine and Pharmacology, Philadelphia, USA <sup>2</sup>, Children's Hospital of Philadelphia, Department of Oncology, Philadelphia, USA<sup>3</sup>, Children's Hospital of Philadelphia, Department of Biomedical and Health Informatics, Philadelphia, USA<sup>4</sup>, Children's Hospital of Philadelphia, Department of Oncology, Philadelphia, Children's Hospital of Philadelphia<sup>5</sup>

### Background/Introduction:

While the implementation of GD2 immunotherapy has improved high-risk neuroblastoma outcomes, many patients experience significant acute toxicities and relapse. Thus, identifying new cell-surface targets that are linked to crucial gene regulatory networks could improve patient responses, limit toxic side-effects and mitigate immune evasion via downregulation of the target.

### Aims:

To develop an integrated epigenomics and RNA-sequencing-based approach to identify neuroblastoma-specific surface molecules in adrenergic and/or mesenchymal subtypes that may be amenable to synthetic immunotherapies.

### Methods/Materials:

We utilized singscore (ssGSEA) with published adrenergic and mesenchymal gene signatures to subset high-risk neuroblastoma patient samples (TARGET RNA-seq, adrenergic=130, mesenchymal=11). We used limma voom to determine differentially expressed genes in each neuroblastoma subtype compared to 1641 normal tissue samples from 25 organs (GTEx). We processed ChIP-seq data of transcription factors in the adrenergic core regulatory circuit (CRC) in two adrenergic neuroblastoma cell lines (KELLY and SKNBE2C) and applied the bedtools and HOMER toolkits to identify CRC-bound differentially expressed genes. Finally, the differentially expressed genes in each neuroblastoma subtype were further analyzed for cell surface localization using the COMPARTMENTS database or referencing the neuroblastoma ligandome via coimmunoprecipitation of MHC Class I and II coupled to mass-spectrometry (see Yarmarkovich, ANR 2020).

### Results:

For the adrenergic subtype, we first showed that genes bound by 6+ CRC transcription factors were significantly more likely to be differentially expressed compared to GTEx normal samples (3.6-fold enrichment; zscore=12.67). Of the 116 adrenergic differentially expressed genes, 41 (35%) were bound by 6+ CRC transcription factors, and 26 of these 41 genes (63%) were either resident cell surface proteins or detected as MHC-bound peptides. These included several proteins currently in clinical development (ALK, L1CAM, and SLC6A2 [NET]). As expected, the mRNA expression of many adrenergic-specific targets was much lower or completely absent in the mesenchymal subtype. We identified 2 mesenchymal-specific genes, and several genes present in both subtypes, that are differentially expressed compared to GTEx normal samples. Notably, the peripherin gene (PRPH) had similar mRNA expression in both adrenergic and mesenchymal patient samples and showed broad H3K27Ac signal in cell lines of each neuroblastoma subtype. Finally, we showed that 8 peptides derived from PRPH were detected in the neuroblastoma ligandome and 4/8 primary neuroblastoma samples presented at least one peptide. Moreover, 3/8 neuroblastoma samples presented a common PRPH peptide that was predicted to bind to HLA-A0201 with an affinity of 9.7 nM (NetMHC4.0). Overall, many candidate immunotherapeutic targets are adrenergic-specific, the exceptions being L1CAM and several MHC-presented peptides derived from PHOX2A, PHOX2B, or PRPH that are present in both adrenergic and mesenchymal neuroblastoma subtypes.

### Summary/Conclusions:

Identification of CRC-bound genes encoding cell surface proteins or MHC-presented peptides can help prioritize immunotherapeutic targets for clinical development. Targeting proteins expressed in both the adrenergic and mesenchymal subtypes could abrogate classical mechanisms of immune escape such as selection of subclones with low target expression.



## **P078 ALYREF/USP3/MYCN signaling axis is a novel molecular vulnerability in high-risk neuroblastoma**

*Nagy, Z.<sup>2</sup>, Seneviratne, J.<sup>3</sup>, Kanikevich, M.<sup>4</sup>, Chang, W.<sup>5</sup>, Mayoh, C.<sup>6</sup>, Du, Y.<sup>7</sup>, Jiang, C.<sup>8</sup>, Koach, J.<sup>9</sup>, Carter, D.<sup>10</sup>, Liu, T.<sup>11</sup>, Haber, M.<sup>12</sup>, Norris, M.<sup>13</sup>, Cheung, B.<sup>14</sup>, Marshall, G.<sup>15</sup>*

Children's Cancer Institute, Embryonal Cancer Therapeutics and Prevention, Sydney, Australia <sup>2</sup>, Children's Cancer Institute, Embryonal Cancer Therapeutics and Prevention, Sydney, Australia<sup>3</sup>, Children's Cancer Institute, Embryonal Cancer Therapeutics and Prevention, Sydney, Australia<sup>4</sup>, Children's Cancer Institute, Embryonal Cancer Therapeutics and Prevention, Sydney, Children's Cancer Institute<sup>5</sup>, Children's Cancer Institute, Bioinformatics Team Computational Biology, Sydney, Australia<sup>6</sup>, School of Life Sciences and Technology Tongji University, School of Life Sciences and Technology, Shanghai, China<sup>7</sup>, School of Life Sciences and Technology Tongji University, School of Life Sciences and Technology, Shanghai, China<sup>8</sup>, Children's Cancer Institute, Embryonal Cancer Therapeutics and Prevention, Sydney, Australia<sup>9</sup>, Children's Cancer Institute, Embryonal Cancer Therapeutics and Prevention, Sydney, Australia<sup>10</sup>, Children's Cancer Institute, Embryonal Cancer Therapeutics and Prevention, Sydney, Australia<sup>11</sup>, Children's Cancer Institute, Embryonal Cancer Therapeutics and Prevention, Sydney, Australia, <sup>12</sup>, Children's Cancer Institute, Professor and Deputy Director, Sydney, Australia <sup>13</sup>, Children's Cancer Institute, Embryonal Cancer Therapeutics and Prevention, Sydney, Australia <sup>14</sup>, Children's Cancer Institute, Embryonal Cancer Therapeutics and Prevention, Sydney, Australia <sup>15</sup>

### Background/Introduction:

Segmental copy-number gains occur most frequently on Chr17q21-ter and strongly predict adverse outcomes in neuroblastoma (NB). Target genes in this region are poorly characterised and actionable molecular targets have yet to be discovered for these patients.

### Aims:

Our aim was to identify novel molecular targets for diagnosis and therapy of high-risk NB with 17q21-ter gain.

### Methods/Materials:

We first used bioinformatic techniques to systematically investigate genes on the 17q21-ter amplicon for independent associations with NB prognosis. For mechanistic studies, we used ChIP sequencing, ubiquitin assay, protein half-life studies, quantitative PCR, western blotting, co-immunoprecipitation, cell viability, cell proliferation and colony formation assay. For in vivo studies, microarray on ganglia from homozygote TH-*MYCN* transgenic mice (Ooi et al, 2018), and Balbc/nude NB xenograft models were used.

### Results:

We investigated genes on the 17q21-ter amplicon and identified ALYREF as a key regulator of NB. High ALYREF expression correlated with poor overall survival in a large cohort of primary NB patient tumour samples (n = 498, SEQC dataset). Multivariate analysis showed that high ALYREF expression was independently prognostic compared with other clinical features in NB. *MYCN* is a major driver of tumorigenesis in NB, and high ALYREF expression correlated with *MYCN* expression. Microarray analysis carried out on the ganglia tissues of homozygote TH-*MYCN* transgenic NB mice showed that ALYREF was significantly up-regulated at *MYCN*-driven tumour initiation. Mechanistic experiments revealed that *MYCN* is functionally dependent on ALYREF and the two proteins act in a positive forward feedback expression loop to promote NB progression. ALYREF exerts its effect on NB progression by directly binding to the region between MB Box III and IV of *MYCN* in the nucleus. Stable genetic suppression of ALYREF expression reduced cell viability and colony formation of *MYCN*-amplified NB cell lines. Most importantly, ALYREF knock-down in *MYCN* amplified NB cells resulted in a significant inhibition of tumorigenesis *in vivo*. Genome-wide profile of ALYREF-chromatin interactions using ChIP sequencing suggested that ALYREF promotes tumour formation by

transcriptionally up-regulating the ubiquitin-specific protease 3 (USP3). USP3 forms a protein complex with *MYCN* and protects it from ubiquitin-mediated degradation.

Summary/Conclusions:

Our findings suggest a crucial role for ALYREF in high risk NB through effects on *MYCN* ubiquitination and suggest that inhibition of ALYREF-mediated *MYCN* turnover is a potential therapy for high-risk NB patients.

## **P079 MYCN-KDM6B axis regulates an Rb-E2F transcription program that is positively correlated with GSK-J4 sensitivity**

*Yang, J.*<sup>2</sup>, *D'Oto, A.*<sup>3</sup>, *Fang, J.*<sup>4</sup>, *Mullasseril, A.*<sup>5</sup>, *Abu-Zaid, A.*<sup>6</sup>, *Singh, S.*<sup>7</sup>, *Xu, B.*<sup>8</sup>, *Von Buttlar, X.*<sup>9</sup>, *Jones, V.*<sup>10</sup>, *Hu, D.*<sup>11</sup>, *Wallace, K.*<sup>12</sup>, *Murphy, A.*<sup>13</sup>, *Davidoff, A.*<sup>14</sup>

ST JUDE CHILDREN'S RESEARCH HOSPITAL, SURGERY, Memphis, USA <sup>2</sup>, ST JUDE CHILDREN'S RESEARCH HOSPITAL, SURGERY, Memphis, USA<sup>3</sup>, ST JUDE CHILDREN'S RESEARCH HOSPITAL, SURGERY, Memphis, USA<sup>4</sup>, ST JUDE CHILDREN'S RESEARCH HOSPITAL, SURGERY, Memphis, ST JUDE CHILDREN'S RESEARCH HOSPITAL<sup>5</sup>, ST JUDE CHILDREN'S RESEARCH HOSPITAL, SURGERY, Memphis, USA<sup>6</sup>, ST JUDE CHILDREN'S RESEARCH HOSPITAL, SURGERY, Memphis, USA<sup>7</sup>, ST JUDE CHILDREN'S RESEARCH HOSPITAL, Computational Biology, Memphis, USA<sup>8</sup>, ST JUDE CHILDREN'S RESEARCH HOSPITAL, SURGERY, Memphis, USA<sup>9</sup>, ST JUDE CHILDREN'S RESEARCH HOSPITAL, SURGERY, Memphis, USA<sup>10</sup>, ST JUDE CHILDREN'S RESEARCH HOSPITAL, SURGERY, Memphis, USA<sup>11</sup>, ST JUDE CHILDREN'S RESEARCH HOSPITAL, SURGERY, Memphis, USA, <sup>12</sup>, ST JUDE CHILDREN'S RESEARCH HOSPITAL, SURGERY, Memphis, USA <sup>13</sup>, ST JUDE CHILDREN'S RESEARCH HOSPITAL, SURGERY, Memphis, USA <sup>14</sup>

### Background/Introduction:

Although outcomes of children with low- or intermediate-risk neuroblastoma are excellent, overall survival rates for those with high-risk neuroblastoma, including N-MYC amplified tumors, remain less than 40%, despite the use of intensive multimodal therapies. Thus, there is a pressing need to identify novel therapeutic targets for *MYCN*-amplified neuroblastoma. The H3K27me3/me2 demethylase KDM6B is a promising therapeutic target although the role of KDM6B in neuroblastoma is not clear.

### Aims:

The objective of this study was to understand how KDM6B connects to key oncogenic pathways and identify the molecular markers predicting sensitivity to the KDM6B inhibitor GSK-J4.

### Methods/Materials:

KDM6B expression in tumor cohorts and normal neural crest tissues was analyzed using the R2 microarray program. RNA-seq or microarray was performed for gene pathway analysis. The impact of epigenome of *MYCN* and KDM6B was analyzed using ChIP-seq and ATAC-seq. Chemoinformatics was used to analyze the association of KDM6B inhibition with the CDK4/6 inhibitor palbociclib. Cell lines with overexpression of *MYCN* or CDK4/6 were generated by transduction of retroviral or lentiviral based vectors. Crystal violet staining was performed to assess the effect on cell viability.

### Results:

*MYCN* broadly binds at KDM6B genomic locus, in line with a broad occupancy of active transcription marks, H3K4me1 and H3K27Ac. Genetic or pharmacologic inhibition of KDM6B leads to downregulation of the E2F transcriptome. Chemical genetics data showed that high E2F activity of cancer cells but not KDM6B itself is positively correlated with sensitivity to the KDM6 inhibitor GSK-J4. KDM6B inhibition transcriptomically phenocopies the specific CDK4/6 inhibitor palbociclib and vice versa. Mechanistically, inhibition of KDM6B activity reduced the chromatin accessibility of E2F target genes, accompanying with elevated repressive transcription mark H3K27me3. Overexpression of CDK6 conferred neuroblastoma cell resistance to palbociclib but these cells retained sensitivity to the KDM6B inhibitor GSK-J4.

### Summary/Conclusions:

*MYCN*-KDM6B interacts with the CDK4/6-Rb-E2F pathway in neuroblastoma cells, revealing a new alternative strategy to target KDM6B for cancer cells resistant to CDK4/6 inhibitors.



## **P080 Modifying methylation-sensitive binding of *MYCN* by targeted DNA demethylation using CRISPR/Cas9-TET1 fusion proteins**

*Wehrmann, L.*<sup>2</sup>, *Henrich, K.O.*<sup>3</sup>, *Okonechnikov, K.*<sup>4</sup>, *Herrmann, C.*<sup>5</sup>, *Pfister, S.*<sup>6</sup>, *Westermann, F.*<sup>7</sup>

Hopp-Children's Cancer Center (KiTZ) & German Cancer Research Center (DKFZ), Neuroblastoma Genomics, Heidelberg, Germany <sup>2</sup>, Hopp-Children's Cancer Center (KiTZ) & German Cancer Research Center (DKFZ), Neuroblastoma Genomics, Heidelberg, Germany<sup>3</sup>, Hopp-Children's Cancer Center (KiTZ) & German Cancer Research Center (DKFZ), Pediatric Neurooncology, Heidelberg, Germany<sup>4</sup>, University Clinics Heidelberg, Health Data Science Unit, Heidelberg, University Clinics Heidelberg<sup>5</sup>, Hopp-Children's Cancer Center (KiTZ) & German Cancer Research Center (DKFZ), Pediatric Neurooncology, Heidelberg, Germany<sup>6</sup>, Hopp-Children's Cancer Center (KiTZ) & German Cancer Research Center (DKFZ), Neuroblastoma Genomics, Heidelberg, Germany<sup>7</sup>

### Background/Introduction:

Amplified *MYCN* is found in 25% of neuroblastoma (NB) cases and is associated with high-risk disease and poor outcome. DNA methylation based-clustering identifies patient subgroups strongly associated with NB key clinical and biological features. The group of *MYCN*-amplified NBs is characterized by aberrant methylation of regulatory DNA elements being in line with an impact of *MYCN* on DNA methylation patterns. This aberrant methylation leads to pronounced transcriptomic and chromatin landscape changes that mediate tumor de-differentiation. Further, recent data suggest *MYCN*-binding itself to be highly responsive to DNA methylation creating an image of *MYCN* guiding its own binding profile via methylation.

### Aims:

Elucidate how methylation-sensitive binding of *MYCN* shapes *MYCN*-regulated gene expression programs. With an integrated approach analyzing DNA methylation, gene expression and *MYCN*-binding we plan to considerably contribute to the understanding of epigenetic patterns in high-risk NB and potentially identify candidate genes deregulated by aberrant *MYCN* E-Box methylation.

### Methods/Materials:

We used drug-mediated demethylation of SK-N-BE(2)C cells and globally assessed *MYCN*-binding via ChIP-sequencing analysis. High resolution DNA methylation changes were assessed by whole-genome bisulfite sequencing. The consequences of DNA methylation-associated *MYCN* redistribution were monitored by RNA-sequencing and chromatin mark ChIP-sequencing.

In order to validate candidate elements controlled by *MYCN*, we established a system for targeted epigenetic editing using CRISPR/Cas9-TET1 fusion proteins allowing us to specifically demethylate regions of interest.

### Results:

We could show methylation-sensitive binding of *MYCN* leading to thousands of new *MYCN* binding sites upon demethylation. *MYCN* binding interestingly occurred independently of a classical E-box preference, which is the only E-Box harboring a central CpG, or accumulation at distinct genomic regions. In accordance with other published *MYCN* profiles, *MYCN* binding was only associated with changes in gene expression in the minority of cases.

The bulk of newly occurring *MYCN* binding sites harbored CpGs that were already hypomethylated before pointing towards a hierarchy of additional regulatory mechanisms including chromatin compaction and/or methylation-dependent binding partners that guide *MYCN* to its target sequences.

New *MYCN* binding sites that also correlated with differential gene expression were predominantly found at distal elements, whereas promoter-associated binding sites seemed to be already established before and less affected by demethylation.



We could successfully de-repress *MYCN*-regulated genes by dCas9-TET1 mediated targeted demethylation of priori identified methylation-sensitive binding sites to further study *MYCN*-regulated networks.

#### Summary/Conclusions:

With this integrated analysis we considerably contribute to the understanding of epigenetic patterns in high-risk NB by showing methylation-sensitive binding of *MYCN* in NB cell lines. Further, we could potentially identify candidate genes involved in pro-differentiation programs deregulated by aberrant methylation having significant clinical relevance for high-risk NB patients.

## **P081 The histone demethylase KDM6B modulates Neuroblastoma stemness and cancer stem cell survival**

*Huang, T.*<sup>2</sup>, *Rozen, E.*<sup>3</sup>, *Wigglesworth, K.*<sup>4</sup>, *Shohet, J.*<sup>5</sup>

Umass Medical School, Dept of Pediatrics, Worcester, USA <sup>2</sup>, Umass Medical School, Dept of Pediatrics, Worcester, USA<sup>3</sup>, Umass Medical School, Dept of Pediatrics, Worcester, USA<sup>4</sup>, Umass Medical School, Dept of Pediatrics, Worcester, Umass Medical School<sup>5</sup>

### Background/Introduction:

We have previously characterized a cancer stem cell (CSC) subpopulation in neuroblastoma based on expression of the G-CSF receptor (CD114). These CSCs are present in all neuroblastoma cell lines, PDXs, and primary tumor specimens typically representing less than 1% of the total tumor cell population. FACS purified CD114+ NB cells self-renew, differentiate and recapitulate heterogeneous tumors *in vivo*. They are approximately 10 fold more tumorigenic than CD114 negative tumor cells based on limiting dilution assays. Previously we demonstrated the KDM6B (aka JMJD3 - Jumonji D3 protein) is over expressed in CD114+ CSCs and we and others have shown that KDM6B inhibition a) dramatically reduces CD114+ subpopulation b) induces tumor regression *in vivo*. This histone demethylase specifically targets the repressive chromatin modification of H3K27me3 formed by PRC2 activity.

### Aims:

We therefore sought to evaluate how gain of function of KDM6B impacts cancer stem cell survival and stemness phenotypes in NB cell lines and xenografts.

### Methods/Materials:

We used the dCAS9-VPR system to specifically up-regulate the endogenous KDM6B locus. Stable SHSY5Y and NGP NB cell lines expressing dCAS9 fused to VP64-p65-Rta (dCAS9-VPR) were generated by lentiviral transduction. Subsequent introduction of sgRNA guides targeting the KDM6B promoter or control guides allowed us to isolate stable lines expressing control or elevated levels of endogenous KDM6B. Western Blot and qPCR assays demonstrated a modest 2-fold increase in JMJD3 expression.

### Results:

To determine if KDM6b expression altered the CD114+ subpopulation, we assessed CD114 expression for flow cytometry in JMJD3 high and control NB cell lines. We found that increased KDM6B expression consistently increased the proportion of CD114+ cells in SH-SY5Y to 0.8% from 0.3-0.4% for control SH-SY5Y cells, and in NGP cells to 1.1% from 0.7% for control NGP cells.

Next, we evaluated the ability of the KDM6B high and low cell lines to establish colonies from single cells under standard culture conditions and from tumor spheres in stem cell media at limiting dilution. We found that the modest increase in KDM6B expression dramatically enhanced both these *in vitro* surrogate assays for stemness. Single cell Colony formation on 96 well plate increased from 20 to 31 ( $p < 0.05$ ) and tumor sphere formation increased 2.5 folds ( $p < 0.001$ ). a representative illustration is below.

### Summary/Conclusions:

Up-regulation of endogenous KDM6B increases the *in vitro* stemness phenotypes of NGP and SHY5Y as assayed by colony formation and sphere formation. Ongoing *in vivo* tumorigenesis assays by orthotopic injection and tail vein injection models are testing the relative tumorigenicity of the KDM6B-high and control cell lines. We also demonstrate the utility of dCAS-VPR for sgRNA mediated target gene regulation in neuroblastoma cell lines.

Picture 1: [https://www.eventure-online.com/parthen-uploads/89/0ANR/add\\_1\\_614765\\_be6dc96f-a669-4a25-97cc-05dea159d9c0.png](https://www.eventure-online.com/parthen-uploads/89/0ANR/add_1_614765_be6dc96f-a669-4a25-97cc-05dea159d9c0.png)

## **P082 Synergistic activity of HDAC inhibitor Panobinostat and ALK inhibitor Lorlatinib in neuroblastoma cell lines harboring mutated ALK**

*Linke, R.B.<sup>2</sup>, Finkler, S.<sup>3</sup>, Wünschel, J.<sup>4</sup>, Peitz, C.<sup>5</sup>, Astrahantseff, K.<sup>6</sup>, Kuenkele, A.<sup>7</sup>, Toedling, J.<sup>8</sup>, Bluethgen, N.<sup>9</sup>, Kopp-Schneider, A.<sup>10</sup>, Lodrini, M.<sup>11</sup>, Eggert, A.<sup>12</sup>, Deubzer, H.E.<sup>13</sup>*

Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin, Germany <sup>2</sup>, Charité Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin, Germany<sup>3</sup>, Charité Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin, Germany<sup>4</sup>, Charité Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin, Germany<sup>5</sup>, Charité Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin, Germany<sup>6</sup>, Charité Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin, Germany<sup>7</sup>, Charité Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin, Germany<sup>8</sup>, Charité Universitätsmedizin Berlin, Computational Modelling in Medicine, Berlin, Germany<sup>9</sup>, German Cancer Research Center, Biostatistics, Heidelberg, Germany<sup>10</sup>, Charité Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin, Germany<sup>11</sup>, Charité Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin, Germany, <sup>12</sup>, Charité Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin, Germany <sup>13</sup>

### Background/Introduction:

Targeted treatment approaches for patients with high-risk neuroblastoma are required in case treatment failure occurs after first- or second-line therapies. These approaches should ideally be well tolerated as many patients have already experienced significant therapy-related morbidity at that stage.

### Aims:

Inhibition of histone deacetylase (HDAC) activity by the clinically approved HDAC inhibitor Panobinostat has significant anti-tumor effects in preclinical models of *MYCN*-amplified and *TERT*-rearranged neuroblastoma, however, effects as stand-alone therapy are not curative in most cases. The purpose of this study was the identification of drugs that act synergistically with Panobinostat in neuroblastoma cells.

### Methods/Materials:

We screened four chemotherapeutic drugs implemented in first-line therapies and five targeted drugs, which are used in second-line therapies or are currently being tested in phase II or III clinical trials, for synergistic effects with Panobinostat in two neuroblastoma cell lines using the Cell Titer Glo Luminescent *Cell Viability assay*. Each drug was applied in six different concentrations as stand-alone or combination therapy, and results were tested for synergy using the *SynergyFinder* software. Cell death assays were conducted with the top six drug combinations. A two-factorial mixed linear model with interaction and random effect for each experimental run was used to assess results for potential synergistic effects of the two-drug combinations using SAS PROC MIXED.

### Results:

Altogether, two chemotherapeutic agents (doxorubicine, temozolomide) and four targeted drugs inhibiting tyrosine kinases, mTOR, the proteasome or ALK attenuated neuroblastoma cell proliferation synergistically when combined with Panobinostat. Strongest synergistic killing effects were observed in combination treatment with the ALK inhibitor Lorlatinib. Findings were confirmed in six neuroblastoma cell lines harboring mutated *ALK*. Effects of the two-drug combination on cell cycle, DNA damage and replication stress response as well as on phosphorylation levels of selected proteins is investigated at the single-cell level using a *CyTOF* mass cytometer in ongoing experiments.

### Summary/Conclusions:

Panobinostat is effective against preclinical high-risk neuroblastoma models, and its effects can be strongly enhanced through simultaneous targeting of mutated *ALK*. This promising combination could be an important step towards improved second-line treatments for patients with high-risk neuroblastoma in case of treatment failure and warrants further testing in the framework of a clinical trial.

## **P083 Adrenergic-lineage genes are epigenetically repressed in immature MES-type neuroblastoma cells**

*Valentijn, L.J.<sup>2</sup>, Muñoz-Lasso, D.C.<sup>3</sup>, Zwijnenburg, D.A.<sup>4</sup>, Hasselt, N.E.<sup>5</sup>, Haneveld, F.<sup>6</sup>, Van Nes, J.<sup>7</sup>, Koster, J.<sup>8</sup>, Versteeg, R.<sup>9</sup>*

Amsterdam UMC, University of Amsterdam, Oncogenomics, Amsterdam, Netherlands<sup>2</sup>, Amsterdam UMC, University of Amsterdam, Oncogenomics, Amsterdam, Netherlands<sup>3</sup>, Amsterdam UMC, University of Amsterdam, Oncogenomics, Amsterdam, Netherlands<sup>4</sup>, Amsterdam UMC, University of Amsterdam, Oncogenomics, Amsterdam, Amsterdam UMC, University of Amsterdam<sup>5</sup>, Amsterdam UMC, University of Amsterdam, Oncogenomics, Amsterdam, Netherlands<sup>6</sup>, Amsterdam UMC, University of Amsterdam, Oncogenomics, Amsterdam, Netherlands<sup>7</sup>, Amsterdam UMC, University of Amsterdam, Oncogenomics, Amsterdam, Netherlands<sup>8</sup>, Amsterdam UMC, University of Amsterdam, Oncogenomics, Amsterdam, Netherlands<sup>9</sup>

### **Background/Introduction:**

As reported recently, neuroblastoma tumors include two types of tumor cells with diverging characteristics. The majority of the tumor typically consists of adrenergic (ADRN) cells, which are chemosensitive and characterized by lineage markers like PHOX2A, PHOX2B and DBH. A minor proportion of the tumor cells are mesenchymal (MES) and have immature features and lack lineage differentiation markers. Interestingly, MES and ADRN cells can spontaneously interconvert into one another, which may have far reaching consequences on the resilience towards clinical intervention of neuroblastoma tumors. During reprogramming MES and ADRN cells undergo a major shift in epigenetic modification status.

### **Aims:**

To understand the role of epigenetic modification on establishing the MES- and ADRN-phenotypes and their transdifferentiation potential.

### **Methods/Materials:**

Four isogenic pairs of MES and ADRN cell lines were analyzed by ChIP-sequencing with anti-H3K27me3 and anti-H3K4me3. The HiSeq sequencing result was analyzed and visualized on the R2 genomics analysis and visualization platform (<http://r2.amc.nl/>).

### **Results:**

Polycomb complexes mediate large-scale gene repression by H3K27me3 modification of chromatin. We therefore analyzed differential H3K27me3 modifications of MES- and ADRN-type neuroblastoma cell lines. ADRN-specific lineage commitment genes like ASCL1 and DBH are silent in MES-type cells. We observed that the transcription start site (TSS) of these genes have a H3K27me3 silencing mark in MES cells. Also ADRN-specific neurogenesis genes, like NEFL, NEFM and NRCAM, carry an H3K27me3 modifications at their TSS sites in MES cells. The reverse pattern was not apparent: MES-specific genes that are silenced in ADRN-type cells have no H3K27me3 marks at their TSS site in ADRN cells.

It has been observed that in early embryogenesis, regulatory genes that are silent but have to be expressed later in lineage development may carry a bivalent modification of their TSS. This modification consists of a suppressive H3K27me3 mark and an activating H3K4me3 mark. We therefore further analyzed the TSS of the ADRN-specific genes that are silent in MES cells. Many of the silent regulatory ADRN genes have this bivalent modification in MES cells. However, MES-specific genes that are silenced in ADRN cells lack a bivalent mark. This suggests that MES and ADRN cells have maintained some of the normal epigenetic processes that control lineage development.

### **Summary/Conclusions:**

ADRN-specific genes are suppressed in MES-type cells by H3K27me3 modification of the TSS. However, these TSS sites can also carry a H3K4me3 modification, thus creating a bivalent mark typical for genes that are to be expressed in subsequent developmental stages. This suggests that basic regulatory principles of lineage development are preserved in MES- and ADRN-type neuroblastoma cells.

**P084 T-SNE and DeMixT applied to identify core biological processes and subsets of cells from which the signals originate.**

Von Stedingk, K.J.M.<sup>2</sup>, Ingrid, I.<sup>3</sup>, Versteeg, R.<sup>4</sup>, Koster, J.<sup>5</sup>

University of Amsterdam, Department of Oncogenomics, Academic Medical Center, Amsterdam, Netherlands <sup>2</sup>, Lund University, Department of Paediatrics, Clinical Sciences Lund, Lund, Sweden<sup>3</sup>, University of Amsterdam, Department of Oncogenomics, Academic Medical Center, Amsterdam, Netherlands<sup>4</sup>, University of Amsterdam, Department of Oncogenomics, Academic Medical Center, Amsterdam, University of Amsterdam<sup>5</sup>

**Background/Introduction:**

Many mRNA profiling studies have highlighted the strong expression differences between low- and high-risk neuroblastoma. Nonetheless, both disease entities are histopathologically very similar and are both thought to arise from the sympatho-adrenergic lineage.

**Aims:**

We therefore analyzed neuroblastoma mRNA profiles not only for differences, but also for commonalities between both low- and high-risk tumours to identify key transcriptional networks underlying different molecular subtypes of neuroblastoma.

**Methods/Materials:**

To achieve this, we performed gene-based T-SNE dimension reduction analysis on mRNA profiling datasets of NB followed by cellular deconvolution using the algorithm DeMixT.

**Results:**

We identify 3 central dimensions of the disease, consisting of gene-networks regulating epithelial to mesenchymal transition (EMT), immune cell/inflammatory response and finally cell cycle/*MYCN*. These core networks were validated and remained stable across 3 independent NB datasets consisting of over 900 tumours and were identifiable independent of patient stage, risk-status or *MYCN*-amplification. Application of DeMixT suggests that signals from these networks originated from different cellular subsets within the tumour, including adrenergic, mesenchymal and stromal cellular components. Furthermore, clustering of patients based on these 3 core networks identified subdivision of patients within the each of the clinically relevant NB subgroups (low-risk, high-risk and *MYCN*-amplified).

**Summary/Conclusions:**

This suggests underlying biological subtypes of NB that differ based on root biological networks independent of the aggressiveness of the disease. In addition, these NB subtypes are likely reflective of different cellular subtype composition within the tumour. These findings may provide insight into identifying key therapeutic networks and targets within both high-risk and *MYCN*-amplified patients that may have been previously overlooked.



## **P085 6p22.3 Locus lncRNAs regulate *MYCN* stability to determine the neuroblastoma susceptibility**

Juvvuna, P.K.<sup>2</sup>, Di Marco, M.<sup>3</sup>, MONDAL, T.<sup>4</sup>, Kosalai, S.T.<sup>5</sup>, Ménard, M.<sup>6</sup>, Weiss, W.A.<sup>7</sup>, KANDURI, C.<sup>8</sup>

Institute of Biomedicine, Gothenburg University, Dept of Medical Biochemistry and Cell Biology, Gothenburg, Sweden <sup>2</sup>, Institute of Biomedicine, Gothenburg University, Dept of Medical Biochemistry and Cell Biology, Gothenburg, Sweden<sup>3</sup>, Institute of Biomedicine, Gothenburg University, Dept of Medical Biochemistry and Cell Biology, Gothenburg, Sweden<sup>4</sup>, Institute of Biomedicine, Gothenburg University, Dept of Medical Biochemistry and Cell Biology, Gothenburg, Institute of Biomedicine, Gothenburg University<sup>5</sup>, University of California, Departments of Neurology, Pediatrics, and Neurological Surgery, San Francisco, USA<sup>6</sup>, University of California, Departments of Neurology, Pediatrics, and Neurological Surgery, San Francisco, USA<sup>7</sup>, Institute of Biomedicine, Gothenburg University, Dept of Medical Biochemistry and Cell Biology, Gothenburg, Sweden<sup>8</sup>

### Background/Introduction:

Neuroblastoma (NB) is a childhood cancer and the most common extra cranial solid tumor, arises from neural crest cells of the developing sympathetic nervous system. Non-random chromosomal aberrations, including *MYCN* amplification, are commonly seen NBs. In NB, *MYCN* amplification correlates with tumor aggressiveness, and as a transcriptional driver, it is thought to determine cancer initiation and progression. In our previous findings we demonstrated lncRNAs CASC15-003 and NBAT1 (6p22 lncRNA) encoded by NB risk associated locus 6p22.3 as NBs-specific independent prognostic biomarkers (Pandey et al., 2014; Tanmoy et al., 2018). Here we attempt to understand the functional connection between 6p22 lncRNAs and *MYCN* dependent oncogenic pathways.

### Aims:

6p22.3 lncRNA levels negatively correlate with *MYCN* protein levels. This intrigued us to find the functional nexus between 6p22 lncRNAs and *MYCN* dependent oncogenic program.

### Methods/Materials:

Methods: Immunocytochemistry (ICC), Xenografts, Western blot analysis, Co-immunoprecipitation (Co-IP) assay, ChIP, RNA sequencing and transcriptomic analysis, Ubiquitination assays.

Materials: NB tissues from patients and NB cell lines (*MYCN* amplified and non-*MYCN* amplified cell lines)

### Results:

Ectopic expression of 6p22 lncRNAs in *MYCN* amplified and non-amplified cell lines decreased the *MYCN* protein levels, whereas their depletion in the NB cell lines resulted in increased levels of *MYCN* protein. We show that 6p22 lncRNAs and their common interacting ribonucleoprotein USP36, ubiquitin-specific protease, regulate *MYCN* protein levels. Consistent with functional connection between USP36 and *MYCN*, Co-IP experiments demonstrate the interaction between *MYCN* and USP36 and also, they co-localize at the nucleolus, as judged by ICC. Knockdown of USP36 in *MYCN* amplified NB cells resulted in reduced levels of *MYCN* protein and decreased cell proliferation. Ectopically expressed USP36 reduced *MYCN* ubiquitination, suggesting that USP36 deubiquitinates *MYCN*. Transcriptomic analysis revealed a set of common genes regulated by 6p22.3 lncRNAs/USP36/*MYCN* which are involved in cell proliferation and cell adhesion, there by contributing to NB disease progression. Knock down of 6p22 lncRNAs/USP36/*MYCN* regulated genes, such as Col18A1, in the 6p22 lncRNA depleted NB cells rescues molecular and phenotypic effects, which occur due to the loss of 6p22 lncRNAs.

### Summary/Conclusions:

In summary, our data demonstrate the functional link between 6p22 lncRNAs and *MYCN*. Identification of 6p22 lncRNAs/USP36/*MYCN* regulated genes uncovers novel *MYCN* dependent oncogenic program, leading to new *MYCN* dependent therapeutic options for high-risk NBs.

## **P086 A single amino acid mutation in the RNF121 protein blocked tumour neuroblastoma development in TH-MYCN mice**

*Cheung, B.*<sup>2</sup>, *Nagy, Z.*<sup>3</sup>, *Salib, A.*<sup>4</sup>, *Raipuria, M.*<sup>5</sup>, *Murray, J.*<sup>6</sup>, *Wong, I.*<sup>7</sup>, *Restuccia, D.*<sup>8</sup>, *Sutton, S.*<sup>9</sup>, *Milazzo, G.*<sup>10</sup>, *Perini, G.*<sup>11</sup>, *Zhu, S.*<sup>12</sup>, *Solomon, B.*<sup>13</sup>, *Parker, M.*<sup>14</sup>, *Liu, T.*<sup>15</sup>, *Norris, M.*<sup>16</sup>, *Haber, M.*<sup>17</sup>, *Carter, D.*<sup>18</sup>, *Marshall, G.*<sup>19</sup>

Children's Cancer Institute Australia, Embryonal Cancer Therapy and Prevention, Sydney, Australia<sup>2</sup>, Children's Cancer Institute Australia, Embryonal Cancer Therapy and Prevention, Sydney, Australia<sup>3</sup>, Children's Cancer Institute Australia, Embryonal Cancer Therapy and Prevention, Sydney, Australia<sup>4</sup>, Children's Cancer Institute Australia, Embryonal Cancer Therapy and Prevention, Sydney, Children's Cancer Institute Australia<sup>5</sup>, Children's Cancer Institute Australia, Molecular Diagnostics, Sydney, Australia<sup>6</sup>, Children's Cancer Institute Australia, Embryonal Cancer Therapy and Prevention, Sydney, Australia<sup>7</sup>, Children's Cancer Institute Australia, Embryonal Cancer Therapy and Prevention, Sydney, Australia<sup>8</sup>, Children's Cancer Institute Australia, Embryonal Cancer Therapy and Prevention, Sydney, Australia<sup>9</sup>, University of Bologna, Department of Pharmacy and Biotechnology, Bologna, Italy<sup>10</sup>, University of Bologna, Department of Pharmacy and Biotechnology, Bologna, Italy<sup>11</sup>, Mayo Clinic, Department of Biochemistry and Molecular Biology, Rochester, USA,<sup>12</sup> Peter MacCallum Cancer Centre, Melbourne, Cancer Therapeutics Program, Melbourne, Australia<sup>13</sup>, The University of Melbourne, Bio21, Molecular Science and Biotechnology Institute, Melbourne, Australia<sup>14</sup>, Children's Cancer Institute Australia, Histone Modification Group, Sydney, Australia<sup>15</sup>, Children's Cancer Institute Australia, Professor and Deputy Director, Research, Sydney, Australia<sup>16</sup>, Children's Cancer Institute Australia, Office of Executive Director, Sydney, Australia<sup>17</sup>, Children's Cancer Institute Australia, Embryonal Cancer Therapy and Prevention, Sydney, Australia<sup>18</sup>, Children's Cancer Institute Australia, Embryonal Cancer Therapy and Prevention, Sydney, Australia<sup>19</sup>

### Background/Introduction:

Embryonal cells produced in excess to requirements for organogenesis are normally deleted in a programmed manner following trophic factor withdrawal, whereas failure of this process is the first step in embryonal cancer initiation.

### Aims:

*MYCN* amplification is a poor prognostic indicator in neuroblastoma, occurring in approximately 20% of patients. Identification of co-factors of *MYCN* are valuable as novel therapeutic targets in this disease.

### Methods/Materials:

We used ENU mutagenesis on neuroblastoma-prone *TH-MYCN* transgenic mice in a forward genetic screen to discover genes whose loss of function inhibited neuroblastoma tumourigenesis.

### Results:

We generated founder lines with heritable, decreased tumour incidence, and used large-scale mapping, focussed exome and next-generation sequencing of the mutant mouse genomes to identify Ring Finger Protein 121 (RNF121) as a candidate mutant gene linked to loss of tumour formation. The point mutation in RNF121 is located in a transmembrane domain of the protein, and the molecular modelling indicated that it can result in loss of function due to steric clashes with surrounding transmembrane domains. Immunofluorescence demonstrated RNF121 is localized in the cis-Golgi membrane of neuroblastoma cells, which was further supported by cell fractionation experiments. The point mutation in RNF121 disrupts localisation of RNF121 to cis-Golgi. Analysis of patient datasets and prognosis indicated that high RNF121 expression is associated with worse prognosis, particularly in *MYCN*-amplified patients, and higher RNF121 expression is observed in human *MYCN*-amplified neuroblastoma cell lines. Suppression of RNF121 by siRNAs in *MYCN*-amplified cell lines significantly impaired cell viability and colony formation, and induced G2/M arrest, whilst overexpression of RNF121 enhanced cell viability and proliferation. Consistent with an association of RNF121 with

*MYCN* amplification, suppression of RNF121 by siRNAs decreases *MYCN* protein half-life and stability in *MYCN*-amplified cell lines.

Furthermore, we have shown that the amplification of RNF121 (18%) and c-MYC (41%) in esophageal tumours samples. Amplification of RNF121 was found in TCGA datasets of Head and Neck Cancer (13%), Ovarian (8%) and *MYCN*-driven neuroendocrine prostate cancer (NEPC) (20%). To examine the functional role of RNF121 *in vivo*, we have generated RNF121 a conditional knockout mouse model. The RNF121 homozygous knockout mice are embryonic lethal at E12.5. The model is currently being crossed with the *TH-MYCN* mice.

Summary/Conclusions:

We found that RNF121 is: a cis-Golgi trans-membrane protein, a prognostic factor in NB, amplified in head and neck cancer and *MYCN*-driven neuroendocrine prostate cancer, and increase *MYCN* protein stability. The importance of this completely novel target lies in the fact that the mutant mice were not only cancer-free despite the presence of the *MYCN* transgene, but healthy, suggesting RNF 121 inhibitors will have a wide therapeutic index.

## **P087 LIN28B increases neural crest cell migration and leads the transformation of trunk sympathoadrenal precursors**

Corallo, D.<sup>2</sup>, Donadon, M.<sup>3</sup>, Pantile, M.<sup>4</sup>, Sidarovich, V.<sup>5</sup>, Cocchi, S.<sup>6</sup>, Ori, M.<sup>7</sup>, De Sarlo, M.<sup>8</sup>, Candiani, S.<sup>9</sup>, Frasson, C.<sup>10</sup>, Distel, M.<sup>11</sup>, Quattrone, A.<sup>12</sup>, Zanon, C.<sup>13</sup>, Basso, G.<sup>14</sup>, Tonini, G.P.<sup>15</sup>, Aveic, S.<sup>16</sup>

IRP - Istituto di Ricerca Pediatrica Fondazione Città della Speranza, Neuroblastoma Laboratory, Padova, Italy <sup>2</sup>, IRP - Istituto di Ricerca Pediatrica Fondazione Città della Speranza, Neuroblastoma Laboratory, Padova, Italy<sup>3</sup>, IRP - Istituto di Ricerca Pediatrica Fondazione Città della Speranza, Neuroblastoma Laboratory, Padova, Italy<sup>4</sup>, University of Trento, Department of Cellular and Integrative Biology (CIBIO), Trento, University of Trento<sup>5</sup>, University of Trento, Department of Cellular and Integrative Biology (CIBIO), Trento, Italy<sup>6</sup>, University of Pisa, Department of Biology, Unit of Cell and Developmental Biology, Pisa, Italy<sup>7</sup>, University of Pisa, Department of Biology, Unit of Cell and Developmental Biology, Pisa, Italy<sup>8</sup>, University of Genova, Department of Earth, Environmental and Life Sciences (DISTAV), Genova, Italy<sup>9</sup>, IRP - Istituto di Ricerca Pediatrica Fondazione Città della Speranza, Department of Haematology-Oncology, Padova, Italy<sup>10</sup>, Children's Cancer Research Institute (CCRI), Innovative Cancer Models, Wien, Austria<sup>11</sup>, University of Trento, Department of Cellular and Integrative Biology (CIBIO), Trento, Italy, <sup>12</sup>, IRP - Istituto di Ricerca Pediatrica Fondazione Città della Speranza, Neuroblastoma Laboratory, Padova, Italy <sup>13</sup>, University of Padua, Department of Women and Child Health, Haematology-Oncology Clinic, Padova, Italy <sup>14</sup>, IRP - Istituto di Ricerca Pediatrica Fondazione Città della Speranza, Neuroblastoma Laboratory, Padova, Italy <sup>15</sup>, IRP - Istituto di Ricerca Pediatrica Fondazione Città della Speranza, Neuroblastoma Laboratory, Padova, Italy <sup>16</sup>

### Background/Introduction:

The RNA-binding protein LIN28B regulates developmental timing and stem cell identity by suppressing the *Let-7* family of microRNAs. The post-embryonic reactivation of LIN28B is a hallmark of poor prognosis and metastasis in different types of cancer, including neuroblastoma, a pediatric solid tumor deriving from neural crest cells (NCC). However, the extent to which LIN28B activation participates in the modulation of NCC behavior in neuroblastoma remains unclear.

### Aims:

The aim of this study was to assess the molecular mechanisms by which LIN28B influences the viability of the NCC toward a malignant and metastatic phenotype.

### Methods/Materials:

The effects of transient LIN28B overexpression on NCC migration and differentiation were analyzed in the two vertebrate models *Danio rerio* and *Xenopus laevis*. The phenotype and the expression of related genes were analyzed by time-lapse microscopy, in situ hybridization, immunolabelling, and western blot analyses. The doxycycline-inducible neuroblastoma SH-SY5Y<sup>LIN28B</sup> cell line was obtained by lentiviral infection. The cell migratory and invasive capabilities were detected through transwell migration assay, matrigel invasion assay, scratch test and *in vivo* xenotransplantation model. Flow cytometric analyses, western blot, and real-time PCR were used to characterize the LIN28B-related molecular phenotypes.

### Results:

In this study, we provided evidence that the overexpression of LIN28B inhibits the sympathoadrenergic cell differentiation and increases the speed of NCC migration *in vivo*. Moreover, we show that LIN28B sustains the invasive motility of neuroblastoma cells *in vitro* and in zebrafish xenotransplanted embryos, where tumor cells show increased spread throughout the animal body. Our data support a growing body of evidence that LIN28B may re-establish neuroblastoma heterogeneity through the epithelial-to-mesenchymal transition (EMT), leading to tumor progression and metastatic tumor cell invasion. The EMT/PI3K/AKT regulatory axis is triggered by the up-

regulation of the two integrins, ITGA5 and ITGA6, upon LIN28B overexpression, sustaining the metastatic phenotypes of this disease.

**Summary/Conclusions:**

Taken together, our results establish the relevance of LIN28B in the regulation of NCC migration during embryonic development. Our data suggest a mechanism by which a long-term overexpression of LIN28B in neuroblastoma cells supports the integrin-mediated cell spreading and migration by facilitating the formation of focal contacts.

## **P088 CD44-high neural crest stem-like cells are associated with tumour aggressiveness and poor survival in neuroblastoma tumours**

Vega, F.M.<sup>2</sup>, Gómez-Muñoz, M.A.<sup>3</sup>, Colmenero-Repiso, A.<sup>4</sup>, Rodríguez-Prieto, I.<sup>5</sup>, Ramírez, G.<sup>6</sup>, Márquez, C.<sup>7</sup>, Cabello, R.<sup>8</sup>, Pardal, R.<sup>9</sup>

Universidad de Sevilla, Cell Biology, Seville, Spain <sup>2</sup>, Universidad de Sevilla, Medical Physiology and Biophysics, Seville, Spain<sup>3</sup>, Universidad de Sevilla, Medical Physiology and Biophysics, Seville, Spain<sup>4</sup>, Universidad de Sevilla, Medical Physiology and Biophysics, Seville, Universidad de Sevilla<sup>5</sup>, Hospital Universitario Virgen del Rocío, Pediatric Oncology, Seville, Spain<sup>6</sup>, Hospital Universitario Virgen del Rocío, Pediatric Oncology, Seville, Spain<sup>7</sup>, Hospital Universitario Virgen del Rocío, Pediatric Surgery, Seville, Spain<sup>8</sup>, Universidad de Sevilla, Medical Physiology and Biophysics, Seville, Spain<sup>9</sup>

### Background/Introduction:

Neuroblastoma is a paediatric tumour originated from sympathoadrenal precursors and characterized by its heterogeneity and poor outcome in advanced stages. Intra-tumoral cellular heterogeneity has emerged as an important feature in neuroblastoma, with a potential major impact on tumour aggressiveness and response to therapy. CD44 is an adhesion protein involved in tumour progression, metastasis and stemness in different cancers; however, there have been controversies about the significance of CD44 expression in neuroblastoma and its relationship with tumour progression.

### Aims:

We aim to explore the function of CD44 in neuroblastoma and its role as a potential marker for neural crest stem-like cells within neuroblastoma tumours

### Methods/Materials:

We have performed transcriptomic analysis on patient tumour samples studying the outcome of patients with high CD44 expression. Adhesion, invasion and proliferation assays were performed in sorted CD44high neuroblastoma cells. Tumoursphere cultures have been used to enrich in undifferentiated stem-like cells and to assess self-renewal and differentiation potential. We have finally performed in vivo tumorigenic assays on cell line-derived or Patient-derived xenografts.

### Results:

We show that high CD44 expression is associated with low survival in human neuroblastoma, independently of *MYCN* amplification. CD44 is expressed in a cell population with neural crest stem-like features, and with the capacity to generate multipotent, undifferentiated tumourspheres in culture. These cells are more invasive and proliferative *in vitro*. CD44 positive cells obtained from tumours are more tumorigenic and metastatic, giving rise to aggressive neuroblastic tumours at high frequency upon transplantation.

### Summary/Conclusions:

We describe an unexpected intra-tumoral heterogeneity within cellular entities expressing CD44 in neuroblastoma, and propose that CD44 has a role in neural crest stem-like undifferentiated cells, which can contribute to tumorigenesis and malignancy in this type of cancer.

## **P089 Short-term Efficacy Of Autologous Stem Cell Transplantation With Busulfan And Melphalan Regimen In The Treatment Of High-risk Neuroblastoma**

Wang, D.<sup>2</sup>, Zhao, Q.<sup>3</sup>, Yan, J.<sup>4</sup>

Tianjin Medical University Cancer Institute & Hospital, Pediatric cancer, Tianjin, China<sup>2</sup>, Tianjin Medical University Cancer Institute & Hospital, Pediatric cancer, Tianjin, China<sup>3</sup>, Tianjin Medical University Cancer Institute & Hospital, Pediatric cancer, Tianjin, China<sup>4</sup>

### Background/Introduction:

To investigate the clinical outcome of children with high-risk neuroblastoma and the prognostic function of Busulfan and melphalan as high-dose chemotherapy for high-risk neuroblastoma.

### Aims:

### Methods/Materials:

The clinical and follow-up data of patients who suffered from high-risk neuroblastoma admitted from January 2014 to December 2018 were retrospectively reviewed. The survival rate was calculated and Cox proportional hazard model was used to determine the clinical and pathological factors ,especially the factors of Autologous peripheral blood stem cell transplantation (APBST) on overall survival(OS) of high-risk neuroblastoma .

### Results:

68 case was included ,among them 38 case received Busulfan and melphalan as high-dose chemotherapy and APBST. The Kaplan-Meier curve revealed regular therapy significantly improve the prognosis of high-risk neuroblastomaThe 3-year survival rate was 65.6%. Busulfan and melphalan as highdose chemotherapy for high-risk neuroblastoma improved event-free survival. 3-year event-free survival 67.8%vs 39.8% $P<0.05$ . 3-year overall survival 82.1%vs 42.1% $P<0.05$ .

### Summary/Conclusions:

Busulfan and melphalan improved event-free survival in children with high-risk neuroblastoma and caused fewer severe adverse events. Busulfan and melphalan should thus be considered standard high-dose chemotherapy and ongoing randomized studies will continue to aim to optimize treatment for high-risk.



## **P090 Clinical significance of detection of small lesions based on GD2 immunocytology in high-risk neuroblastoma**

Li, J.<sup>2</sup>

Tianjin Medical University Cancer Institute and Hospital, Department of Pediatric Oncology, Tianjin, China<sup>2</sup>

### Background/Introduction:

Neuroblastoma is the most common extracranial solid tumor in children, and the most common malignancy in infants. Outcomes vary significantly among patients with this disease, as subsets of very young children fare well with little or no treatment, whereas older children with metastatic disease have an event-free survival (EFS) of approximately 50% despite aggressive, multimodality therapy that results in significant long-term side-effects.

### Aims:

To explore the predictive effect of GD2-based immunocytology on the prognosis of children with NB, and to compare the sensitivity of bone marrow smear and GD2 staining in the detection of NB small lesions. Meanwhile, to analyze the effect of stem cell transplantation on prognosis of children with high-risk NB.

### Methods/Materials:

The clinical and pathological data of 56 patients with high-risk neuroblastoma from Tianjin Medical University Cancer Hospital from February 2011 to September 2018 were retrospectively analyzed. The clinical significance of GD2-based immunocytological detection of small lesions in children with high-risk NB is discussed from the following aspects:

Comparative study on sensitivity of detection of small lesions: sensitivity differences of NB small lesions detected by bone marrow smear method and GD2-based immunocytography at the time of diagnosis and the end of chemotherapy were analyzed.

Analysis of pathological factors of GD2 positive in bone marrow at diagnosis: Pathological factors such as gender, age, tumor site, abnormal amplification of *MYCN* gene and initial NSE level were further studied. In addition, total survival (OS) and disease-free survival (DFS) of children with initial GD2 positive and negative in bone marrow were compared and analyzed.

Correlation between the status of GD2 in bone marrow at each treatment stage and the recurrence: statistical methods were used to analyze the potential relationship between GD2 status of bone marrow and recurrence and prognosis in children with NB at each treatment stage.

Efficacy analysis of stem cell transplantation: To analyze the effect of stem cell transplantation on the prognosis of high-risk NB children, and to group the children with GD2 positive and negative at the end of chemotherapy, then to explore the efficacy of stem cell transplantation within the groups, respectively.

### Results:

Comparative study on sensitivity of detection of small lesions: The differences in sensitivity of bone marrow smear and GD2 staining were statistically significant at the time of diagnosis and at the end of chemotherapy in children with high-risk NB, and the sensitivity of detection of microscopic lesions using GD2 staining was better than that of traditional bone marrow smear.

Analysis of pathological factors of GD2 positive in bone marrow at diagnosis: There was no significant difference in gender, age, tumor site, abnormal amplification of *MYCN* gene and initial NSE level between children with GD2 positive and negative in bone marrow at the time of diagnosis ( $P > 0.05$ ). The difference of disease-free survival and total survival compared between the two groups was also not statistically significant ( $P > 0.05$ ).

Correlation between the status of GD2 in bone marrow at each treatment stage and the recurrence: At the time of diagnosis, and after 2 courses chemotherapy, GD2 positive had no effect on the recurrence and overall survival of high-risk NB children. However, GD2 is still positive after 4 courses chemotherapy and at the end of chemotherapy, which is a risk factor for recurrence and prognosis.

Efficacy analysis of stem cell transplantation: The difference was statistically significant in the effect of stem cell transplantation on the prognosis of children with high-risk NB, and the recurrence rate of children with high risk NB showed a trend of decrease after stem cell transplantation.

Summary/Conclusions:

The detection of microscopic lesions based on GD2 immunocytology was more sensitive than bone marrow smear. At the time of diagnosis and after 2 courses chemotherapy, the status of GD2 was not related to the prognosis of children with high-risk NB, but the GD2 staining was still positive after 4 courses chemotherapy, indicating poor prognosis and prone to recurrence. hematopoietic stem cell transplantation can reduce the recurrence rate and the effect is more obvious for children with GD2 positive at the end of chemotherapy.

## **P091 Targeting the c-MYC oncogenic signal through novel PA2G4 inhibitors**

Massudi, H.<sup>2</sup>

Childrens Cancer Institute, Woman and Childrens Health, Kensington, Australia <sup>2</sup>

### Background/Introduction:

MYC (*MYCN* and c-MYC) oncoprotein expression is pivotal in over 70% of human cancers and is associated with poor prognosis and disease aggression, making it an attractive target for pharmacological inhibition. *MYCN* drives 20% of the high-risk paediatric neuroblastoma (NB) patients, whereas, c-MYC has recently emerged as a potent driver gene in a subset of 10% of high-risk NB. We identified a novel oncogenic co-factor, proliferation-associated protein 2G4 (PA2G4), which bound *MYCN* to increase its stability and thus promoting cancer cell growth. Most importantly, the PA2G4-*MYCN* protein-protein interaction could be disrupted by a novel small molecule, WS6, which markedly reduced tumorigenesis in several *in vivo* NB models.

### Aims:

To evaluate whether c-MYC bound PA2G4 and increased its stability/oncogenic function.

To assess the efficacy of WS6 and analogues to target the putative PA2G4-c-MYC interaction in NB and other c-MYC driven malignancies.

To determine the mechanism of a putative PA2G4-c-MYC interaction.

### Methods/Materials:

Publicly available R2 gene expression database (n = 649, Kocak dataset) was used for gene expression analysis. Mechanism of PA2G4/c-MYC interaction was investigated using plasmid DNA overexpression, siRNA mediated knockdown, cycloheximide chase assay, western blots and colony formation assays. Cell viability and drug treatment assays were conducted to measure drug IC-50 and therapeutic window. Molecular modelling, surface plasmon resonance, differential scanning fluorimetry, and Lapinski's drug like properties assessments were conducted to rank WS6 analogues for potency and efficacy.

### Results:

For the first time, we showed that PA2G4 and c-MYC mutually stimulate high levels of each protein. High PA2G4 expression was a prognostic marker in c-MYC high NB patient tumor subset. High expression of PA2G4 and c-MYC predicted poor overall survival in *MYCN* non-amplified, high c-MYC NB patients. In addition, PA2G4 knockdown significantly reduced c-MYC protein stability and half-life. WS6 treatment showed selective toxicity and sensitivity for high c-MYC expressing NB cells as well as other c-MYC driven cancer cells (B cell leukemia/lymphoma, medulloblastoma). Most importantly, we have identified selective and potent analogues of WS6, which have significant cytotoxicity to NB cells with high PA2G4 expression and less toxicity for normal cells. WS6 analogue treatments reduce the protein levels of PA2G4/c-MYC and significantly reduced colony formation.

### Summary/Conclusions:

These data strongly support PA2G4's role as an oncogenic cofactor in c-MYC driven cancers. WS6 and its potent selective analogues target the c-MYC oncogenic signal, providing critical data to guide future clinical trials.

## **P092 Oncogenic ALK signaling represses the pro-differentiation DLG2 tumor suppressor to promote an undifferentiated state in neuroblastoma**

*Siaw, J.*<sup>2</sup>, *Van den Eynden, J.*<sup>3</sup>, *Lind, D.*<sup>4</sup>, *Lai, W-Y.*<sup>5</sup>, *Martinsson, T.*<sup>6</sup>, *Palmer, R.*<sup>7</sup>, *Hallberg, B.*<sup>8</sup>

Institute of Biomedicine, Sahlgrenska Academy, University of Gothenburg, Department of Medical Biochemistry and Cell Biology, Gothenburg, Sweden <sup>2</sup>, 2Department of Human Structure and Repair, Anatomy and Embryology Unit, Department of Human Structure and Repair, Anatomy and Embryology Unit, Ghent Un, Ghent, Belgium<sup>3</sup>, Institute of Biomedicine, Sahlgrenska Academy, University of Gothenburg, Department of Medical Biochemistry and Cell Biology, Gothenburg, Sweden<sup>4</sup>, Institute of Biomedicine, Sahlgrenska Academy, University of Gothenburg, Department of Medical Biochemistry and Cell Biology, Gothenburg, Institute of Biomedicine, Sahlgrenska Academy, University of Gothenburg<sup>5</sup>, Institute of Biomedicine, Sahlgrenska Academy, University of Gothenburg, Department of Laboratory Medicine, Gothenburg, Sweden<sup>6</sup>, Institute of Biomedicine, Sahlgrenska Academy, University of Gothenburg, Department of Medical Biochemistry and Cell Biology, Gothenburg, Sweden<sup>7</sup>, Institute of Biomedicine, Sahlgrenska Academy, University of Gothenburg, Department of Medical Biochemistry and Cell Biology, Gothenburg, Sweden<sup>8</sup>

### Background/Introduction:

High-risk neuroblastomas (NBs) typically display an undifferentiated/poorly differentiated morphology. Here we report a novel mechanism by which gain of function activity of the Anaplastic Lymphoma Kinase (ALK) Receptor Tyrosine Kinase (RTK) can counteract differentiation of neuroblastoma cells. ALK activity inhibits the expression of a previously described tumor suppressor gene, *DLG2*. In keeping with a role in NB, expression of *DLG2* is low in high risk NB and associated with worse prognosis of these patients. We show here that *DLG2* drives the neuronal differentiation process and identify the molecular mechanisms that down-regulate the expression of *DLG2* by ALK. *DLG2* is expressed in the 'bridge signature' that represents the transcriptional transition state when neural crest cells or Schwann Cell Precursors (SCP) become chromaffin cells of the adrenal gland. A synergistic increase in neuronal differentiation, close to 100%, is observed when *DLG2* is expressed with known inducers of differentiation such as retinoic acid (RA) or nerve growth factor (NGF). This is reflected in the robust androgenic signature that is observed in NB cells expressing *DLG2*, in comparison with the more mesenchymal signature seen in cells treated with RA. Taken together, our data show that ALK activation suppresses expression of the *DLG2* tumor suppressor to promote an undifferentiated phenotype in neuroblastoma.

### Aims:

To reveal the development of neuroblastoma

### Methods/Materials:

biochemistry, cell culture, xenograf, NGS

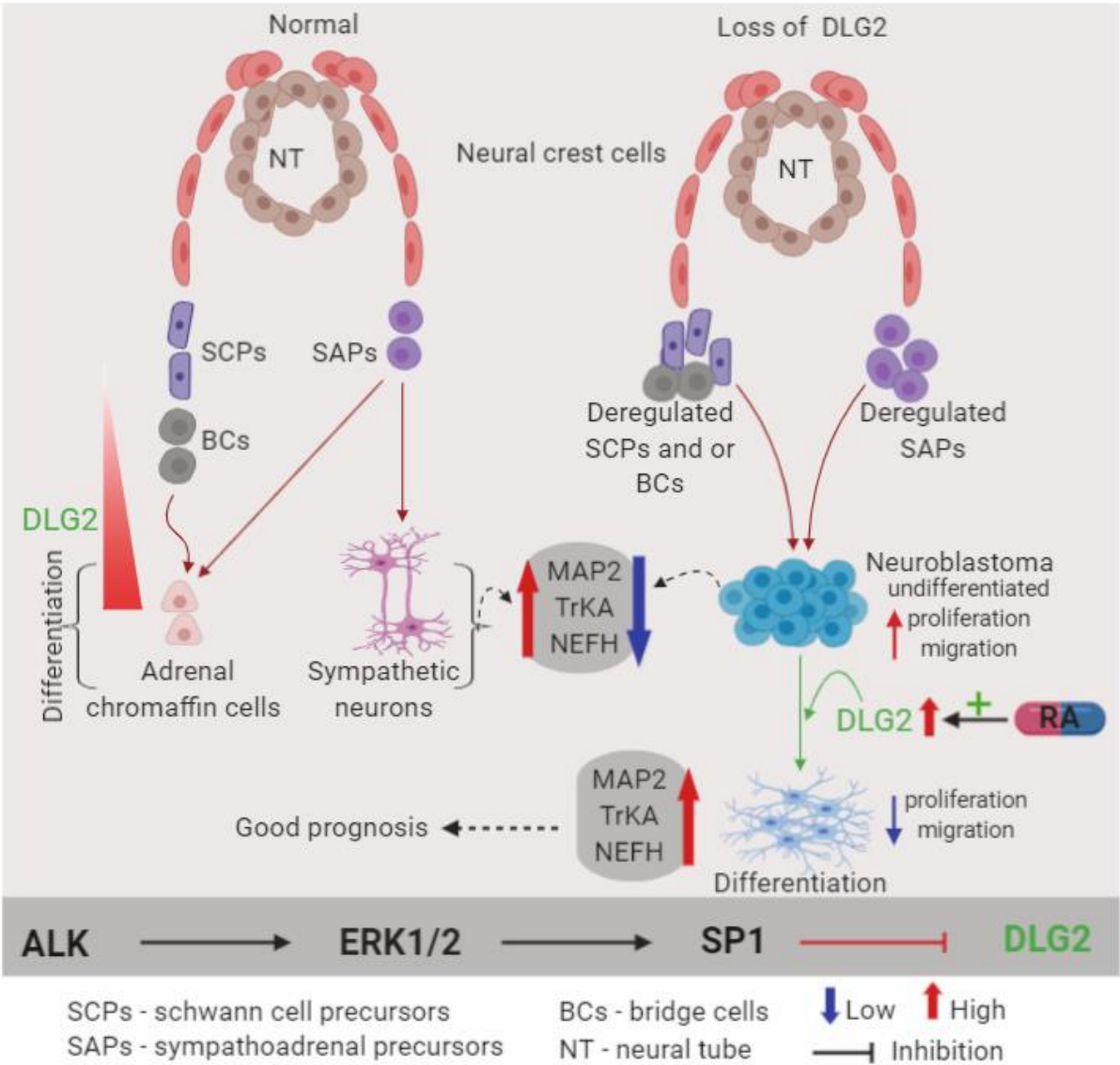
### Results:

Taken together, our data show that ALK activation suppresses expression of the *DLG2* tumor suppressor to promote an undifferentiated phenotype in neuroblastoma.

### Summary/Conclusions:

In summary, our data suggest that an important role for oncogenic ALK-ERK1/2-SP1 signaling may be the maintenance of an undifferentiated state of transformed NC-derived progenitors that is achieved by repression of *DLG2*. The importance of SP1 and *DLG2* in this process highlighted by the findings that restoring *DLG2* expression, e.g. by targeting SP1, spontaneously drives NB differentiation. While targeting SP1 presents challenges, these results motivate further exploration of SP1 as therapeutic target as well as evaluation of *DLG2* and SP1 as prognostic markers for NB. Finally, our data also suggest that further exploration of other 'bridge genes' may help to understand the mechanisms underlying the differentiation of SCPs and SA cells and their contribution to NB

Picture 1:



Caption 1: ALK regulate DLG2

## **P093 Embryonic mutations reveal independent tumour formation in bilateral neuroblastoma**

Coorens, T.H.H.<sup>2</sup>, Sarah, S.J.<sup>3</sup>, Mitchell, T.J.<sup>4</sup>, Lee, S.<sup>5</sup>, Hubank, M.<sup>6</sup>, Sebire, N.J.<sup>7</sup>, Anderson, J.<sup>8</sup>, Behjati, S.<sup>9</sup>

Wellcome Sanger Institute, Cancer, Ageing, and Somatic Mutations, Hinxton, United Kingdom <sup>2</sup>, University of Cambridge, Cambridge University Hospitals NHS Foundation Trust, Cambridge, United Kingdom<sup>3</sup>, Wellcome Sanger Institute, Cancer, Ageing, and Somatic Mutations, Hinxton, United Kingdom<sup>4</sup>, Wellcome Sanger Institute, Cancer, Ageing, and Somatic Mutations, Hinxton, Wellcome Sanger Institute<sup>5</sup>, The Royal Marsden NHS Foundation Trust, Clinical Genomics, London, United Kingdom<sup>6</sup>, UCL Great Ormond Street Institute of Child Health, Paediatric Pathology, London, United Kingdom<sup>7</sup>, UCL Great Ormond Street Institute of Child Health, Developmental Biology and Cancer, London, United Kingdom<sup>8</sup>, Wellcome Sanger Institute, Cellular Genetics, Hinxton, United Kingdom<sup>9</sup>

### Background/Introduction:

Multifocal cancer is most typically regarded as comprising primary tumours with metastatic deposits. However, in some forms of childhood cancer, synchronous lesions located in separate anatomical regions may represent independent oncogenic events as has been described in Wilms' tumour and retinoblastoma. Bilateral/Multifocal neuroblastoma is a well recognised but little-studied phenomenon.

### Aims:

We aimed to study two cases of neuroblastoma with bilateral adrenal tumours to determine their respective embryological origins and thereby distinguish two potential models: separate events initiating tumourigeneses versus a common precursor.

### Methods/Materials:

Two patients presenting with bilateral adrenal neuroblastoma tumours underwent diagnostic biopsies and/or surgical resection of the primary tumours. Whole-genome sequencing was performed on DNA derived from each tumour and from peripheral blood of the child and of both parents. Using a validated variant-calling pipeline we determined mutations of all classes, subdivided into inherited germline, *de novo* germline, mosaic (embryonic), and somatic variants. From these catalogues of mutations, we reconstructed the phylogenetic development of the respective tumours.

### Results:

In each case, we identified germline variants predisposing to neuroblastoma (SMARCA4 and CHEK2 in one family and PHOX2B in the second). In each case, analysis of *de novo* mosaic mutations in blood and tumour indicated separate embryonic origins with no evidence of somatic mutations in common. Remarkably in the first case the two embryonic lineages evolved in parallel into high risk tumours with apparent identical second hits on cytogenetic analysis, which sequencing revealed to have no molecular features in common.

### Summary/Conclusions:

Our findings show that bilateral neuroblastoma in our two cases are independent neoplasms that separated early in embryogenesis, thus providing direct phylogenetic proof of the suspicion that some multisite tumours in children do not represent metastatic disease. Whilst parallel convergent development of different clones within a tumor is a well-described phenomenon, the development of independent synchronous tumors that mirror each other is an unprecedented observation. We speculate that the pre-existing two oncogenic germline mutations forced tumor formation down a restricted conglomeration of required second hits. As whole-genome DNA sequencing is becoming a routine clinical test, our analytical strategy may guide treatment and provide further insights into the early embryonic development of childhood cancer.



## **P094 DDX3X and infant neuroblastoma: an oncogenic driver or coincidence?**

*Ash, S.<sup>2</sup>, Johnson-Kerner, B.L.<sup>3</sup>, Amar, S.<sup>4</sup>, Sherr, E.H.<sup>5</sup>*

Schneider Children's Medical Center of Israel, The Rina Zaizov Pediatric Hematology Oncology Division, Petach Tikva, Israel <sup>2</sup>, UCSF Benioff Children's Hospital Oakland, Division of Child Neurology, San Francisco, USA<sup>3</sup>, Schneider Children's Medical Center of Israel, The Rina Zaizov Pediatric Hematology Oncology Dept., Petach Tikva, Israel<sup>4</sup>, UCSF Benioff Children's Hospital Oakland, Division of Child Neurology, San Francisco, UCSF Benioff Children's Hospital Oakland<sup>5</sup>

### **Background/Introduction:**

DDX3X syndrome is a recently identified genetic disorder associated with brain malformations and likely accounts for 1-3% of girls with otherwise unexplained intellectual disability (ID), and can also impact boys. Affected individuals present with ID, hypotonia brain anomalies such as dysgenesis of the corpus callosum and polymicrogyria, and systemic malformations including cardiac and ophthalmologic. DDX3X, located on the X-chromosome (Xp11.4), belongs to the DEAD-box RNA helicase family and acts as a key RNA-binding protein to exert its regulatory functions in various cellular processes including transcription, splicing, translation, and transport. DDX3 mutations have been reported to have a role various cancers, including colorectal cancer, breast cancer, lung cancer, hepatocellular carcinoma, Ewing sarcoma and glioblastoma multiforme.

### **Aims:**

### **Methods/Materials:**

### **Results:**

We present three cases of infant females with DDX3X-related neurodevelopmental disorder who were diagnosed with localized neuroblastoma; two of which were found incidentally while undergoing imaging for their neurodevelopmental delays. The first patient was diagnosed at the age of 4 months with mediastinal neuroblastoma. She underwent resection of the mass without recurrence. The second patient was diagnosed at the age of 7 months with unresectable presacral neuroblastoma invading the foramina. She received 6 courses of chemotherapy and surgery. The third patient was diagnosed at the age of 6 months with an unresectable abdominal mass. She received 4 courses of chemotherapy. She had a recurrence one year later, which was completely resected. All three individuals after treatment were tumor-free at annual follow-ups.

### **Summary/Conclusions:**

This is the first report on a possible correlation between individuals with DDX3X-related neurodevelopmental disorder syndrome and neuroblastoma. All patients were female infants with localized disease, completely treated with no evidence of disease. Patients with DDX3X-related neurodevelopmental disorder have not been reported to have other cancers other than infantile neuroblastoma to date. Future studies are planned to explore the pathogenic mechanisms or the oncogenic role of DDX3X in neuroblastoma development.



## **P095 TAp63; A Molecular Switch between Spontaneous Regression and Aggressive Growth in Neuroblastoma**

Nakagawara, A.<sup>2</sup>, Suenaga, Y.<sup>3</sup>

Saga HIMAT Foundation, Heavy particle beam radiation cancer therapy center, Tosu, Japan <sup>2</sup>, Chiba Cancer Center Research Institute, Division of Molecular Carcinogenesis, Chiba, Japan<sup>3</sup>

### Background/Introduction

The clinical feature of neuroblastoma (NB) is very variable and enigmatic because it spans from developmentally programmed spontaneous regression (s-regression) in infants to aggressive growth in children. The nerve growth factor (NGF)/TrkA signaling pathway may be the major target for both the s-regression and the malignant transformation. To unveil the molecular mechanism, we have long worked to identify many novel genes and their products by reverse translational research and genomics using primary NBs and cell lines. They included TrkA, KIF1Bb, TAp63, UNC5D, BMCC1 and NLRR3 as the major regulators for inducing s-regression, most of which were upregulated by NGF depletion, and TrkB, N-CYM (a *de novo* evolved cis-antisense gene product of MYCN which stabilizes MYCN through GSK3b), NLRR1, ALK and ncRAN as those for promoting the aggressiveness in NB. However, the mechanism how s-regression and aggressiveness are molecularly linked remains elusive.

### Aims

We aimed to identify the molecule(s) to connect or switch the two poles in NB.

### Methods/Materials

Total RNA was prepared from 106 primary NB tissues. Expression levels of mRNA were measured by quantitative real-time RT-PCR. To block the p53 binding site, a CRISPR-dCas9 vector was constructed. To measure the MYCN promoter activity, chromatin immunoprecipitation assay was used.

### Results

Among the p53 family, high expression of only TAp63 mRNA was significantly correlated with favorable outcome in NB (n=106; P=0.00696). Furthermore, expression of TAp63 was inversely correlated with that of MYCN (P=0.0144, R=-0.239) and NCYM (P=0.000418; R=-0.344). Overexpression of TAp63 suppressed MYCN expression, whereas knockdown of TAp63 had the opposite effect. By binding to exon 1 of MYCN gene, TAp63 suppressed the promoter activities of MYCN and NCYM. All-trans-retinoic acid (ATRA) treatment of CHP134 cells induced TAp63 and reduced p53 expression, accompanied by downregulation of MYCN/NCYM expressions. Meanwhile, TAp63 knockdown inhibited ATRA-induced repression of NCYM gene expression. Blocking the p53 family binding site by CRISPR-dCas9 system in CHP134 cells induced MYCN/NCYM expression and promoted apoptosis. TAp63 also repressed transcription of c-MYC in glioblastoma. Thus, TAp63 repressed MYCN/NCYM bidirectional transcription, contributing to inhibition of NB growth.

### Summary/Conclusions

p63 was originally cloned as p51 by our group (Nat. Med., 1998). TAp63, the major isoform of p63, is induced during programmed cell death of developing normal sympathetic neurons in mice. Our previous and present studies revealed that, TAp63 as well as KIF1Bb, UNC5D and BMCC1. is upregulated after NGF depletion in favorable NB cells and primary NBs. The induced TAp63 further targets UNC5D, a second dependence receptor gene downstream of TrkA, for its transcriptional activation to enhance expression of pro-apoptotic genes. Thus, these and other evidences suggest that TAp63 is one of the key regulators to induce s-regression downstream of TrkA. On the other hand, our present study also clearly showed that TAp63 is down-regulated in aggressive NBs and that TAp63 directly inhibits transcription of both MYCN and NCYM, a 100% co-amplified gene with MYCN, to further enhance degradation of MYCN. Therefore, TAp63 may be a molecular switch to regulate the cellular machinery of s-regression and aggressive growth in NB.

## **P096 Linking telomere structures and processing to neuroblastoma tumor cell differentiation and malignancy**

*Yu, E.Y.<sup>2</sup>, Zahid, S.<sup>3</sup>, Cheung, I.<sup>4</sup>, Cheung, N.K.V.<sup>5</sup>, Lue, N.F.<sup>6</sup>*

Weill Cornell Medicine, Microbiology & Immunology, New York, USA <sup>2</sup>, Weill Cornell Medicine, Microbiology & Immunology, New York, USA<sup>3</sup>, Memorial Sloan Kettering Cancer Center, Pediatrics, New York, USA<sup>4</sup>, Memorial Sloan Kettering Cancer Center, Pediatrics, New York, Memorial Sloan Kettering Cancer Center<sup>5</sup>, Weill Cornell Medicine, Microbiology & Immunology, New York, USA<sup>6</sup>

### Background/Introduction:

Neuroblastoma (NB) tumor cells were previously shown to manifest three distinct neural crest differentiation states: neuroblastic (N-type), substrate-adherent non-neuronal (S-type), and intermediate (I-type). These lineage-specific cell types can inter-convert and the resulting phenotypic diversity appears to contribute to tumor progression and relapse. Recent studies also showed that telomere maintenance mechanisms (TMMs) are critical for high-risk NB and positive TMMs are strong prognostic indicators for poor outcomes. However, the impacts of telomeres and TMMs on the development/differentiation of neural crest and neuroblastoma cells are poorly understood.

### Aims:

Profile telomere structures in NB tumors/cell lines and normal neural tissues to identify telomere features in NB that are related to its developmental program.

Analyze telomere structures, TMMs, and telomere protein compositions in matched N, S and I clones to explore the roles of telomeres in NB cell differentiation.

### Methods/Materials:

Previous studies of NB telomeres were mostly limited to the determination of average telomere lengths and telomerase activity. We conducted a detailed survey of telomeres in 5 cell lines and 30 surgical samples from high-risk NB patients using a combination of assays that assess different aspects of telomere structure and processing (Clinicaltrials.gov, NCT00588068) with IRB approval. Most importantly, the levels of telomere trimming activity (a telomere shortening pathway) was examined using in-gel hybridization and C-circle assays. We also used STELA, a PCR-based assay that captures individual telomeres, to gain a high-resolution view of telomere length distributions. The results from NB samples were compared to those from normal neural tissues.

The same telomere assays were applied to a series of longitudinal tumor samples from individual patients to identify aspects of telomere structures or processing that are related to tumor progression.

The telomere protein compositions and telomerase activity in matched N, S and I cells were compared by Western and TRAP assays.

### Results:

We discovered robust “telomere trimming” activity (evidenced by high levels of C-strand single-stranded DNA and low levels of C-circles) in NB samples and normal neural tissues, but not in other cancers. This suggests that telomere trimming is active in normal neural development and is retained in the majority of NB tumors. Furthermore, we found that the trimming activity tends to diminish during tumor relapse, suggesting that tumors with low trimming activity may be more resistant to therapy. Hence, TMMs may be especially critical for in high-risk NB tumors growth by countering a developmental program of telomere shortening. We also observed strong connections between telomeres and the morphologically distinct, lineage-specific NB cells. Specifically, in comparison to highly malignant N-type cells, S-type cells consistently manifested low levels of telomerase activity and telomere proteins. Conversion of I-type cells to S-type cells through BrdU treatment also triggered reductions in telomerase activity and telomere proteins. Our data indicate that telomere remodeling is tightly linked to the developmental and differentiation pathways in NB tumor cells.

Summary/Conclusions:

Our observations reinforce crucial connections between telomeres, NB tumor cell differentiation, and tumor malignancy. Better understanding of the mechanistic basis of these connections may lead to new biomarkers and therapies for NB.

## **P097 Paternal pre-conception stress as a risk factor for neuroblastoma.**

*Kitlinska, J.*<sup>2</sup>, *Hong, S.H.*<sup>3</sup>, *Da Cruz, R.S.*<sup>4</sup>, *Galli, S.*<sup>5</sup>, *De Assis, S.*<sup>6</sup>

Georgetown University, Biochemistry and Molecular & Cellular Biology, Washington, USA <sup>2</sup>,  
Georgetown University, Biochemistry and Molecular & Cellular Biology, Washington, USA<sup>3</sup>,  
Georgetown University, Oncology, Washington, USA<sup>4</sup>, Georgetown University, Biochemistry and  
Molecular & Cellular Biology, Washington, Georgetown University<sup>5</sup>, Georgetown University, Oncology,  
Washington, USA<sup>6</sup>

### Background/Introduction:

While neuroblastoma (NB) appears to have a genetic background, its etiology cannot be explained solely by genetics. Yet, the role of environment in its development remains understudied. Consequently, there are no established risk factors for NB aside from rare genetic predisposition and it is not clear what determines its malignancy. NBs arise due to defects in sympathetic neuron (SN) differentiation. We hypothesize that pathological states disrupting this process lead to accumulation of neuroblasts, which in conjunction with preexisting or new genetic aberrations promotes NB formation. Since these defects in SN differentiation occur in utero, environmental factors triggering them are most likely to be prenatal or pre-conception exposures. Indeed, maternal stress during pregnancy induces neurodevelopmental defects in offspring. In line with this, we have previously shown that in a mouse model of NB, prenatal stress interferes with SN differentiation in offspring, leading to increased frequency and malignancy of NB. This effect was driven by activation of maternal sympathetic nervous system, which causes uterine vasoconstriction and transient fetal hypoxia. Strikingly, paternal stress prior to conception causes neurodevelopmental and behavioral changes in the offspring that are very similar to those caused by maternal stress during pregnancy. These paternal effects were associated with epigenetic changes in sperm and offspring, which are known to regulate both neuronal differentiation and tumorigenesis. The role of paternal exposures in NB has also been suggested by epidemiological data correlating increased risk of this disease with certain occupations in fathers.

### Aims:

The goal of the current study was to determine the impact of paternal stress prior conception on NB development and phenotype.

### Methods/Materials:

Hemizygous TH-*MYCN* males were subjected to 6 weeks of chronic unpredictable stress (CUS) prior to conception and then mated with wild type females. Sperm epigenetic profiles, placenta morphology and NB frequency and phenotypes in TH-*MYCN* offspring were compared between the control and stress groups.

### Results:

CUS triggered changes in non-coding RNA content of the germline, including miRNAs and tsRNAs. Paternal stress decreased also the rate of successful pregnancies from 75% in controls to 37.5% and increased frequency of death in utero from 5.5% to 29.2%. The male TH-*MYCN* offspring of stressed fathers exhibited accelerated NB development and higher tumor frequency, as well as a more metastatic phenotype. In contrast, in female offspring, NB development remained unaffected by paternal stress, yet the tumors appeared to be more metastatic. In line with these data, we have observed morphological abnormalities in placentas of the stressed fathers' offspring, which suggested insufficient perfusion of the labyrinth tissue, the main site of the oxygen exchange between maternal and fetal blood. These changes were more profound in male fetuses, where they associated with increased area of the junctional zone of the placenta, which stained positively for hypoxia markers.

### Summary/Conclusions:

Our results indicate that paternal pre-conception stress affects placental development via epigenetic mechanisms, which in turn impairs SN differentiation and promotes development of aggressive NB in

a sex-specific manner. Moreover, our data implicate fetal hypoxia as a common mechanism underlying impact of parental stress on NB risk.

## **P098 Kalirin-RAC controls nucleokinetic migration in ADRN-type neuroblastoma**

*Afanasyeva, E.*<sup>2</sup>, *Westermann, F.*<sup>3</sup>, *Gartlgruber, M.*<sup>4</sup>, *Ryl, T.*<sup>5</sup>, *Decaestecker, B.*<sup>6</sup>, *Denecker, G.*<sup>7</sup>, *Speleman, F.*<sup>8</sup>

DKFZ, Neuroblastoma Genomics, Heidelberg, Germany <sup>2</sup>, DKFZ, Neuroblastoma Genomics, Heidelberg, Germany<sup>3</sup>, DKFZ, Neuroblastoma Genomics, Heidelberg, Germany<sup>4</sup>, University of Duisburg Essen, Department of Neurosurgery, Essen, University of Duisburg Essen<sup>5</sup>, Ghent University, Center for Medical Genetics, Ghent, Belgium<sup>6</sup>, Ghent University, Center for Medical Genetics, Ghent, Belgium<sup>7</sup>, Ghent University, Center for Medical Genetics, Ghent, Belgium<sup>8</sup>

### Background/Introduction:

Neuroblastoma (NB) is thought to be a result of impaired differentiation of neural crest-derived progenitor cells, which promotes the expansion of a population of cells susceptible to the secondary transforming events (Pei et al., 2013). NB has been recently resolved as a biphasic malignancy with primary tumors containing the cells of ADRN (noradrenergic) type and MES (mesenchymal) type (Boeva et al., 2017; van Groningen et al., 2017). Both ADRN and MES lineages produce aggressive metastatic tumors, yet ADRN identity could be reprogrammed towards MES “toolkit”. The migrational propensity of NB is affected by the identity, but the mechanisms behind the divergence remain unknown.

Differentiation failure in ADRN NB has been traced at the epigenetic, genetic and transcriptional level and manifests as the down-regulation of the genes involved in maintaining neuronal morphology (Henrich et al., 2016). Yet, many neuritogenesis genes are essential for cell migration and failure in their regulation might be involved in NB invasion program. The question remains open as to how differentiation block and migratory propensity are balanced in the ADRN lineage’s tumors and whether ADRN cells are a subject to EMT process. From a point of clinical relevance, the closest compartment that may reflect migration and dormancy, are disseminated cancer cells detected as part of minimal residual disease (MRD) (Raimondi et al., 2010). Previous studies in NB identified doublecortin (*DCX*) mRNA encoding the core component of neuronal motility, as a MRD marker associated with poor survival in NB patients (Hartomo et al., 2013; Viprey et al., 2014).

### Aims:

Based on these facts, we reasoned that understanding the mechanisms implicated in migration of DCX-positive NB can shed light on initial steps of the metastatic process in NB.

### Methods/Materials:

### Results:

Our data show that *DCX* expression is associated with ADRN identity. Further, ADRN-type cells exhibit nucleokinetic (NUC) migration, resembling motility observed in neurons. Time-lapse movie analysis with ADRN- and MES-type cell lines expressing a nuclear marker allows us to distinguish between mesenchymal (MES) and NUC mode of migration. Using RNAi along with above-described approach, we show that NUC migration relies on integral components of neuronal migration, including the above-mentioned *DCX* and lissencephaly1 (*LIS1*). We propose that parallelism between migration modes in neurons and ADRN NB involves transcriptional control and suggest several candidate TFs including the SOXC factor, *SOX11*, which regulates *DCX* expression in ADRN-type cells. We demonstrate that nucleokinesis is RAC1- and kalirin-dependent in ADRN NB, the latter mediating NUC activity of RAC1.

### Summary/Conclusions:

Taken together, these data provide a rationale for further investigation of vulnerabilities associated with NUC migration in ADRN-type cells.



## **P099 Low dose induction-differentiation therapy for Neuroblastoma in children**

*Zhang, J.H.<sup>2</sup>, Zhang, S.J.<sup>3</sup>, WANG, Y.Z.<sup>4</sup>, GONG, B.C.<sup>5</sup>, Li, Z.J.<sup>6</sup>, Chen, L.Y.<sup>7</sup>*

Shengjing Hospital of China Medical University, Medical research center, Liaoning, China <sup>2</sup>, The Second affiliated Hospital of Jilin University, Hematologic oncology, Jilin, China<sup>3</sup>, The first affiliated Hospital of China Medical University, Hematologic oncology, Liaoning, China<sup>4</sup>, Shengjing Hospital of China Medical University, Medical research center, Liaoning, Shengjing Hospital of China Medical University<sup>5</sup>, Shengjing Hospital of China Medical University, Medical research center, Liaoning, China<sup>6</sup>, Shengjing Hospital of China Medical University, Medical Iconography, Liaoning, China<sup>7</sup>

### **Background/Introduction:**

Neuroblastoma (NB) is the most common pediatric solid tumor that originates in the neural crest. The international therapeutic schedule for neuroblastoma is mainly the maximum tolerance dose chemotherapy + surgery + stem cell transplantation + immunotherapy and other methods, but the maximum tolerance dose chemotherapy makes the hemogram of children worse, the immune system became collapse, and ultimately leads to irreversible consequences. However, low-dose chemotherapy, high-frequency, long-term chemotherapy can not only kill tumor cells, but also effectively protect the immune system, activate the immune response of the body itself, effectively inhibit the growth of tumor. On the other hand, it can also induce tumor differentiation to a benign direction. Finally, these children received surgical resection, or for those patients could not receive a surgery, they showed a balance between the body and tumor, and coexistence with it.

### **Aims:**

The aim of this study is to report the efficacy of low dose induction-differentiation chemotherapy in the treatment for neuroblastoma (NB).

### **Methods/Materials:**

From 1992 to 2018, 232 NB patients enrolled into this study. Patients were separated into two groups, the initial treatment group: never received the chemotherapy; treated group: relapse patients or received chemotherapy in other hospitals. All the 232 cases were treated by low dose induction-differentiation therapy and traditional Chinese medicine, or low dose chemo+1<sup>st</sup> initial surgery (mass only partially resected) +low dose chemo continuously+2<sup>nd</sup> surgery.

### **Results:**

Among the 232 NB patients, 75 patients were completely release (CR), 52 patients were partially release (PR), 36 patients were stable disease (SD), 8 patients were progressive disease (PD), 52 patients were dead, and 9 patients loss of follow-up. K-M survival analysis showed that the 5-year survival rate of the initial treatment group was higher than the treated group (68.4% vs 43.5%,  $p=0.025$ ). And 13 patients showed amazing therapeutic outcome, there were 10 patients who were treated by low dose induction-differentiation chemotherapy and traditional Chinese medicine only, the tumor disappeared completely. In these 10 cases, five were Stage IVs, four were Stage IV and one was Stage III. And the three stage III NB patients were treated with low dose induction-differentiation chemotherapy + surgery (only partial resection) after surgery low dose chemotherapy continued, finally, a second surgery was performed, and the mass was completely removed, each tumor mass was transformed into benign GN confirmed by pathology.

### **Summary/Conclusions:**

The low dose induction-differentiation therapy is an efficient method for the treatment of NB in children, and it can improve prognosis of the NB children.

## **P100 MYT1 attenuates neuroblastoma cell differentiation by interacting with the LSD1/CoREST complex**



*Wu, Z.<sup>2</sup>, Chen, K.<sup>3</sup>, Wu, Y.<sup>4</sup>*

Department of pediatric surgery, Xinhua hospital, Department of pediatric surgery, Shanghai, China <sup>2</sup>,  
Department of pediatric surgery, Xinhua hospital, Department of pediatric surgery, Shanghai, China<sup>3</sup>,  
Department of pediatric surgery, Xinhua hospital, Department of pediatric surgery, Shanghai, China<sup>4</sup>

#### Background/Introduction:

Recent studies have shown that myelin transcription factor 1 (MYT1) promotes vertebrate neurogenesis by regulating gene expression. It can act as either a transcriptional repressor or activator depending on the cell context. However, the roles of MYT1 in regulating cell developments in neuroblastoma remains unknown.

#### Aims:

To understand the molecular mechanisms of MYT1 in regulating cell differentiation of neuroblastoma.

#### Methods/Materials:

MYT1 expression across the clinical samples was evaluated by mass-spectrometry based quantitative proteomics. Tissue array and relevant clinical data were used to investigate the potential roles of MYT1 in neuroblastoma. Further biological mechanism of MYT1 in regulating cell differentiation was investigated with RNA-seq and cell experiments

#### Results:

MYT1 was differentially expressed among neuroblastoma patients with different pathological diagnoses. Analysis of clinical data showed that overexpression of MYT1 was associated with a significantly shorter 3-year overall survival rate and poor differentiation in neuroblastoma specimens. MYT1 knockdown inhibited proliferation and promoted the expression of multiple differentiation-associated proteins. Integrated omics data indicated that many genes involved in neuro-differentiation were regulated by MYT1. Interestingly, many of these genes are targets of the LSD1/CoREST complex and we show that MYT1 physically interacts with this complex. Depletion of LSD1 or inhibition of LSD1 by ORY-1001 decreased the expression of MYT1, providing an alternative approach to target MYT1. Taken together, our results indicate that MYT1 significantly attenuates cell differentiation by interacting with the LSD1/CoREST complex. MYT1 is, therefore, a promising therapeutic target for enhancing the neurite-inducing effect of retinoic acid and for inhibiting the growth of neuroblastoma.

#### Summary/Conclusions:

Our results identify an association between MYT1 expression and the differentiation status of neuroblastoma cells and provide evidence that MYT1 knockdown in combination with inhibition of either retinoic acid or LSD1 can significantly enhance neuroblastoma differentiation. Mechanistically, MYT1 may stabilize REST expression, inhibit differentiation-related gene expression by forming a complex with LSD1 and coREST, and reduce the expression of RARs. Thus, LSD1 inhibitor may alternative provide potential therapeutic benefit for high-risk neuroblastoma patients with high levels of MYT1 expression.

## **P101 Screening of the embryonic sympathoadrenal secretome to identify microenvironmental triggers of neuroblastoma metastatic dissemination reveals key contribution of Noelin1/OLFM1**

*Ben Amar, D.*<sup>2</sup>, *Thoinet, K.*<sup>3</sup>, *Couté, Y.*<sup>4</sup>, *Costechareyre, C.*<sup>5</sup>, *Delloye-Bourgeois, C.*<sup>6</sup>, *Castellani, V.*<sup>7</sup>

NeuroMyoGene Institute (CNRS UMR5310-INSERM U1217-UCBL), Neurodevelopment, Cancer and Signaling, Lyon, France <sup>2</sup>, NeuroMyoGene Institute (CNRS UMR5310-INSERM U1217-UCBL), Neurodevelopment, Cancer and Signaling, Lyon, France<sup>3</sup>, Biosciences and Biotechnology Institute of Grenoble (BIG)- U1038 INSERM/CEA/UGA, Exploring the Dynamics of Proteomes, Grenoble, France<sup>4</sup>, OncoFactory SAS, OncoFactory, Lyon, OncoFactory SAS<sup>5</sup>, NeuroMyoGene Institute (CNRS UMR5310-INSERM U1217-UCBL), Neurodevelopment, Cancer and Signaling, Lyon, France<sup>6</sup>, NeuroMyoGene Institute (CNRS UMR5310-INSERM U1217-UCBL), Neurodevelopment, Cancer and Signaling, Lyon, France<sup>7</sup>

### Background/Introduction:

Neuroblastoma (NB), the most common extracranial solid tumor in childhood, is a neural crest-derived malignancy that arises from the sympatho-adrenal lineage. NB aggressive forms are usually diagnosed with already established metastases, resulting in low survival rate and lack of understanding of early steps of the dissemination process. Our previous work conducted in a novel avian NB model revealed that the sympathetic embryonic environment profoundly impacts the gene program of NB cells, switching-off cell-cell cohesion pathways in the primary tumor to trigger the metastatic process (Delloye-Bourgeois et al., Cancer Cell, 2017).

### Aims:

The first objective of our work was to characterize the impact of the embryonic sympathetic secretome on NB cell-cell cohesion and migratory behavior. Our second aim was to provide an exhaustive description of the sympathetic secretome with the underlying idea of identifying key molecular signals of the microenvironment that impact on NB cell gene program to trigger their detachment from the primary tumor and switch on their migratory potential.

### Methods/Materials:

We produced media conditioned by sympathetic ganglia (SG<sup>CM</sup>) from chick embryos dissected out at different developmental stages and from mouse embryos, and assessed their functional properties on NB cell-cell cohesion, migratory and invasive behaviors. We analyzed by mass spectrometry the proteomic content of SG<sup>CM</sup> and the transcriptomic profiles of SG<sup>CM</sup>-treated NB cells. We extracted a list of relevant candidates and further functionally characterized their effect on NB cell-cell cohesion and migration *in vitro* and *in vivo* in our avian model of metastatic NB.

### Results:

We observed that SG<sup>CM</sup> profoundly affects NB cell-cell cohesion properties and increases their invasive behavior. Proteomic analysis of SG<sup>CM</sup> and RNAseq data of NB cells exposed to SG<sup>CM</sup> allowed to identify a molecular signature centered on the glycoprotein OLFM1 alias Noelin1, whose developmental role in neural crest migration has previously been described. Functional *in vitro* studies combined to *in vivo* experiments consisting in blocking endogenous OLFM1 in our avian tumoral model confirmed that OLFM1 plays a major contribution to the onset of NB metastasis.

### Summary/Conclusions:

Our study provides the first picture of the embryonic sympatho-adrenal microenvironment in which NB primary tumors develop and its impact on NB gene activity and cell behavior. The identification of OLFM1/Noelin1 as a key microenvironment-derived trigger of NB metastatic dissemination underlines the central implication of developmental signaling in the course of the disease and opens the avenue on novel therapeutic strategies for aggressive NB.



## **P102 Uncovering the molecular dialogs of neuroblastoma cells with their different embryonic microenvironments during primary tumorigenesis and metastatic disease progression**

*Delloye-Bourgeois, C.*<sup>2</sup>, *Ben Amar, D.*<sup>3</sup>, *Thoinet, K.*<sup>4</sup>, *Costechareyre, C.*<sup>5</sup>, *Jarrosso, L.*<sup>6</sup>, *Tourniaire, G.*<sup>7</sup>, *Croze, S.*<sup>8</sup>, *Lachuer, J.*<sup>9</sup>, *Castellani, V.*<sup>10</sup>

NeuroMyoGene Institute (CNRS UMR5310-INSERM U1217-UCBL), Neurodevelopment, Cancer and Signaling team, Lyon, France <sup>2</sup>, NeuroMyoGene Institute (CNRS UMR5310-INSERM U1217-UCBL), Neurodevelopment, Cancer and Signaling team, Lyon, France<sup>3</sup>, NeuroMyoGene Institute (CNRS UMR5310-INSERM U1217-UCBL), Neurodevelopment, Cancer and Signaling team, Lyon, France<sup>4</sup>, OncoFactory SAS, OncoFactory, Lyon, OncoFactory SAS<sup>5</sup>, OncoFactory SAS, OncoFactory, Lyon, France<sup>6</sup>, Cellenion SASU, Cellenion, Lyon, France<sup>7</sup>, ProfilExpert/Viroscan3D, UCBL UMS 3453 CNRS - US7 INSERM, Lyon, France<sup>8</sup>, ProfilExpert/Viroscan3D, UCBL UMS 3453 CNRS - US7 INSERM, Lyon, France<sup>9</sup>, NeuroMyoGene Institute (CNRS UMR5310-INSERM U1217-UCBL), Neurodevelopment, Cancer and Signaling team, Lyon, France<sup>10</sup>

### Background/Introduction:

Aggressive neuroblastoma (NB) is usually diagnosed with already established metastases, resulting in a low survival rate despite intensive treatments. This feature underlines a very efficient, early metastatic process occurring in a still developing organism, making it difficult to have a clinical access to the etiological course of the dissemination. We previously reported the setting of an avian model of NB, in which human NB cells are grafted back within their original micro-environment, the neural crest. In this embryonic context, NB cells are driven to form sympatho-adrenal primary tumors and to disseminate into the embryonic tissues, thus mimicking key steps of the NB disease. Comparative RNAseq analyses of pre-grafted NB cells and primary tumors revealed a profound impact of the sympathetic microenvironment, driving changes of NB gene program notably to release NB cell-cell cohesion to allow their secondary dissemination (Delloye-Bourgeois et al, 2017).

### Aims:

We first aimed at analyzing NB metastatic progression at the whole embryo scale using lightsheet microscopy to image the dissemination routes and the metastatic foci in the distal organs at different time points after the graft. Our second aim was to characterize the genome/epigenome states of the different cellular contingents composing NB tumors, when cells colonize primary tumor sites, migrate along distinct metastatic routes -ie, the peripheral nerves or the dorsal aorta-, and settle within distant secondary sites. The underlying objective was to identify genetic and epigenetic signatures reflecting choices taken by NB cells during their metastatic progression and to correlate these features with the cellular and molecular heterogeneity of NBs.

### Methods/Materials:

We engrafted human NB cell lines within the chick embryonic neural crest and processed the embryos for clearing procedure and immunofluorescence prior to light sheet imaging. Next, we micro-dissected tumor foci twelve days post-graft within sympathetic ganglia and adrenal glands (primary tumors), migrating along peripheral nerves and within the dorsal aorta (en route towards secondary tumor sites), and within the bone marrow (metastatic target site). We performed single cell and bulk whole RNA sequencing of cells at each of these anatomic sites.

### Results:

First, we observed huge dissemination along the peripheral nerves and vessels, with sheets of NB cells reaching the thoracic cavity. Later on, NB cells were observed to colonize the skeletal bones and to settle within the bone marrow. Second, data from our RNAseq program were analyzed to extract signatures reflecting the dialogs of NB cells with the embryonic microenvironment.

### Summary/Conclusions:

Our approach, which makes use of a novel NB model, will provide an unprecedented and comprehensive view of NB metastatic process. The data will bring a novel basis for better understanding the processes of NB metastatic dissemination, as well as for determining to which extent the mechanisms of neural crest cell development have been retained and exploited by NB cells to migrate towards distal organs.

## **P103 Tumor suppressive functions of an identified trunk neural crest specific gene**

Fredlund, E.<sup>2</sup>, Persson, C.U.<sup>3</sup>, Van Nes, J.<sup>4</sup>, Mohlin, S.<sup>5</sup>

Lund University, Translational Cancer Research and Division of Pediatrics, Lund, Sweden <sup>2</sup>, Lund University, Translational Cancer Research, Lund, Sweden<sup>3</sup>, Amsterdam UMC University of Amsterdam, Department of Oncogenomics, Amsterdam, Netherlands<sup>4</sup>, Lund University, Translational Cancer Research and Division of Pediatrics, Lund, Lund University<sup>5</sup>

### Background/Introduction:

Neuroblastoma (NB) is a childhood cancer with origin in the developing sympathoadrenal lineage in the early neural crest.

### Aims:

To investigate the role of and potential tumor suppressive functions in NB of a gene recently identified as trunk neural crest (NC) cell specific.

### Methods/Materials:

*In silico* of gene expression in NBs from publicly available RNA sequencing data. We overexpressed the identified NC specific gene in NB cell lines and performed RNA sequencing, analyzed tumor growth *in vivo*, and cell movement, clonogenic capacity, differentiation and proliferation *in vitro*. *In vivo* experiments modulating gene expression in chick embryo NC cells were also performed.

### Results:

The trunk NC specific gene is expressed in a time- and spatially restricted pattern during early human (embryonic week 5 and 6) and avian (E1.5 and E3) NC development. Tissue specific knockdown of the gene during normal avian development resulted in delayed embryonic growth. Gene overexpression in NB cells with no endogenous expression resulted in less tumor burden by decelerated tumor growth and prolonged survival *in vivo* as well as decreased proliferation *in vitro*. Through RNA sequencing we found a defined set of eight genes involved in cell invasion, cell adhesion, angiogenesis, and differentiation. Our identified trunk NC specific gene further discriminates between the mesenchymal- and adrenergic phenotype in NB being virtually exclusively expressed in mesenchymal, neural crest like NB cells and, in addition, in Schwann cell precursor cells.

### Summary/Conclusions:

We present evidence of tumor suppressive functions of this trunk NC specific gene. Gene knockout in chick embryonic NC cells with endogenous expression as well as overexpression in NB cells lacking the endogenous protein suggest important functions during normal and tumor development. The genetic profile depicts a sympathoadrenal origin and our data can aid in defining a subtype of NB driven by loss of trunk NC specific genes.

## P104 ARID1A and a mesenchymal program in neuroblastoma

*Freeman, K.W.*<sup>2</sup>, *Wallace, K.A.*<sup>3</sup>, *Miller, L.*<sup>4</sup>, *Garcia-Lopez, J.*<sup>5</sup>, *Alleboina, S.*<sup>6</sup>, *DeVaux, C.*<sup>7</sup>, *Davidoff, A.*<sup>8</sup>, *Holt, J.*<sup>9</sup>, *Hayes, N.*<sup>10</sup>

University of Tennessee Health Science Center, Genetics, Genomics and Informatics, Memphis, USA<sup>2</sup>, University of Tennessee Health Science Center, Genetics, Genomics and Informatics, Memphis, USA<sup>3</sup>, University of Tennessee Health Science Center, Genetics, Genomics and Informatics, Memphis, USA<sup>4</sup>, St. Jude Children's Research Hospital, Developmental Neurobiology, Memphis, St. Jude Children's Research Hospital<sup>5</sup>, University of Tennessee Health Science Center, Genetics, Genomics and Informatics, Memphis, USA<sup>6</sup>, University of Tennessee Health Science Center, Genetics, Genomics and Informatics, Memphis, USA<sup>7</sup>, St. Jude Children's Research Hospital, Surgery, Memphis, USA<sup>8</sup>, University of Tennessee Health Science Center, Pathology, Memphis, USA<sup>9</sup>, University of Tennessee Health Science Center, Pathology, Memphis, USA<sup>10</sup>

### Background/Introduction:

NBL arises from immature cells during commitment of neural crest cells (NCCs) to a sympathoadrenal lineage. During development, NCCs undergo an epithelial-mesenchymal transition (EMT) and migrate throughout the embryo before terminally differentiating. EMT is a known pro-metastatic program in adult cancers that is part of a stem cell signature and likely contributes to aggressive metastasis in NBL. N-Myc amplified NBL is a high-risk subset of NBL that frequently associates with loss of heterozygosity (LOH) at 1p36. ARID1A is a subunit of the SWI/SNF chromatin remodeling complex which regulates fate decisions during development. In multiple cancers, ARID1A mutations associate with a mesenchymal state that increases cancer aggressiveness. We recently reported that ARID1A is a critical 1p36 tumor suppressor that collaborates with oncogenic N-Myc in NBL, but the mechanism of action is unknown.

### Aims:

To determine if loss of *Arid1a* facilitates N-MYC transformation in NBL by retention of a mesenchymal program and to identify therapeutic vulnerabilities based on *Arid1a* status.

### Methods/Materials:

We innovated an approach for accurately modeling human neuroblastoma that involves N-Myc transformation of primary murine neural crest cells (NCCs). Using NCCs with floxed *Arid1a*, we generated NCCs that are wild-type, have loss of one copy or loss of both copies of *Arid1a*. These NCCs were transfected with N-Myc and injected into mice for tumor formation. The resulting tumors were analyzed by RNA-seq and immunohistochemistry. Tumor derived cell lines were also generated to identify, base on *Arid1a* status, sensitivity to small molecule inhibitors and they were whole exome sequenced to find mutations that are selected for during tumorigenesis.

### Results:

N-Myc/ *Arid1a* NCCs generated tumors on average 40 days earlier than N-Myc/ *Arid1a*<sup>+/+</sup> NCCs, while N-Myc/ *Arid1a*<sup>-/-</sup> NCCs did not show reduce tumor latency. Analysis of these tumor by RNA-seq showed a mesenchymal signature in the *Arid1a* het tumors that was not seen in the wild-type or null tumors. Both the wild-type and null tumors were more adrenergic and glial. These differences were confirmed by immunohistochemistry.

Our preliminary data demonstrates that deletion of ARID1A increases expression of the EMT master transcriptional factors TWIST1, SOX9 and SNAI1 expression in our NBL model. While het tumors show a 5-fold increase in TWIST1, null tumors show a 60-fold increase in TWIST1. It is known that TWIST1 dosage dictates different lineage commitments since TWIST1 functions as a homodimer at low levels of expression but interacts with other bHLH transcription factors at higher expression. Further, our small molecule screening has identified increased sensitivity to inhibitors of mesenchymal

growth factor receptors and the PI3K pathway when Arid1a is lost. We also observe selection for mutations in the PI3K pathway with loss of ARID1A.

Summary/Conclusions:

NCC undergo specification during differentiation by multiple bivalent decisions including an autonomic/mesenchymal fate decision. Our preliminary findings support ARID1A as a repressor of a mesenchymal program found to increase cancer aggressiveness in multiple cancers. We are currently investigating if ARID1A loss maintains a mesenchymal program via overexpression of EMT factors in NBL and if TWIST1 dosage is critical for transformation by *MYCN*.



## **P105 *In vitro* modelling of neuroblastoma initiation using human pluripotent stem cells**

Tsakiridis, A.<sup>2</sup>, Guerrero, I.<sup>3</sup>, Frith, T.<sup>4</sup>, Stavish, D.<sup>5</sup>, Tarelli, S.<sup>6</sup>, Barbaric, I.<sup>7</sup>, Andrews, P.<sup>8</sup>

University of Sheffield, Biomedical Science, Sheffield, United Kingdom <sup>2</sup>, University of Sheffield, Biomedical Science, Sheffield, United Kingdom<sup>3</sup>, University of Sheffield, Biomedical Science, Sheffield, United Kingdom<sup>4</sup>, University of Sheffield, Biomedical Science, Sheffield, University of Sheffield<sup>5</sup>, University of Sheffield, Biomedical Science, Sheffield, United Kingdom<sup>6</sup>, University of Sheffield, Biomedical Science, Sheffield, United Kingdom<sup>7</sup>, University of Sheffield, Biomedical Science, Sheffield, United Kingdom<sup>8</sup>

### Background/Introduction:

Neuroblastoma (NB) is considered as a developmental tumour; due to its embryonic origins: NB tumours are primarily found in the adrenal gland/sympathetic ganglia and therefore are thought to arise during early embryonic development as a result of oncogenic transformation of multipotent trunk neural crest (NC) cells or their derivatives such as sympathoadrenal progenitors (SAP). Consequently, transformed trunk NC/SAP cells fail to differentiate properly, a phenomenon that is likely to contribute to the wide spectrum of cellular phenotypes defining the inter- and intra-tumoural heterogeneity observed in NBs. Since NB heterogeneity has been linked to non-uniform response to treatment and recurrence, it is essential to define precisely how NB tumourigenesis correlates with the distorted differentiation trajectories adopted by transformed trunk NC cells. Current models for studying NB biology involve the use of transgenic animals/primary explants, patient-derived cell lines and xenograft models. Although such approaches have provided valuable insights, they do not fully recapitulate the pathology of human NB due to species differences and only provide a snapshot of late disease stages. Thus these models do not allow the temporal dissection of the events associated with tumour initiation during human trunk NC development, while the limited availability of early-stage human foetal material precludes the direct examination of non-transformed trunk NC/SAP cells in comparison to transformed cells. An attractive solution involves the use of human pluripotent stem cells (hPSCs), which can generate every cell type in the body and therefore represent an attractive *in vitro* model of early human development. However, conventional hPSC differentiation approaches induce only a modest yield of trunk NC and this limitation is a major obstacle to the hPSC-based modelling of NB tumourigenesis.

### Aims:

Here, we aimed to define an efficient protocol for the *in vitro* generation of trunk NC cells and their derivatives from hPSCs.

### Methods/Materials:

### Results:

We show that cultures of hPSC-derived, Brachyury-positive neuromesodermal progenitors, the posteriorly-located drivers of embryonic axis elongation, contain trunk NC precursors. These can be directed to efficiently generate functional trunk NC cells as well as sympathoadrenal progenitors and sympathetic neurons.

### Summary/Conclusions:

This strategy represents a considerable improvement over current differentiation approaches and is an ideal platform for the mechanistic dissection of NB initiation at the cellular and molecular level. To this end, we are currently investigating the behaviour of trunk NC/SAP cells derived from hPSCs carrying two principal NB-associated genetic aberrations: gain of chromosome arm 17q and inducible *MYCN* overexpression to mimic *MYCN* gene amplification.

## **P106 Combined Loss of 1p36 Gene KIF1Bbeta and NF1 Causes Neuroblastoma, Pheochromocytoma and Composite Tumors in Mice**

*Li, W.<sup>2</sup>, Bedoya-Reina, O.<sup>3</sup>, Fell, S.<sup>4</sup>, Plescher, M.<sup>5</sup>, Arsenian Henriksson, M.<sup>6</sup>, Adameyko, I.<sup>7</sup>, Larsson, C.<sup>8</sup>, Juhlin, C.<sup>9</sup>, Schlisio, S.<sup>10</sup>*

Karolinska Institutet, Microbiology, Tumor and Cellbiology, Solna, Sweden <sup>2</sup>, Karolinska Institutet, Microbiology, Tumor and Cellbiology, Solna, Sweden<sup>3</sup>, Karolinska Institutet, Cell and Molecular Biology, Solna, Sweden<sup>4</sup>, Karolinska Institutet, Microbiology, Tumor and Cellbiology, Solna, Karolinska Institutet<sup>5</sup>, Karolinska Institutet, Microbiology, Tumor and Cellbiology, Solna, Sweden<sup>6</sup>, Karolinska Institutet, Physiology and Pharmacology, Solna, Sweden<sup>7</sup>, Karolinska Institutet, Oncology-Pathology, Solna, Sweden<sup>8</sup>, Karolinska Institutet, Oncology-Pathology, Solna, Sweden<sup>9</sup>, Karolinska Institutet, Microbiology, Tumor and Cellbiology, Solna, Sweden<sup>10</sup>

### **Background/Introduction**

We recently discovered a tumor suppressor gene KIF1B $\beta$  located on 1p36 that is required for sympathoblast differentiation and developmental apoptosis during NGF competition. Thus, we generated conditional gene targeting of KIF1Bb in the mouse sympatho-adrenal lineage and generated crosses with mice expressing Cre-recombinase under the control of the gene for dopamine beta hydroxylase (DBH-Cre). We discovered that KIF1B $\beta$  is required for nerve growth factor (NGF)-dependent neuronal differentiation through anterograde transport of the NGF receptor TRKA and that pathogenic KIF1B $\beta$  mutations identified in neuroblastoma impair TRKA transport. Although the expression of neuronal differentiation markers is ablated in KIF1B $\beta$ -deficient mouse neuroblasts, our KIF1B $\beta$ fl/fl;DbhCre mice did not develop any sympathoadrenal malignancies. We predicted that loss of KIF1Bb alone might not be sufficient to cause neuroblastoma in the KIF1B $\beta$ fl/flCRE mice. Recently, a novel pathogenic KIF1Bb missense mutation together with a NF1 loss of function mutation was identified in a patient diagnosed with neural crest tumors and loss of NF1 has been associated with neuroblastoma and pheochromocytoma. We therefore asked if loss of KIF1Bb together with loss of NF1 will mirror neuroblastoma and pheochromocytoma development.

### **Aims**

Developing novel neuroblastoma and pheochromocytoma mouse models for identifying cancer cell of origin. Profiling tumor heterogeneity and immaturity on single cell resolution of mouse neuroblastoma and pheochromocytoma tumors.

### **Methods/Materials**

We generated homozygous KIF1B $\beta$ dbcbKO mice and used tumors for histological, immunohistochemical, and cytogenetic studies to characterize proliferation and differentiation status. We perfused and dissected tumors and adrenal glands, FACS-sorted approximately 400 single cells per tumor/gland and performed single cell sequencing. We performed targeted in situ sequencing (ISS), allowing the generation of targeted, multiplexed expression profiles within fixed tumor tissues at single-cell resolution.

### **Results**

We observed sympathetic ganglion (SCG) hyperplasia in the first month after birth and tumors arising at the adrenal gland of adult double cKO and NF1cKO mice. Double-cKO mice developed neuroblastoma, pheochromocytoma and composite tumors originating from immature chromaffin cells as were control breeding have not resulted in such tumor promoting phenotype. We profiled tumor heterogeneity on single cell resolution. Single cell analysis of the tumors are currently analyzed with respect to regulatory patterns identified in early or late embryonic mouse sympathoblast /crest populations.

Single-cell transcriptomics requires isolation of discrete cells and information about the original location within tissue is lost. Therefore, it is not possible to draw cellular maps of the tumor architecture and their positioning in relation to other cells. In order to map the cells within their physiological, morphological, and anatomical context and space, we performed in situ transcriptomics in these tumors, a method that involves targeted in situ sequencing (ISS).

### **Summary/Conclusions**

Our analysis of our double-cKO mice that develop both, pheochromocytoma and neuroblastoma,

provokes the question if NB and PCC might have a common progenitor or if these are parallel events during sympatho-adrenal development.

## **P107 ASPM is a regulator of neuroblastoma cell proliferation and differentiation**

*Szymansky, A.*<sup>2</sup>, *Becker, L-L*<sup>3</sup>, *Overath, M.D.*<sup>4</sup>, *Winkler, A.*<sup>5</sup>, *Ahrens, K.*<sup>6</sup>, *Dorado García, H.*<sup>7</sup>, *McGearey, A.*<sup>8</sup>, *Astrahantseff, K.*<sup>9</sup>, *Künkele, A.*<sup>10</sup>, *Henssen, A.G.*<sup>11</sup>, *Hertwig, F.*<sup>12</sup>, *Eggert, A.*<sup>13</sup>, *Toedling, J.*<sup>14</sup>, *Kaindl, A.M.*<sup>15</sup>, *Schulte, J.H.*<sup>16</sup>

Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin, Germany<sup>2</sup>, Charité – Universitätsmedizin Berlin, Department of Pediatrics, Division of Neurology, Berlin, Germany<sup>3</sup>, Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin, Germany<sup>4</sup>, Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin, Charité – Universitätsmedizin Berlin<sup>5</sup>, Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin, Germany<sup>6</sup>, Experimental and Clinical Research Center (ECRC) of the Charité and MDC, Clinical research, Berlin, Germany<sup>7</sup>, Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin, Germany<sup>8</sup>, Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin, Germany<sup>9</sup>, Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin, Germany<sup>10</sup>, Experimental and Clinical Research Center (ECRC) of the Charité and MDC, Clinical research, Berlin, Germany<sup>11</sup>, Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin, Germany,<sup>12</sup> Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin, Germany<sup>13</sup>, Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin, Germany<sup>14</sup>, Charité – Universitätsmedizin Berlin, Department of Pediatrics, Division of Neurology, Berlin, Germany<sup>15</sup>, Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin, Germany<sup>16</sup>

### Background/Introduction:

ASPM is a microtubule-associated protein involved in mitotic spindle assembly during cell division. Biallelic mutations in *ASPM* are the most common cause of autosomal recessive primary microcephaly (MCPH), a neurodevelopmental disorder characterized by intellectual disability and reduced brain volume ensuing from defective neural stem cell proliferation. In the other extreme, elevated ASPM levels during development are associated with hyperproliferation and may, therefore, have a causative role in neuro-embryonal malignancies.

### Aims:

Due to their role in regulating proliferation in neuronal progenitor cells, we hypothesized that genes associated with MCPH, and particularly *ASPM*, might drive neuroblastoma cell proliferation. We aimed to evaluate whether *ASPM* overexpression constitutes an independent risk factor in neuroblastoma.

### Methods/Materials:

Expression of all genes associated with MCPH was analyzed in 25 publicly available data sets from tumors arising in either adults or children/adolescents and normal tissues. The association of *ASPM* expression levels with clinical covariates and outcome was investigated in a large, representative neuroblastoma cohort (n=498). Event-free and overall survival in patients with tumors expressing high or low *ASPM* levels were compared using Kaplan-Meier analyses. Multivariate regression analyses based on Cox proportional hazard models were performed to assess the value of *ASPM* expression as a prognostic factor in the context of established neuroblastoma risk criteria (*MYCN* amplification, INSS stage, age, *TERT* expression). The effect of siRNA-mediated *ASPM* knockdown on cell viability, proliferation, cell death and differentiation was investigated.

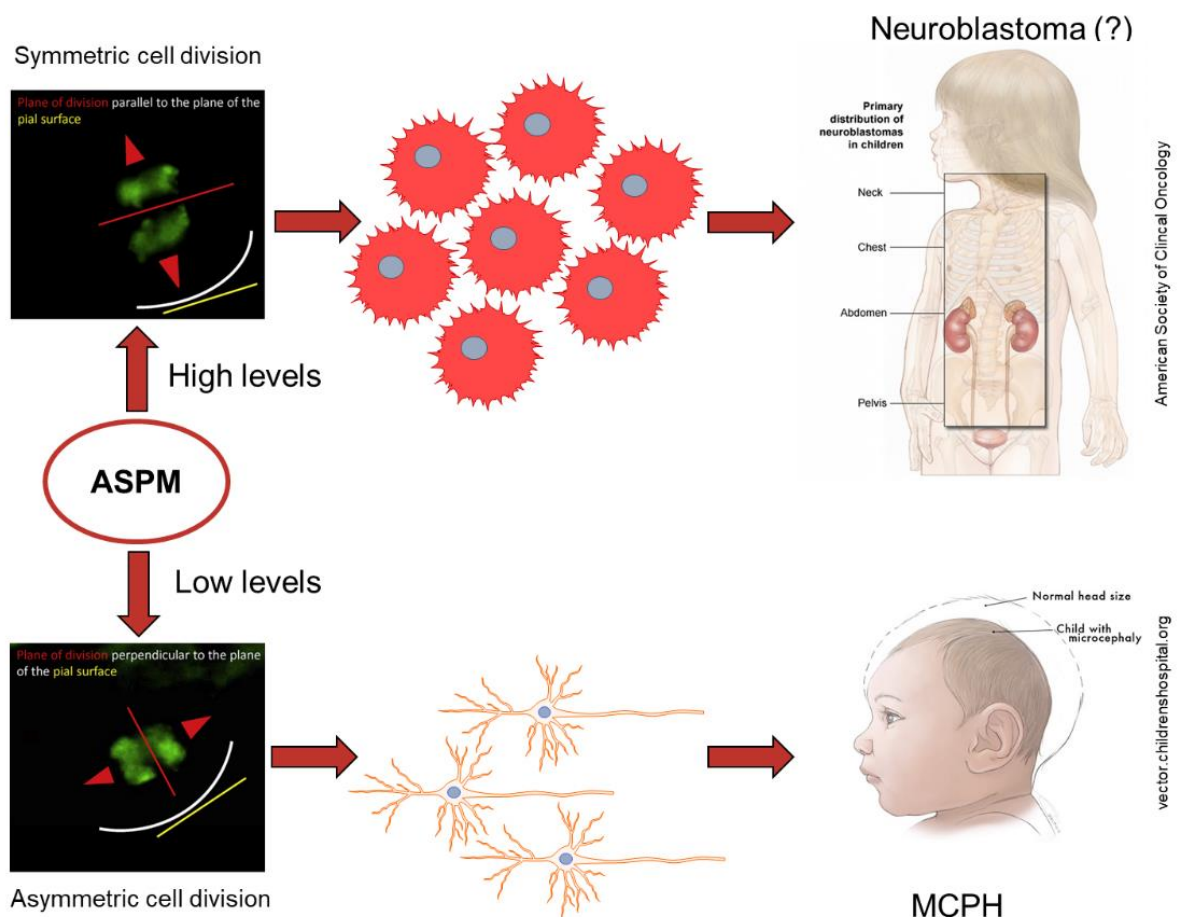
### Results:

Comparisons of multiple microarray gene expression data sets revealed the highest *ASPM* expression levels to be in neuroblastomas and other neuro-pediatric tumors in contrast to tumors arising in adulthood and normal tissues. Elevated *ASPM* levels were significantly associated with adverse

clinical outcome and unfavorable clinical covariates, indicating a functional role of ASPM in aggressive, undifferentiated neuroblastoma biology and a crucial function for proliferation. Multivariate regression analysis revealed that high *ASPM* expression constitutes an independent risk factor in addition to established neuroblastoma risk factors. *ASPM* knockdown in the NGP neuroblastoma cell line reduced cell viability and proliferation. Features of neuronal differentiation also appeared four days following *ASPM* knockdown, such as neurite-like outgrowths, cytoskeletal changes and increased expression of differentiation markers, including microtubule associated protein 2 (MAP2), tubulin beta 3 class III (TUBB3) and the gene encoding the neuronal migration protein, doublecortin (DCX). Differentiation-associated apoptosis was also flow cytometrically detected that accompanied reduced cell proliferation.

Summary/Conclusions:

Our data are in accordance with ASPM playing a functional role in maintaining the undifferentiated phenotype of highly aggressive neuroblastoma cells. ASPM function in the spindle apparatus suggest spindle-checkpoint and spindle-assembly inhibitors may be potential treatment options for neuroblastomas with high-level *ASPM* expression.



Caption 1: Is the microtubule-associated protein ASPM a possible link between microcephaly and neuroblastoma?

**P109 Modification of Home-Made HPLC Method Allowed the Detection of Interference in The Measurement of Catecholamines and Metanephrines**

Tamir, R.<sup>2</sup>

Rambam Medical Center, Clinical Biochemistry Lab, Haifa, Israel <sup>2</sup>

#### Background/Introduction:

BRAF is a serine/threonine kinase immediately downstream of KRAS in the MAPK signaling pathway. The mutation most commonly expressed in human neoplasia, V600E, is expressed in a variety of common malignancies, among them are colon cancer, melanoma, thyroid cancer, glioma and more. Previous studies demonstrated that in some tumors this mutation is a prognostic marker and can be utilized for targeted therapy. In general, few studies investigated the presence of this mutation in pediatric tumors, and in particular, the evaluation of neuroblastoma, has been minimal.

#### Aims:

The aim of this study was to investigate the expression of the most common BRAF mutation - V600E, in a variety of neuroblastic tumors.

#### Methods/Materials:

We used the antibody Mouse anti-human BRAF V600E monoclonal antibody (clone VE1). Demographic as well as clinical and pathologic data was collected for these patients including age, gender, stage and outcome. The immunohistochemical stains were evaluated using a 3-tiered semiquantitative method.

#### Results:

The study included tumor samples from sixty four patients: 55 Neuroblastomas, 7 Ganglioneuroblastomas and 2 Ganglioneuromas. The immune stains from all samples were negative for the BRAF. The controls were all positive.

#### Summary/Conclusions:

These results cannot support the existence of BRAF mutation (V600E) in neuroblastic tumors. Therefore, this mutation cannot be considered as a target for treatment of neuroblastic tumors.

## **P110 The role of the neurotrophin receptors, TrkA/NTRK1 and TrkB/NTRK2, in modulation of radiation responses in neuroblastoma.**

Hassiepen, C.H.<sup>2</sup>

Universitätsklinikum Essen/ Innere Klinik, Molecular Oncologie, Essen, Germany <sup>2</sup>

### Background/Introduction:

Radiotherapy is an integral part of treatment in many cancer types including neuroblastoma. Neuroblastoma is the most common extracranial tumor of childhood and shows high diversity in disease outcome. While in some patients tumors differentiate spontaneously, in others the disease spreads aggressively and even after successful initial therapy tumor relapse might occur. High expression of TrkA/NTRK1 has been found to be associated with a good prognosis, while high TrkB/NTRK2 expression on the other hand is associated with an unfavorable outcome. Both, TrkA/NTRK1 and TrkB/NTRK2 are members of the neurotrophin receptor family and are involved in multiple processes during neuronal development including differentiation and apoptosis. *In vitro* models of neuroblastoma with different TrkA/NTRK1 and TrkB/NTRK2 levels suggested a role for neurotrophin receptors in modulation of chemo- and radiation resistance.

### Aims:

The aim of the project is to investigate if TrkA/NTRK1 and TrkB/NTRK2 or their downstream targets are involved in radioresistance in neuroblastoma.

### Methods/Materials:

We previously established inducible TrkA/NTRK1 or TrkB/NTRK2 overexpression in the human neuroblastoma cell line SH-SY5Y. To investigate the effects of TrkA/NTRK1 and TrkB/NTRK2 expression and activation in the context of response to irradiation (IR), we monitored changes in cell-cycle distribution by PI-staining and H3pS10-assay. The effects of TrkA/NTRK1 and TrkB/NTRK2 overexpression and activation in combination with different irradiation intensities were analyzed by MTT and colony formation assay.

### Results:

We generated human neuroblastoma SH-SY5Y cells with inducible or stable overexpression of TrkA/NTRK1 or TrkB/NTRK2 by using tetracycline-controlled transcriptional activation. Expression of NTRKs was unaffected by irradiation. Phenotypic and molecular characterization of the radiation response indicated a defective G2/M checkpoint in NTRK1-overexpressing cells, indicated by a higher mitotic index upon ionizing radiation (IR). This effect could be rescued by addition of the NTRK-inhibitor, Loxo-101, suggesting a direct impact of NTRK1 on the response to IR. However, DNA damage-sensing is still intact in SY5Y cells as ATM- or ATR-inhibition is sufficient to abrogate IR induced checkpoint.

Previously, TrkB/NTRK2 expression had been linked with chemo-resistance in NB cells by NTRK2-dependent activation of the PI3K-pathway and by up-regulating MDR (multidrug-resistance) genes (Jaboin et al., Cancer Research, 2002). Our results also point to a TrkB/NTRK2-mediated enhancement in clonogenic survival upon IR. This effect could be confirmed by assessing cell viability in a short term survival assay when TrkB/NTRK2 was either stably or inducibly expressed

### Summary/Conclusions:

Our experimental data indicate that both, TrkA/NTRK1 and TrkB/NTRK2, are modulators of the radiation response. While increased TrkA/NTRK1 activity in SH-SY5Y cells led to DNA damage independent cell-cycle progression, increased TrkB/NTRK2 signaling was characterized by an increased long term survival upon irradiation. Both findings are in line with clinical data, which correlated high TrkA/NTRK1 expression with a favorable and high TrkB/NTRK2 expression with an unfavorable prognosis.

However, further investigation of the molecular downstream mechanisms of these receptors is important to understand underlying principles and to identify potential therapeutic targets.



## **P111 Metallothionein-3 is an important factor for development of chemoresistance to CDDP and extravasation and intravasation of neuroblastoma cells**

*Merlos Rodrigo, M.A.<sup>2</sup>, Casar, B.<sup>3</sup>, Hana, H.<sup>4</sup>, Strmiska, V.<sup>5</sup>, Adam, V.<sup>6</sup>, Heger, Z.<sup>7</sup>*

Mendel University in Brno, Chemistry and Biochemistry, Brno, Czech Republic <sup>2</sup>, Instituto de Biomedicina y Biotecnología de Cantabria (IBBT) - CSIC, Cell & Molecular Signalling, Cantabria, Spain<sup>3</sup>, Mendel University in Brno, Chemistry and Biochemistry, Brno, Czech Republic<sup>4</sup>, Mendel University in Brno, Chemistry and Biochemistry, Brno, Mendel University in Brno<sup>5</sup>, Mendel University in Brno, Chemistry and Biochemistry, Brno, Czech Republic<sup>6</sup>, Mendel University in Brno, Chemistry and Biochemistry, Brno, Czech Republic<sup>7</sup>

### Background/Introduction:

Neuroblastoma (Nbl) is a challenging childhood malignancy, with a very high percentage of patients relapsing following acquisition of drug resistance, thereby necessitating the identification of mechanisms of drug resistance as well as new biological targets. Cisplatin (CDDP) is one of the most commonly used drugs in the treatment of Nbl. Human metallothionein-3 (hMT-3) is presumed to participate in the processes of heavy metal detoxification and protection against oxidative damage of free radicals in the central nervous system. It is worth noting that the concentration of MTs increases in the moment of administration of the platinum-based drugs. Such stimulated MTs can also rapidly bind the administered CDDP, which can result in decrease of drug concentration below the effective level. However, the primary functions of hMT-3 and the mechanism underlying its multiple functions in neuroblastoma have not been elucidated so far.

### Aims:

We aim to gain further understanding of the mechanisms underlying development of CDDP resistance using a chick chorioallantoic membrane xenograft model (CAM assay).

### Methods/Materials

The CAM sustains rapid tumor formation within 5-7 days after neuroblastoma cells grafting. This feature provides a unique experimental model for a rapid study of the intravasation and colonization steps of the metastatic cascade. Furthermore, using quantitative PCR to detect species-specific sequences, such as Alu, the chick embryo CAM model can be used to monitor and quantify the presence of the xenografted, ectopic tumor cells in distant tissues.

### Results

CAM (UKF-NB-4 cells) treated with 100  $\mu$ M CDDP during 24 h exhibited inhibition of the weight of primary tumors. The number of UKF-NB-4 cells in CAM distal, liver, lung and brain decreased significantly, compared with the control (no treatment). In contrast, CAM induced with CDDP resistant cells (UKF-NB-4CDDP) did not show significant differences between 0 – 100  $\mu$ M CDDP. The obtained results indicate that, due to the acquired chemoresistance, UKF-NB-4CDDP cells also exhibit high rate of chemoresistance in CAM xenograft model. Also, we used the CAM assay to examine whether CDDP had any effect on metastatic spreading after hMT-3 overexpression in UKF-NB-4 cells. The tumors were very reliably grown after 6 days. Then, the 100  $\mu$ M CDDP was added topically on the upper CAM during 24 h. The data showed no significant differences in primary tumor weight in mock vs. hMT-3 in UKF-NB-4 cell line upon treatment with 100  $\mu$ M CDDP, (91.6 mg vs. 86.7 mg, respectively). However, hMT-3 overexpression in UKF-NB-4 cells increased the capacity of intravasation, extravasation and metastatic spreading of Nbl cells.

### Summary/Conclusions

We identified a significant increase in chemoresistance to CDDP due to hMT-3 overexpression in CDDP sensitive UKF-NB-4 cell line in in vitro and in vivo model. In addition, hMT-3 markedly stimulated the ability of UKF-NB-4 cells to extravasate and intravasate in CAM. The results provide new insights into better understanding of the role of hMT-3 in progression of Nbl.

## **P112 Clinical significance of PHOX2B gene expression for predicting metastasis and prognostic evaluation in newly diagnosed neuroblastoma patients**

*Fan, F.<sup>2</sup>, Xing, T.<sup>3</sup>, Hong, H.<sup>4</sup>, Duan, C.<sup>5</sup>, Zhao, W.<sup>6</sup>, Wang, X.<sup>7</sup>, Zhao, Q.<sup>8</sup>, Jin, M.<sup>9</sup>, Su, Y.<sup>10</sup>, Ma, X.<sup>11</sup>*

Beijing Children's Hospital, Capital Medical University, Hematology Oncology Center, Beijing, China <sup>2</sup>, Beijing Children's Hospital, Capital Medical University, Hematology Oncology Center, Beijing, China<sup>3</sup>, Beijing Children's Hospital, Capital Medical University, Hematology Oncology Center, Beijing, China<sup>4</sup>, Beijing Children's Hospital, Capital Medical University, Hematology Oncology Center, Beijing, Beijing Children's Hospital, Capital Medical University<sup>5</sup>, Beijing Children's Hospital, Capital Medical University, Hematology Oncology Center, Beijing, China<sup>6</sup>, Beijing Children's Hospital, Capital Medical University, Hematology Oncology Center, Beijing, China<sup>7</sup>, Beijing Children's Hospital, Capital Medical University, Hematology Oncology Center, Beijing, China<sup>8</sup>, Beijing Children's Hospital, Capital Medical University, Hematology Oncology Center, Beijing, China<sup>9</sup>, Beijing Children's Hospital, Capital Medical University, Hematology Oncology Center, Beijing, China<sup>10</sup>, Beijing Children's Hospital, Capital Medical University, Hematology Oncology Center, Beijing, China<sup>11</sup>

### **Background/Introduction**

Paired-like homeobox 2B (PHOX2B) is specifically expressed in nervous system including neuroblastoma cells, but little is known about the clinical significance of the expression of PHOX2B in bone marrow (BM) and peripheral blood (PB) samples of neuroblastoma patients.

### **Aims**

The aim of this study was to test the clinical significance of PHOX2B gene expression in newly diagnosed neuroblastoma patients.

### **Methods/Materials**

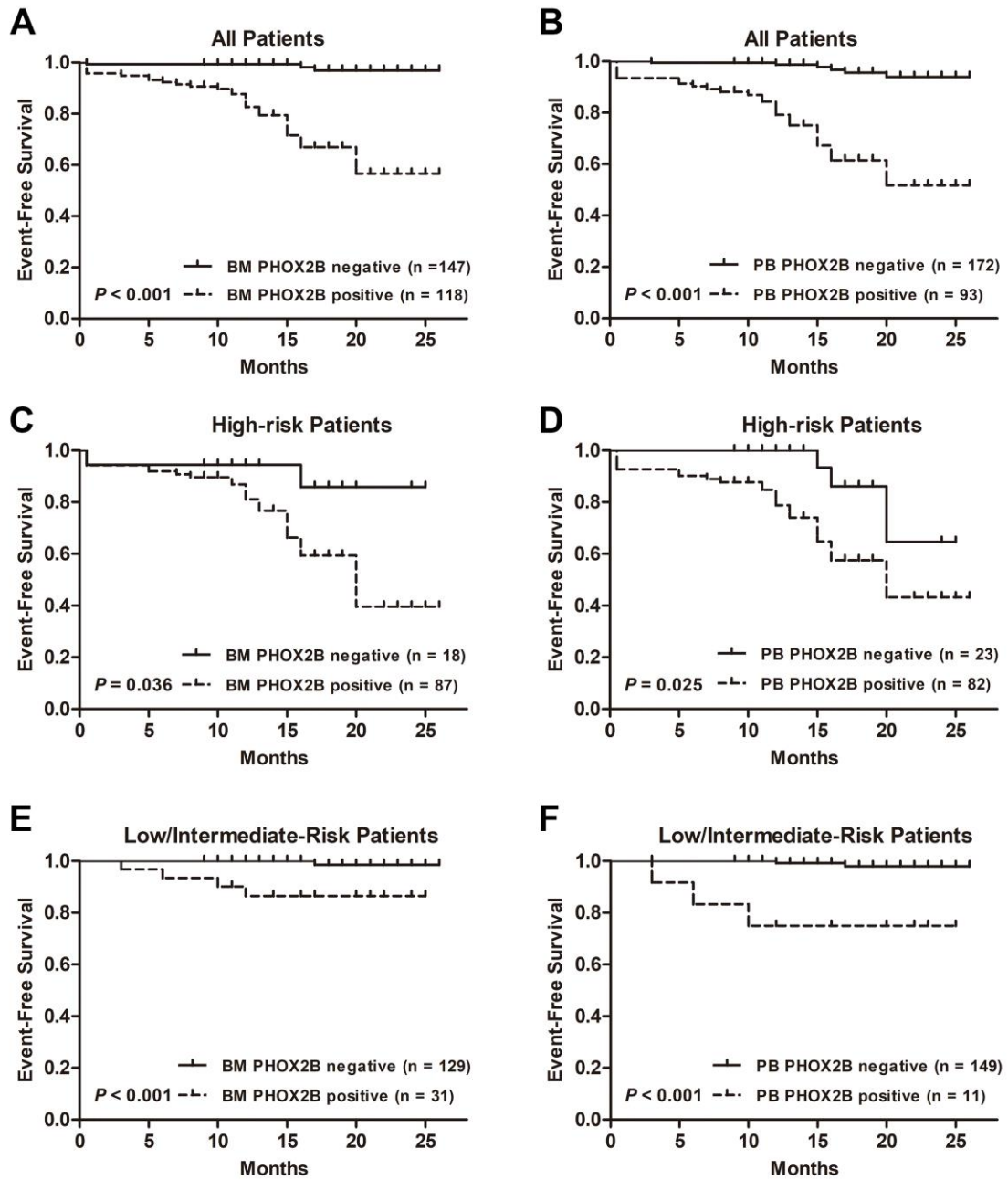
The expression of PHOX2B in samples of BM and PB was tested by quantitative reverse transcriptase polymerase chain reaction (RT-PCR). The predicting metastasis significance of PHOX2B was established through receiver operating characteristic (ROC) analysis. The prognostic significance of BM and PB involvement at diagnosis was assessed by event free survival (EFS).

### **Results**

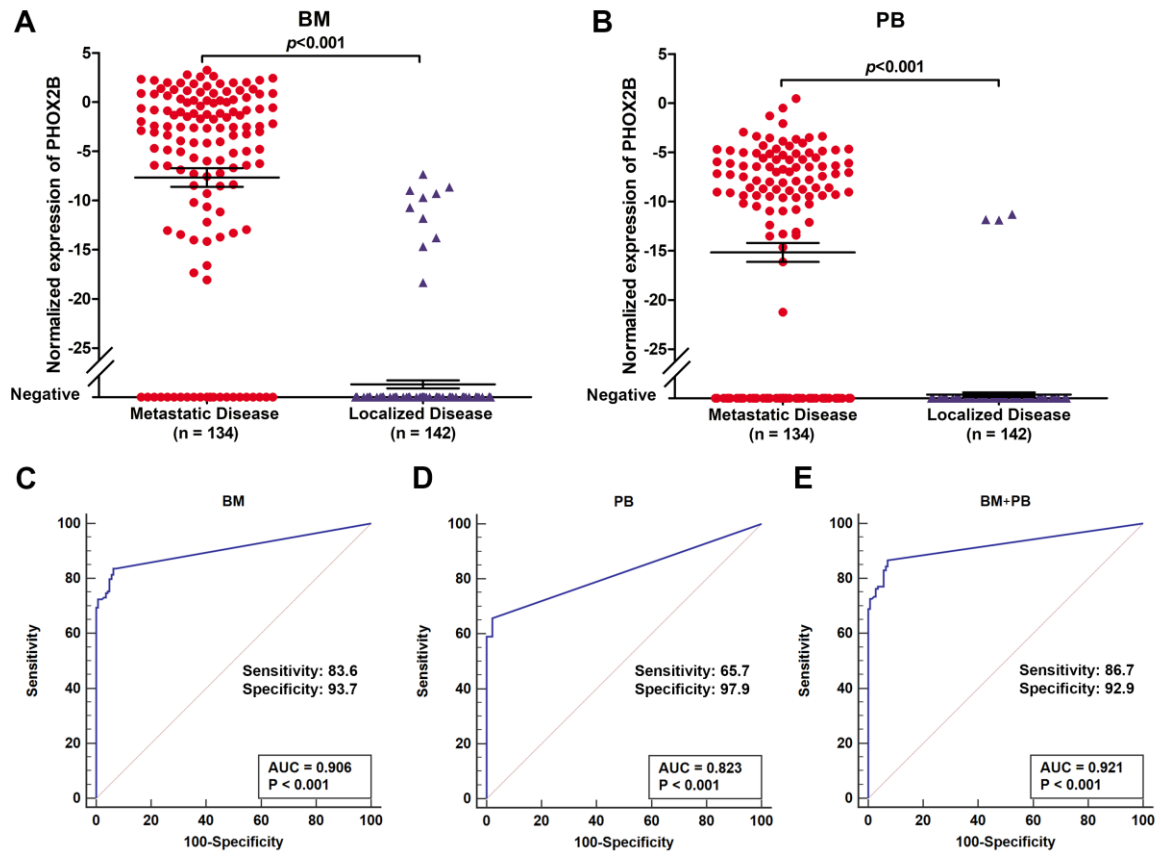
We identified the combined expression of PHOX2B in both BM and PB provided a high diagnostic accuracy for metastasis of neuroblastoma (AUC = 0.920) with the sensitivity and specificity of 86.7% and 92.9%, respectively. At last, 265 children were enrolled for prognostic analysis. The median follow-up time was 16 months. Positive expressions of PHOX2B in BM and PB at diagnosis were associated with worse EFS in patients with neuroblastoma ( $P < 0.05$ ). What's worse, 19.4% (31/160) and 6.9% (11/160) patients with positive expression of PHOX2B in BM and PB at diagnosis in low/intermediate-risk group with favorable prognosis also had worse EFS ( $P < 0.05$ ).

### **Summary/Conclusions**

The expressions of PHOX2B in BM and PB were high in patients with unfavorable clinically characteristics. PHOX2B could be an appropriate biomarker for predicting metastasis in neuroblastoma patients. The expressions of PHOX2B in BM and PB at diagnosis had adverse prognostic significance in neuroblastoma patients as well as in patients with low-/intermediate-risk group.



Caption 1: The expression of PHOX2B in BM and PB samples for predicting metastasis of neuroblastoma



Caption 2: Event-free survival of positive and negative expression of PHOX2B in BM and PB of neuroblastoma patients

## **P113 Casein Kinase II (CK2) as novel therapeutic target in Neuroblastoma**

Gowda, C.<sup>2</sup>, Desai, D.<sup>3</sup>, Reed, R.<sup>4</sup>, Dovat, D.<sup>5</sup>

Pennsylvania State University College of Medicine, Pediatrics, Hershey, USA <sup>2</sup>, Pennsylvania State University College of Medicine, Pharmacology, Hershey, USA<sup>3</sup>, Pennsylvania State University College of Medicine, Pediatrics, Hershey, USA<sup>4</sup>, Pennsylvania State University College of Medicine, Pediatrics, Hershey, Pennsylvania State University College of Medicine<sup>5</sup>

### Background/Introduction:

Casein kinase II (CK2) is a constitutively active, ubiquitously present serine/threonine kinase that plays a critical role in multiple normal cellular processes. CK2 overexpression has been demonstrated in many types of cancers including neuroblastoma. CK2 promotes tumorigenesis and tumor cell survival via several pathways including phosphorylation of transcription factors such as P53, PTEN and Ikaros. CX-4945 (Silmitasertib), is an orally bioavailable, ATP-competitive CK2 inhibitor that show anti-tumor activity in several cancers including leukemia and neuroblastoma. CX-4945 is currently being tested in Phase I/II clinical trial setting for adult solid tumors and pediatric brain tumors.

### Aims:

Validate CK2 as therapeutic target in neuroblastoma and establish the efficacy of CK2 inhibitor CX-4945 in neuroblastoma preclinical models.

### Methods/Materials:

Oncomine NBL database was used to analyze correlation between CSNK2A1 gene expression and clinical outcome. Neuroblastoma cell lines SH-SY-5Y, SK-N-RA and SMS-KCNR were used for *in vitro* experiments. CK2 retroviral transduction of NBL cell lines was used to overexpress CK2. CK2 ShRNA was used for molecular inhibition and CX-4945 for pharmacological inhibition of CK2. RNA sequencing of CK2 altered neuroblastoma cells were done and gene expression changes and pathway analysis were done using Ingenuity pathway analysis software. Athymic nude mice were subcutaneously injected with NBL cells to establish preclinical models. CX-4945 was purchased from vendor was used at dose of 150mg/kg oral gavage once daily for 14 days. Serial tumor measurement was obtained to compare tumor growth inhibition.

### Results:

We analyzed several publically available neuroblastoma gene expression data sets which showed that CSNK2A1 expression is positively correlated with older age, increased mortality, high INSS stage and *MYCN* amplification. In addition, CSNK2A1 mRNA expression is significantly positively correlated to *MYCN* amplification status, high stage disease, tumor recurrence. In tumors without *MYCN* amplification, high CSNK2A1 expression is still prognostic for poor outcome in Kaplan-Meier analysis.

Overexpression of CK2 in Neuroblastoma cell lines (SK-N-RA, SH-SY-5Y, SMS-KCNR) via lentiviral transduction results in increase cell proliferation and decreased apoptosis (*in vitro*) and rapid tumor growth when subcutaneously implanted in athymic mice. Gene expression analysis of CK2 overexpressed NBL cell lines using RNA sequencing showed upregulation of several cancer related genes. NBL cell lines with molecular silencing of CK2 (CK2ShRNA) show decreased tumor growth. CX4945 showed cytotoxic activity in all NBL cell lines with IC50 ranging from 10-12uM. CX4945 is very well tolerated as single agent in mouse models as well as in humans (published data from Phase I study in adults). CX4945 showed significant tumor inhibition (50-60%) in mouse models of NBL cell lines (SH-SY-5Y and SMS-KCNR) at 150mg/kg/day PO given daily for 14 days. CX4945 also delayed tumor progression (tumor endpoint volume >2000mm<sup>3</sup>) in treated mice.

### Summary/Conclusions:

CK2 overexpression in Neuroblastoma cases correlates with poor outcome and decreased survival independent of *MYCN* status. Inhibition of CK2 is potential therapeutic strategy in neuroblastoma. CX-

4945 shows potent antitumor effect in NBL murine models. Further studies to establish mechanism of action of CK2 inhibitors in NBL are underway.

## **P114 Investigating chemoresistance mechanisms in neuroblastoma using a longitudinal cisplatin resistance cell model**

*Balachandran, A.<sup>2</sup>, Seneviratne, J.<sup>3</sup>, Flemming, C.<sup>4</sup>, Le Grand, M.<sup>5</sup>, Kavallaris, M.<sup>6</sup>, Cheung, B.<sup>7</sup>, Marshall, G.<sup>8</sup>, Carter, D.<sup>9</sup>*

Children's Cancer Institute, Embryonal Cancer Therapy and Prevention, Sydney, Australia <sup>2</sup>, Children's Cancer Institute, Embryonal Cancer Therapy and Prevention, Sydney, Australia<sup>3</sup>, Children's Cancer Institute, Experimental Therapeutics, Sydney, Australia<sup>4</sup>, Children's Cancer Institute, Tumour Biology and Targeting, Sydney, Children's Cancer Institute<sup>5</sup>, Children's Cancer Institute, Tumour Biology and Targeting, Sydney, Australia<sup>6</sup>, Children's Cancer Institute, Embryonal Cancer Therapy and Prevention, Sydney, Australia<sup>7</sup>, Sydney Children's Hospital, Kids Cancer Centre, Sydney, Australia<sup>8</sup>, Children's Cancer Institute, Embryonal Cancer Therapy and Prevention, Sydney, Australia<sup>9</sup>

### **Background/Introduction:**

A significant challenge to anti-cancer therapy is the heterogeneity within tumours driven by genetically, transcriptionally and phenotypically divergent cancer cell populations. This intra-tumoural heterogeneity equips cancers with innate and/or acquired mechanisms of drug resistance, often among minor subclones. Recent studies have uncovered significant aspects of neuroblastoma heterogeneity that impact their response to drug treatment. Neuroblastoma consists of adrenergic and mesenchymal cells, the latter displaying higher resistance to chemotherapy. It has also been shown that neuroblastoma cells can acquire drug resistance through transcriptional changes leading to dysregulation of chromatin structure and downstream gene expression. These findings highlight the importance of understanding cell plasticity and the substantial variation in the types of molecular alterations, at a single cell resolution, that occur in response to anti-cancer therapy.

### **Aims:**

We aimed to assess the transcriptional heterogeneity within and between parental IMR-32 neuroblastoma cell line and its drug-resistant counterparts to identify transcriptional profiles that may explain mechanisms of cisplatin resistance.

### **Methods/Materials:**

To investigate heterogeneity and drivers of chemoresistance in neuroblastoma, we established a longitudinal drug resistance cell model using the IMR-32 cell line. Treatment-naïve IMR-32 cells were pulsed with increasing concentrations of cisplatin, over 13 months. Three increasingly resistant cell lines were established at separate time points during this period. We used the 10X Genomics Chromium™ platform to profile the transcriptomes of 39,000 single cells across these four IMR-32 cell lines. We next examined gene expression signatures of individual cells to distinguish unique transcriptional states, cell clusters and reconstruct transcriptional trajectories. We then functionally validated candidate genes involved in cisplatin resistance using immunofluorescence, western blotting and cell viability assays.

### **Results:**

Through our single cell analysis, we identified three transcriptionally distinct clusters, each with varying abundances of cells from the four cell lines that provide evidence for depletion or enrichment of cell populations with increasing cisplatin resistance. Hence, we inferred that varying transcriptomically-defined phenotypes are involved in governing drug sensitivity or resistance. We further identified a transcriptionally dormant cluster composed predominantly of cells from the resistant cell lines suggesting that transcriptional inactivation could be a potential mechanism for drug resistance. Through trajectory analysis we observed strong transitions in the expression of cytoskeletal genes with increasing cisplatin exposure, especially of the microtubule destabiliser, stathmin-1 (STMN1). Immunocytochemistry of these targets revealed altered microtubule network structures in the resistant cells. We found that shRNA mediated knockdown of STMN1 sensitised cells to cisplatin treatment in a neuroblastoma cell line, SK-N-BE2.

#### Summary/Conclusions:

Our work suggests that upon cisplatin exposure, specific transcriptionally-defined phenotypes of individual cells influence sensitivity or resistance in IMR-32 cells and that the parental cell line contains a transcriptionally dormant subpopulation which is enriched upon cisplatin treatment. Taken together we find that cisplatin treated neuroblastoma cells become resistant using complex and multifaceted mechanisms involving transcriptomic silencing and cytoskeletal adaptation.



## **P115 45A ncRNA EXPRESSION IMPAIRS MICROTUBULES DYNAMICS AFFECTING DRUG RESPONSE IN NEUROBLASTOMA**

*Calderoni, M.*<sup>2</sup>, *Grinovero, N.*<sup>3</sup>, *Viaggi, S.*<sup>4</sup>, *Pagano, A.*<sup>5</sup>

University of Genoa, Department of Experimental Medicine (DIMES), Genoa, Italy <sup>2</sup>, University of Genoa, Department of Experimental Medicine (DIMES), Genoa, Italy<sup>3</sup>, University of Genoa, 2.

Department of Earth Sciences, Environment, and Life (DISTAV), Genoa, Italy<sup>4</sup>, University of Genoa, Department of Experimental Medicine (DIMES), Genoa, University of Genoa<sup>5</sup>

### Background/Introduction:

45A non-coding (nc) RNA overexpression induces deep modifications of neuroblastoma (NB) cell cytoskeleton leading to a cascade of reactions which interferes with proliferation control, cell migration, tumorigenic potential and cell adhesion properties. In detail, 45A-overexpressing cells show a non-specific metastatic engraftment whereas the silencing of its expression drives to the production of liver-specific metastasis. The reasons behind this modification remains still unclear.

### Aims:

We demonstrate the tuned regulation of several proteins involved in microtubules dynamics with relevant effects on cancer development driven by 45A ncRNA expression. Indeed, the proper function of these proteins is fundamental for the cytoskeleton organization and its impairment leads to the particularly dangerous condition known as chromosomal instability (CIN).

### Methods/Materials:

We investigated by Real-time qPCR, western blot and immunocytochemical analysis the different expression and/or localization of GTSE1 (G2 And S-Phase Expressed 1), MCAK (Mitotic Centromere-Associated Kinesin), Aurora B, p53 and the altered organisation of tubulin in different neuroblastoma cell (SKNBE2) models stably overexpressing or downregulating 45A ncRNA.

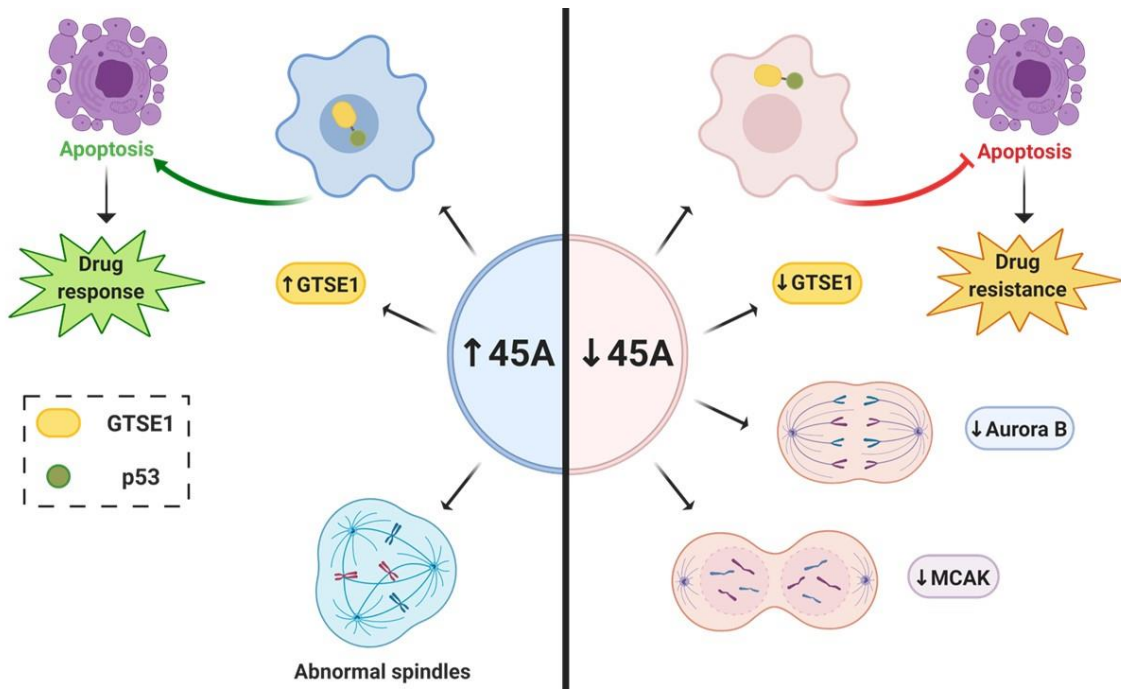
### Results:

We demonstrate that 45A ncRNA not only directly regulates the expression of aforementioned proteins but can even affect the subcellular localization of GTSE1: in 45A-overexpressing cells the protein is accumulated in nuclei, while 45A-downregulation leads to a significant GTSE1 cytoplasm relocation (nuclear GTSE1 being 86% vs 14% respectively,  $p < 0.01$ ). The simultaneous cytoplasmic sequestration of p53, driven by GTSE1, affects apoptosis leading to the observed resistance of 45A-downregulating cells to spindle poisons (e.g. paclitaxel, vincristine, vinblastine).

Furthermore, 45A-overexpression leads to an increased number of abnormal spindles, thus promoting CIN and possibly explaining the increased tumorigenic potential exhibited by 45A-overexpressing cells.

### Summary/Conclusions:

These data highlight the role of 45A ncRNA in the regulation of the expression of several proteins involved in microtubules dynamics, causing variations in drug response and suggesting its possible relevance in NB prognosis and/or therapy.



Caption 1: Graphical abstract

## **P116 The effect of PLK4 inhibitor Centrinone combined with Isotretinoin on neuroblastoma**

Liu, Y.<sup>2</sup>

Tianjin Medical University Cancer Institute and Hospital, Pediatric oncology, Tianjin, China <sup>2</sup>

### Background/Introduction:

Neuroblastoma is a malignancy of the sympathetic nervous system that almost occurs in early childhood. It has unique clinical biological heterogeneity. Neuroblastoma patients with spontaneous regression or differentiation have good prognosis. However, the prognosis of patients who have an extensive metastatic disease is still unsatisfactory and prone to recurrence even after intensive multimodal treatment. The existence of minimal residual disease is one of the reasons for the recurrence, and isotretinoin (13-cis retinoic acid) is used for the elimination of minimal residual disease. But for patients receiving isotretinoin treatment, the 5-year overall survival rate is still only approximately 60%. In addition, NB has strong intratumoral heterogeneity. All of these provide challenges for the treatment of NB. PLK4 plays an important role in the regulation of centrosome replication, and its abnormal expression is associated with poor prognosis in breast cancer, liver cancer, ovarian cancer, NB and so on. Based on this phenomenon, researchers have developed a variety of inhibitors against PLK4 which as a target of clinical treatment, such as CFI-400945, YLT-11, Centrinone etc. Centrinone can inhibit centriole replication and delay cell mitosis. The purpose of this study was to investigate the effects of the inhibitor Centrinone and its combination with all-trans retinoic acid on centromere replication, DNA damage and cell function in NB cells.

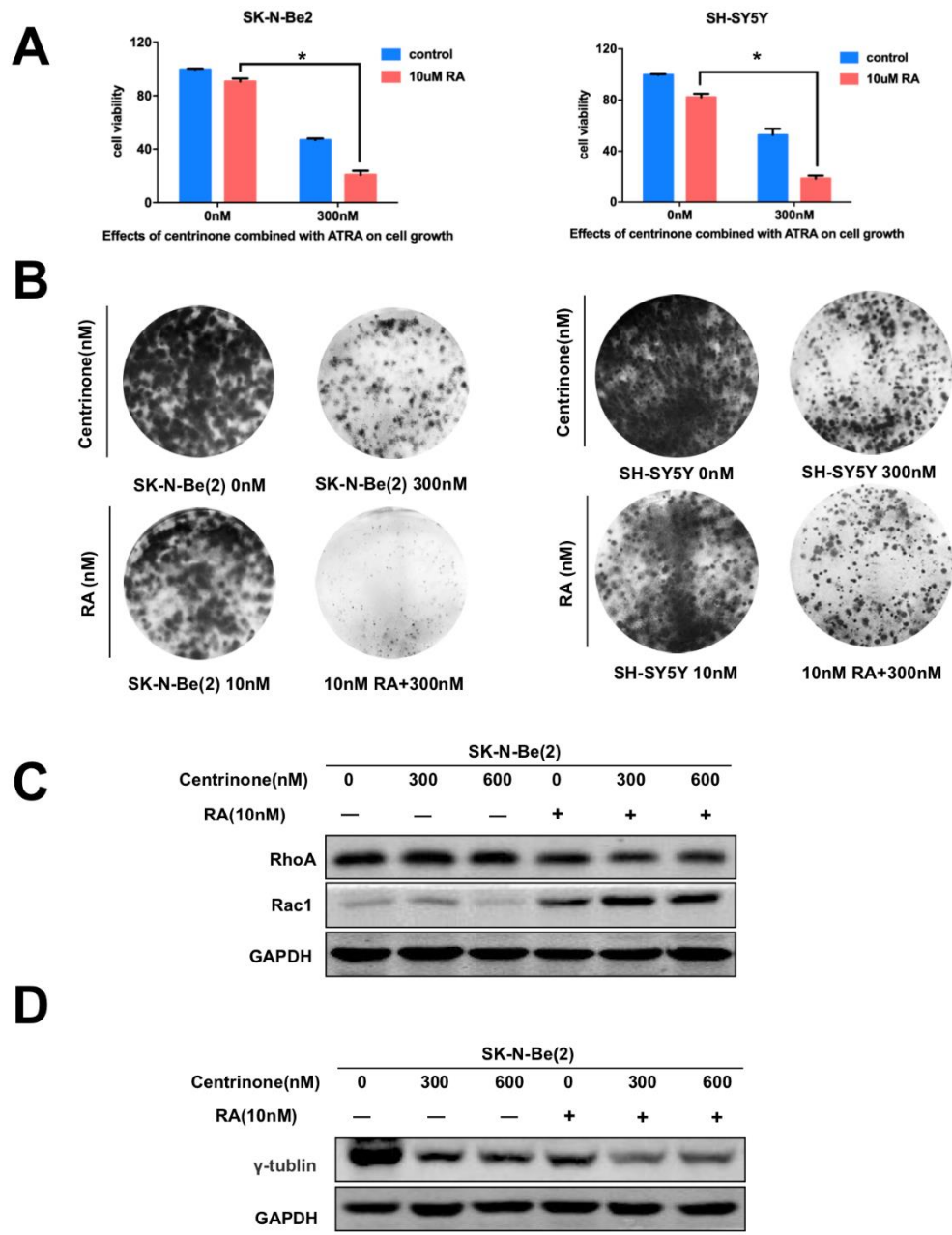
### Aims:

However, despite its widespread use in neuroblastoma for the past decade, there remain a number of drawbacks to its clinical use. PLK4 (Polo-like kinase 4) plays an important role in centriole duplication. The deregulation of PLK4 can lead to mitotic defect and contribute to tumorigenesis. Centrinone is an inhibitor against PLK4, that can inhibit centriole duplication and delay cell mitosis. The purpose of this study was to investigate the effects of the Centrinone and its combination with Isotretinoin on centriole duplication, DNA damage and apoptosis in NB cells.

### Methods/Materials:

Method: 1. To analyze the expression of PLK4 in neuroblastoma and its prognostic relationship with NB Adopting immunohistochemistry and western blot to verify the expression of PLK4 in NB tissues and cells, then the TCGA database was used to analyze the relationship between the expression level of PLK4 and the prognosis of NB patients. 2. To verify the effect of inhibitor Centrinone on the expression of PLK4 and P-PLK4(S305) in NB cells by western blot test. 3. To verify the effect of Centrinone on the function of NB cells (1) To detect the IC50 of NB cell line after adding Centrinone by MTT assay. (2) To verify the effects of Centrinone on proliferation, differentiation and apoptosis of NB cell lines by plate cloning and Western blot. 4. To verify the effect of Centrinone on centriole duplication and DNA damage repair by western blot. 5. To induce differentiation of NB cell lines by using all-trans retinoic acid (ATRA) ,then to detect the expression of Rac1, RhoA, S-100, NSE and other differentiation-related indicators, and after the differentiation model of NB cells was established, test the expression of PLK4, P-PLK4 (S305) and  $\gamma$ -tublin. 6. The centrinone was combined with ATRA and added into NB cells. To verify the effects of the combination on proliferation, migration, differentiation and apoptosis of NB cells by western blot , and test the expressions of  $\gamma$ -tublin, 53BP1 and BRCA1. Results: This study showed the Centrinone can inhibit cell proliferation, centriole duplication, and promote cell apoptosis and DNA damage. Importantly, The combination between Centrinone and Isotretinoin results in more durable and synergistic tumor regression than either single agent alone. Summary/Conclusions: Therefore, targeting PLK4 in combination with Isotretinoin may result in increased antitumor efficacy in Neuroblastoma.

**Fig3**



## **P117 MCM2 and CA9 are novel potential targets for neuroblastoma pharmacological treatment**

*Baldini, F.*<sup>2</sup>, *Garbati, P.*<sup>3</sup>, *Barbieri, R.*<sup>4</sup>, *Cangelosi, D.*<sup>5</sup>, *Zanon, C.*<sup>6</sup>, *Costa, D.*<sup>7</sup>, *Thellung, S.*<sup>8</sup>, *Tonini, G.P.*<sup>9</sup>, *Florio, T.*<sup>10</sup>, *Pagano, A.*<sup>11</sup>

University of Genoa, Department of Experimental Medicine (DIMES), Genova, Italy<sup>2</sup>, Ospedale Policlinico San Martino, IRCCS, Genova, Italy<sup>3</sup>, Ospedale Policlinico San Martino, IRCCS, Genova, Italy<sup>4</sup>, IRCCS Giannina Gaslini Institute, Laboratory of Molecular Biology, Genova, IRCCS Giannina Gaslini Institute<sup>5</sup>, Istituto di Ricerca Pediatrica (IRP)-Fondazione Città della Speranza, Haematology Oncology Lab, Padova, Italy<sup>6</sup>, Ospedale Policlinico San Martino, IRCCS, Genova, Italy<sup>7</sup>, University of Genoa, Department of Internal Medicine and Centre of Excellence for Biomedical Research, Genova, Italy<sup>8</sup>, Istituto di Ricerca Pediatrica (IRP)-Fondazione Città della Speranza, Haematology Oncology Lab, Padova, Italy<sup>9</sup>, University of Genoa, Department of Internal Medicine and Centre of Excellence for Biomedical Research, Genova, Italy<sup>10</sup>, University of Genoa, Department of Experimental Medicine, Genova, Italy<sup>11</sup>

### Background/Introduction:

Despite survival rates of Neuroblastoma (NB) patients have been improved significantly by advancements in cancer treatment in recent years, high-risk neuroblastoma (HR-NB) remains associated to high mortality. In order to investigate NB cell differentiation and to overcome the lack of effective pharmacological treatments for HR-NB, the development of *in vitro* and *in vivo* models that better recapitulate the disease are required.

### Aims:

We employed the NDM29-based model in order to identify genes resulting progressively up-regulated proceeding from the non-malignant stage of mature neuron-like cells to the fully malignant stage and those up-regulated during cell differentiation exhibiting the highest expression level in the differentiated non-malignant cells.

### Methods/Materials:

In the present study, human neuroblastoma cancer cells were genetically engineered to overexpress NDM29, a non-coding RNA whose increased synthesis causes the differentiation toward a neuronal phenotype. According to the different numbers of extra copies integrated and the different efficiency of expression, these clones express NDM29 RNA at different levels and exhibit a directly correlated level of differentiation/maturation toward a neural phenotype. This *in vitro* model encompasses NB cell differentiation stages and can be used to identify potential novel pharmacological targets.

### Results:

This analysis allowed us to identify (by low-density RT-PCR arrays) two gene sets, one over-expressed during NB cell differentiation and the other up-regulated in more malignant cells. Challenging two HR-NB gene expression datasets, we found that these two sets of genes are related to high and low survival, respectively. Mouse NB cisplatin-treated xenografts allowed the identification of two genes within the list associated to the malignant stage (MCM2 and CA9) whose expression was positively correlated with tumor growth. Thus, we tested their pharmacological targeting as a potential therapeutic strategy. We measured mice survival and growth rate of human NB xenografts treated with cisplatin in the presence of MCM2/CA9 inhibitors (ciprofloxacin and acetazolamide).

### Summary/Conclusions:

The results obtained show that NDM29-dependent multistep NB differentiation model recapitulates several features of NB cell differentiation and is suitable to identify novel prognostic genes and their inhibitors thus providing novel potential therapeutic molecules to be further investigated in preclinical studies. In our experimental setting, two gene lists represent pro-malignant and pro-differentiation sets where the pro-malignant genes might represent novel targets for innovative NB therapies. We observed that, *in vivo*, the pharmacological inhibition of MCM2 and/or CA9 is efficacious to

inhibit/block tumor growth and increases cisplatin activity, supporting the possible testing of these two genes as targets for NB therapy.

## **P118 Role of Rho-GTPases signalling in Neuroblastoma**

Gómez-Muñoz, M.A.<sup>2</sup>, A. Álvarez, A.<sup>3</sup>, Pardal, R.<sup>4</sup>, Vega, F.<sup>5</sup>

Instituto de Biomedicina de Sevilla, Departamento de Fisiología Médica y Biofísica de la Universidad de Sevilla (US), Sevilla, Spain <sup>2</sup>, Instituto de Biomedicina de Sevilla, Departamento de Biología Celular de la Universidad de Sevilla, Sevilla, Spain<sup>3</sup>, Instituto de Biomedicina de Sevilla, Departamento de Fisiología Médica y Biofísica de la Universidad de Sevilla, Sevilla, Spain<sup>4</sup>, Instituto de Biomedicina de Sevilla, Departamento de Biología Celular de la Universidad de Sevilla, Sevilla, Instituto de Biomedicina de Sevilla<sup>5</sup>

### Background/Introduction:

Neuroblastoma (NB) is the most frequent extracranial solid tumour in childhood, causing 15% of all paediatric oncology deaths. It arises in the embryonic neural crest due to an uncontrolled behaviour of sympathetic nervous system progenitors giving rise to heterogeneous tumours. The disease is characterized for its variable clinical presentation, which ranges from spontaneous regression to incurable aggressive metastatic tumours. During embryonic development, neural crest cells migrate throughout the embryo and ultimately differentiate into multiple cell types such as sympathetic neurons, glial derivatives or pigment cells.

Failures on neural crest cells migration and differentiation processes could result in the development of NB. Recent genomic analysis has identified Rho GTPase signaling pathway as one of the most frequently mutated in neuroblastoma tumours. Rho-GTPases proteins act as molecular switches cycling between GDP inactive state and GTP active state and regulated by multiple positive and negative factors. This family of proteins plays a key role in many physiological and pathological processes like cell proliferation, cell migration or differentiation. As a result, their deregulation could be having an impact on aggressive, resistant or metastatic NB.

### Aims:

To describe the consequences of Rho-GTPases signalling deregulation in high risk NB

### Methods/Materials:

Extensive expression studies were performed using "R2 platform" (<http://r2.amc.nl>). Quantitative PCR was used to analyse Rho-GTPases gene expression in CHLA-20, CHLA-255, IMR-32 and SK-N-SH NB cell lines. NB cell lines stably expressing relevant Rho GTPases were generated. Cell viability, cell differentiation and xenografts assays were carried out using CHLA 20 and CHLA 255 NB cell lines, in immunocompromised mice.

### Results:

*In silico* expression studies show general deregulation of many GTPases in high risk NB patients. RhoB, RhoC, RhoF, RhoH, Cdc42, RhoJ, RhoQ, Rnd2, Rnd3, Rac1, Rac2, RhoU, RhoBTB1/2 seemed to be downregulated in high risk cases, meanwhile RhoA, RhoD, Rnd1, Rac3, RhoG, RhoV were upregulated in 3 and 4 stages, compared to 1 and 2 stages. Moreover, low levels of RhoB and RhoU determined poor overall survival in NB patients. Rho-GTPases expression like RhoB or Rac3 were also found significantly deregulated in NB relapses. In addition, some RhoGTPases were enriched in particular NB cell populations. Some Rho-GTPases modulators and effectors were also deregulated in high risk NB, correlating with Rho-GTPases expression.

Rho-GTPases expression in CHLA 20, CHLA 255, IMR-32 and SK-N-SH NB cell lines were particularly low. Cdc42 overexpression in CHLA 20 and CHLA 255 NB cell lines decreased cell proliferation compared to parental cell lines. In addition, glial differentiation was promoted in Cdc42 overexpressing NB cell lines. Cdc42 overexpressing cell lines gave rise to lower tumour incidence and smaller size. These tumours showed lower expression of proliferative markers, like Ki67, comparing to control xenografts.

#### Summary/Conclusions:

The Rho-GTPases signalling pathway is altered in high risk NB cases, leading us to think of their possible role in aggressive NB. Specially, the overexpression of the Rho-GTPase Cdc42 in NB cells causes a decrease in cell proliferation *in vitro* and *in vivo*. In addition, Cdc42 promotes cell differentiation in NB cell lines.



## **P119 ARNT-dependent HIF-2 transcriptional activity is not sufficient to regulate downstream target genes in neuroblastoma**

Niklasson, C.U.<sup>2</sup>, Von Stedingk, K.<sup>3</sup>, Fredlund, E.<sup>4</sup>, Bexell, D.<sup>5</sup>, Pahlman, S.<sup>6</sup>, Wigerup, C.<sup>7</sup>, Mohlin, S.<sup>8</sup>

Lund University, Translational Cancer Research, Lund, Sweden <sup>2</sup>, University of Amsterdam, Department of Oncogenomic, Amsterdam, Netherlands<sup>3</sup>, Lund University, Translational Cancer Research, Lund, Sweden<sup>4</sup>, Lund University, Translational Cancer Research, Lund, Lund University<sup>5</sup>, Lund University, Translational Cancer Research, Lund, Sweden<sup>6</sup>, Lund University, Translational Cancer Research, Lund, Sweden<sup>7</sup>, Lund University, Translational Cancer Research, Lund, Sweden<sup>8</sup>

### Background/Introduction:

Hypoxia-inducible factor (HIF)-2alpha associates with poor outcome in neuroblastoma and glioblastoma, and gain-of-function mutations in the *EPAS1* gene (encoding HIF-2alpha) have been reported in paragangliomas and pheochromocytomas. Specific targeting of a druggable hydrophobic pocket in the HIF-2alpha PAS-B domain with PT2385 have demonstrated promising clinical results for clear cell renal cell carcinoma (ccRCC).

### Aims:

Investigate the effect of PT2385-mediated inhibition of ARNT dependent HIF-2 activity in neuroblastoma.

### Methods/Materials:

Neuroblastoma patient-derived xenograft (PDX) cells were treated with PT2385 and analyzed for HIF-2-dependent gene expression, HIF activity, HIF-2alpha protein localization, response to chemotherapy and orthotopic tumor growth *in vivo*.

### Results:

We detected high levels of HIF-2alpha protein in perivascular niches in neuroblastoma PDXs *in vivo* and at oxygenated conditions in PDX-derived cell cultures *in vitro* particularly in the cytoplasmic fraction. Nuclear HIF-2alpha expression was reduced following PT2385 treatment, but surprisingly, virtually no effects on tumor growth *in vivo* or expression of canonical HIF downstream target genes *in vitro* were observed. In coherence, RNA sequencing of PT2385-treated PDX cells revealed a virtually unaffected transcriptome. Treatment with PT2385 did not affect cellular response to chemotherapy. In contrast, HIF-2alpha protein knockdown resulted in profound downregulation of target genes.

### Summary/Conclusions:

The lack of effect from PT2385 treatment in combination with high cytoplasmic HIF-2alpha expression at normoxia suggest that HIF-2alpha have additional roles than acting as an ARNT dependent transcription factor. It is important to further unravel the conditions at which HIF-2alpha has transcriptional and non-transcriptional roles in neuroblastoma.

## **P120 Prp19 is an independent prognostic marker and promotes neuroblastoma progression by regulating the YAP signaling pathway**

*Wu, Z.<sup>2</sup>, Cai, Y.<sup>3</sup>, Wu, Y.<sup>4</sup>*

Department of pediatric surgery, Xinhua hospital, Department of pediatric surgery, Shanghai, China <sup>2</sup>,  
Department of pediatric surgery, Xinhua hospital, Department of pediatric surgery, Shanghai, China<sup>3</sup>,  
Department of pediatric surgery, Xinhua hospital, Department of pediatric surgery, Shanghai, China<sup>4</sup>

### Background/Introduction:

Neuroblastoma is the most common extracranial solid tumor in children. Although the overall prognosis of neuroblastoma patients has significantly improved, the prognosis of high-risk patients remains poor. Pre-mRNA processing factor 19(Prp19) was previously reported to involve in tumor progression. However, the Prp19 expression and functions remain elusive in neuroblastoma.

### Aims:

To identify the functions and mechanisms of Prp19 in neuroblastoma.

### Methods/Materials:

The correlation among clinical characteristics, survival and expression of the Prp19 were assessed using a neuroblastic tumor tissue microarray and was validated in two independent patient datasets. The role of Prp19 in neuroblastoma cell proliferation, migration and invasion was examined by overexpression or downregulation of Prp19. Gene expression regulations of Prp19 were analyzed by RNA-sequencing and bioinformatics.

### Results:

Neuroblastic tumor tissue microarrays and two independent validation datasets indicated that Prp19 was associated with high-risk markers and served as a prognostic marker for worse clinical outcome in neuroblastoma. Prp19 promoted proliferation, migration and invasion of neuroblastoma cell lines (SK-N-BE (2) and SK-N-AS). KEGG analysis and differential gene expression analysis showed that the expressions of YAP, a key member of the Hippo-YAP pathway, and its downstream genes were significantly inhibited after downregulation of Prp19. Further experiments showed that Prp19 regulates YAP expression by pre-mRNA splicing of YAP. Downregulating Prp19 may reduce the expression of YAP and consequently inhibit cell proliferation, migration and invasion in neuroblastoma.

### Summary/Conclusions:

Prp19 is associated with high-risk markers and poor prognosis in neuroblastoma and may promote neuroblastoma progression by regulating RNA splicing of YAP.

## **P121 The R2 Platform as a Resource for Neuroblastoma Data Analysis and Visualization**

*Koster, J.<sup>2</sup>, Zwijnenburg, D.A.<sup>3</sup>, Molenaar, P.<sup>4</sup>, Volckmann, R.<sup>5</sup>, Willinge Prins, R.<sup>6</sup>, Hoyng, L.<sup>7</sup>, Versteeg, R.<sup>8</sup>*

Academic Medical Center (AMC), Oncogenomics, Amsterdam, Netherlands <sup>2</sup>, Academic Medical Center (AMC), Oncogenomics, Amsterdam, Netherlands<sup>3</sup>, Academic Medical Center (AMC), Oncogenomics, Amsterdam, Netherlands<sup>4</sup>, Academic Medical Center (AMC), Oncogenomics, Amsterdam, Academic Medical Center (AMC)<sup>5</sup>, Academic Medical Center (AMC), Oncogenomics, Amsterdam, Netherlands<sup>6</sup>, Academic Medical Center (AMC), Oncogenomics, Amsterdam, Netherlands<sup>7</sup>, Academic Medical Center (AMC), Oncogenomics, Amsterdam, Netherlands<sup>8</sup>

### Background/Introduction:

Over the past decade, the R2 platform has proven to be a useful and ever-expanding resource for the Neuroblastoma community. As science progresses and novel technologies become available, so does the development of the R2 platform continues to expand.

### Aims:

### Methods/Materials:

The R2 program, rooted in neuroblastoma research, has a user-friendly interface enabling a wide range of interconnected analyses, which can be harnessed by users with limited or no bioinformatics training. The web-based genomics analysis and visualization platform has been greatly extended over the past period. Novel analyses, easier workflows, and new types of omics data have been introduced.

### Results:

Aside from mRNA expression, copynumber, SNP, Exome and whole genome sequencing data we have greatly extended R2 with intuitive tools for the analysis of single cell, epigenome and ChIPseq analyses. Various different technologies can be combined in integrative analyses and visualizations.

We have added many histone modification profiles of neuroblastoma cell lines and tumor samples. On these profiles peak calling has been performed as well as super enhancer analyses if applicable.

Interactive plots, like ROSE SE visualizations, genome browser views and heatmaps of aligned peaks provide accessible entry points for further exploration of one, or a series of profiles. In addition, integration with other genomic features, such as chromatin states, transcription factor binding sites, gene expression patterns help to test hypotheses. Genome reconstruction has also been added to the repertoire. Finally, Tools to work with single cell sequencing data are now being implemented and added to the repertoire.

Other additions: The popular tSNE dimensionality reduction algorithm has been added and allows for interactive definition of patient subgroups on the basis of the underlying genomics data. UMAP dimensionality reduction is being implemented.

Improved expression signature generation, coupled to various downstream analyses now allows the users to get a better understanding of their cohorts or experiments. The introduction of data scopes, creates easier workflows and allows for more focused analyses within R2.

### Summary/Conclusions:

R2 remains a valuable resource for high throughput data of Neuroblastoma. The R2 program and database has been used in more than 1200 PubMed listed publications and is publicly accessible at <http://r2.amc.nl>. R2 assists researchers in the identification of important genes and biological processes in neuroblastoma.



## **P122 Liquid biopsy in neuroblastoma patients using Tumor Epitope Detection in CD14+/CD16+ Macrophages (EDIM)**

*Warmann, S.<sup>2</sup>, Stagno, M.J.<sup>3</sup>, Handgretinger, R.<sup>4</sup>, Fuchs, J.<sup>5</sup>, Schmid, E.<sup>6</sup>*

University Children's Hospital, Pediatric Surgery and Pediatric Urology, Tuebingen, Germany <sup>2</sup>,  
University Children's Hospital, Pediatric Surgery and Pediatric Urology, Tuebingen, Germany<sup>3</sup>,  
University Children's Hospital, Pediatric Oncology and Hematology, Tuebingen, Germany<sup>4</sup>, University  
Children's Hospital, Pediatric Surgery and Pediatric Urology, Tuebingen, University Children's  
Hospital<sup>5</sup>, University Children's Hospital, Pediatric Surgery and Pediatric Urology, Tuebingen,  
Germany<sup>6</sup>

### **Background/Introduction:**

Epitope Detection In Macrophages (EDIM blood test) has been described to investigate characteristics of solid tumors in the peripheral blood of patients. This blood test allows detection and analysis of the tumor proteins transketolase-like 1 (TKTL1, activation of aerobic glycolysis) and Apo10 (DNaseX, proliferation defect) in a non-invasive way by isolating intracellular tumor epitopes in peripheral blood macrophages after phagocytosis of tumor cells. The ganglioside GD2 is highly expressed in neuroblastoma and thus possibly represents an attractive additional specific biomarker for the EDIM blood test.

### **Aims:**

Aim of this study was to evaluate the role of TKTL1, Apo10, and GD2 as potential targets for EDIM liquid biopsy and as biomarkers for diagnosis, monitoring, and disease progression in neuroblastoma.

### **Methods/Materials:**

Expression levels of TKTL1 and Apo10 in neuroblastoma tumor samples ( $n=6$ ) and cell lines (LAN-1, IMR-32, SK-N-BE, LS, SH-SY5Y) were analyzed using RT-PCR and flow cytometry. Macrophages from the peripheral blood of patients with neuroblastoma ( $n=38$ ) and from healthy individuals (control group,  $n=37$ ) were labeled with CD14+/CD16+, then analyzed using flow cytometry, and assessed for the presence of intracellular epitopes of TKTL1, Apo10, and GD2 using the EDIM blood test technology. LAN-1 cells were co-cultured with blood macrophages in order to verify phagocytosis of tumor compounds as underlying mechanism for the EDIM technology.

### **Results:**

Expression levels of TKTL1 and Apo10 were elevated in all investigated neuroblastoma tumor samples, neuroblastoma cell lines showed differential TKTL1 and Apo10 expression levels. A significant upregulation of TKTL1, Apo10, and GD2 in peripheral blood macrophages co-incubated with LAN-1 neuroblastoma cells was observed compared to peripheral blood macrophages alone or peripheral macrophages incubated with DMEM medium. Using flow cytometry we identified tumor epitopes in peripheral blood macrophages of the investigated neuroblastoma patients (TKTL1 36/38, Apo10 34/38, GD2 16/19). Analysis of tumor epitopes in the macrophages of the control group showed negative results for TKTL1 (0/37) and Apo10 (0/37), as well as positive values for GD2 in only 2/22 patients. Expression scores of these three epitopes in the EDIM blood tests were able to significantly differentiate between neuroblastoma patients and healthy blood donors.

### **Summary/Conclusions:**

Analyzing tumor epitopes in CD14+/CD16+ labeled peripheral macrophages using the EDIM blood test seems to be a promising non-invasive tool for liquid biopsy in neuroblastoma patients. Further studies are currently being undertaken evaluating a potential risk stratification at initial diagnosis, determination of tumor biology, treatment monitoring, and early detection of tumor relapses.



## **P123 Characterization of the immunosuppressive microenvironment during *MYCN*-driven oncogenesis.**

Moreno-Smith, M.,<sup>1</sup> Barbieri, E.,<sup>2</sup> Tao, L.,<sup>3</sup> Wolf, A.B.,<sup>4</sup> Dysthe, M.,<sup>5</sup> Moreno-Smith, M.,<sup>6</sup> Badachhape, A.A.,<sup>7</sup> Ghaghada, K.B.,<sup>8</sup> Bettini, M.L.,<sup>9</sup> Parihar, R.,<sup>10</sup> Barbieri, E.<sup>11</sup>

Baylor College of Medicine, Department of Pediatrics, Dan L Duncan Comprehensive Cancer Center, Houston, USA<sup>1</sup>, Baylor College of Medicine, Department of Pediatrics, Dan L Duncan Comprehensive Cancer Center, Houston, USA<sup>2</sup>, Baylor College of Medicine, Department of Pediatrics, Dan L Duncan Comprehensive Cancer Center, Houston, USA<sup>3</sup>, Baylor College of Medicine, Cancer and Cell Biology Graduate Program, Houston, USA<sup>4</sup>, Baylor College of Medicine, Department of Pediatrics, Cell and Gene Therapy, Houston, Baylor College of Medicine<sup>5</sup>, Baylor College of Medicine, Department of Pediatrics, Dan L Duncan Comprehensive Cancer Center, Houston, USA<sup>6</sup>, Baylor College of Medicine, Department of Radiology, Houston, USA<sup>7</sup>, Baylor College of Medicine, Department of Radiology, Houston, USA<sup>8</sup>, University of Utah, Department of Pathology, Salt Lake City, Utah, USA<sup>9</sup>, Baylor College of Medicine, Department of Pediatrics, Cell and Gene Therapy, Houston, USA<sup>10</sup>, Baylor College of Medicine, Department of Pediatrics, Dan L Duncan Comprehensive Cancer Center, Houston, USA<sup>11</sup>

### Background/Introduction:

Tumors evolve an immunosuppressive microenvironment that prohibits the immune system's natural ability to recognize and destroy tumor cells. The *MYC* oncogene drives both metabolic rewiring and immune evasion by targeting both innate and adaptive immune regulators. *MYCN* amplification occurs in almost 50% of high-risk neuroblastoma (NB) patients and associates with repressed cellular immunity. However, it remains elusive how *MYCN* regulates the immunosuppressive microenvironment during NB progression.

### Aims:

The overall goal of this study was to characterize the immune landscape of NB tumors at different stages of *MYCN*-driven oncogenesis, and determine the immune events driving tumor development. We will further determine how *MYCN* modulates the immunosuppressive microenvironment to promote tumor growth and develop effective therapeutic approaches to revert *MYCN*-driven immunosuppression.

### Methods/Materials:

The TH-*MYCN* GEM model, with targeted *MYCN* expression in the developing neural crest, was used to study immune cells within the microenvironment during NB development. Week 4 and week 6 were defined as early- and late-phase time points of tumor development depending on MRI imaging and tumor weight. Mice (n=4 at week 4 and n=8 at week 8) were perfused to deplete circulating immune cells. Tumor-infiltrating immune cells were isolated by percoll gradient centrifugation, stained and fixed before flow cytometry analysis of T cell and myeloid-derived suppressor (MDSC) cell types. In parallel, global metabolomics profiling (DiscoveryHD4 Platform, Metabolon Inc.) was performed to understand the metabolic landscape of *MYCN*-driven tumors at the same time points.

### Results:

Tumors in TH-*MYCN* mice initiate along the paraspinal region by week 4 (average: 10 mm<sup>3</sup>, 0.06 g) and rapidly grow by week 6 (average: 1160 mm<sup>3</sup>, 2.0 g). There was no difference in infiltrating cytotoxic T cells (CD8<sup>+</sup>) between week-4 and week-6 tumors (3.18% vs. 5.07% of total tumor-infiltrating cells). However, week-6 tumors had lower percentage of CD69, a T cell activation marker, suggesting that T cells are less active at later stages. We did not observe a significant difference in the population of regulatory T cells (CD4<sup>+</sup> Foxp3<sup>+</sup>) or tumor-associated macrophages (CX3CR1<sup>hi</sup> F4/80<sup>hi</sup> MHCII<sup>hi</sup>) between week-4 and week-6 tumors. However, we found that a specific sub-population of MDSCs cells, the polymorphonuclear MDSC (PMN-MDSCs), was remarkably enriched in tumors of week-6 compared to those of week-4 (63.63% vs. 2.77% CD11b<sup>+</sup> Ly6G<sup>hi</sup> Ly6C<sup>lo</sup> CX3CR1<sup>lo</sup> F4/80<sup>lo</sup>

MHCII<sup>lo</sup> cells). In parallel, we have identified distinct metabolic nodes that were highly enriched in week-6 tumors: polyamine (putrescine and spermidine,  $p < 0.05$ ), aspartate (N-acetylaspartate and N-acetyl-aspartyl-glutamate,  $p < 0.0001$ ), and lipid (acyl carnitines and ceramides,  $p < 0.05$ ) metabolism.

Summary/Conclusions:

We have demonstrated a significant enrichment of PMN-MDSCs during tumor progression, suggesting a potential link between PMN-MDSC and NB oncogenesis. We are currently characterizing the role of PMN-MDSC in suppressing T cell functions and promoting oncogenesis. We will further test our hypothesis that the *MYCN*-directed metabolic pathways form a permissive microenvironment for the development of high-risk tumors.



## **P124 Single-cell transcriptomic analysis reveals the early separation of neuroblastoma fate in Th-*MYCN* mice**

*Tsubota, S.*<sup>2</sup>, *DuVerle, D.*<sup>3</sup>, *Srivastava, U.*<sup>4</sup>, *Kashima, Y.*<sup>5</sup>, *Suzuki, Y.*<sup>6</sup>, *Tsuda, K.*<sup>7</sup>, *Kadomatsu, K.*<sup>8</sup>

Nagoya University Graduate School of Medicine, Department of Biochemistry, Nagoya, Japan <sup>2</sup>, Graduate School of Frontier Sciences, the University of Tokyo, Department of Computational Biology and Medical Sciences, Kashiwa, Japan<sup>3</sup>, Graduate School of Frontier Sciences, the University of Tokyo, Department of Computational Biology and Medical Sciences, Kashiwa, Japan<sup>4</sup>, Graduate School of Frontier Sciences, the University of Tokyo, Department of Computational Biology and Medical Sciences, Kashiwa, Graduate School of Frontier Sciences, the University of Tokyo<sup>5</sup>, Graduate School of Frontier Sciences, the University of Tokyo, Department of Computational Biology and Medical Sciences, Kashiwa, Japan<sup>6</sup>, Graduate School of Frontier Sciences, the University of Tokyo, Department of Computational Biology and Medical Sciences, Kashiwa, Japan<sup>7</sup>, Nagoya University Graduate School of Medicine, Department of Biochemistry, Nagoya, Japan<sup>8</sup>

### Background/Introduction:

One of the challenging topics of neuroblastoma research is to understand the enigmatic phenomenon of “spontaneous regression”. Although its plausible mechanisms such as developmental programmed cell death following neurotrophin deprivation have been proposed, the precise molecular mechanisms driving this phenomenon remain a matter of speculation (Brodeur GM, *Cell Tissue Res.* 2018).

### Aims:

The aim of this study is to understand the cellular and molecular bases of neuroblastoma fate separation (i.e. either tumor growth or spontaneous regression) in the Th-*MYCN* mouse model and human specimens.

### Methods/Materials:

We investigated the early-stage tumor tissues in Th-*MYCN* mice and performed droplet-type single-cell RNA sequencing (scRNA-seq) by Chromium Single Cell 3' assay (10x Genomics). We carried out single-molecule fluorescence in situ hybridization (smFISH) to validate the analysis. Besides, we also obtained scRNA-seq data from stage 4 and stage 4S human neuroblastoma specimens.

### Results:

Firstly, survival analysis showed that 80% of Th-*MYCN* hemizygous mice died of tumor at 10 to 20 weeks of age while 20% of them never developed neuroblastoma. Secondly, when we investigated the tumor origin of the mice histologically, all of them had clusters of neuroblastoma cells until 3 weeks of age. Therefore, although neuroblastoma cells appeared in all cases, whether they developed further into a tumor or disappeared was likely determined during early age. We assume that the latter fate was due to a spontaneous regression-like phenomenon in Th-*MYCN* mice. Thirdly, to understand the observed fate determination, we obtained single-cell transcriptomes of early neuroblastoma cells from the tissues of seven 3-week-old and one 6-week-old (tumor-developing) Th-*MYCN* mice. The analysis revealed distinct cell types in the tissues including *MYCN*<sup>+</sup> neuroblastoma cells, ganglion cells, glial cells and so on. Within *MYCN*<sup>+</sup> neuroblastoma cells, there were distinct sub-populations and the proportion of cells was different between individuals, suggesting that different fates were captured at the single-cell level. The sub-populations were characterized by differential expressions of target genes regulated by certain transcription factors. Lastly, smFISH analysis validated the results of single-cell analysis. Recently, we obtained scRNA-seq data from two stage 4 and two stage 4S human neuroblastoma specimens which were dissected at diagnosis. We are currently analyzing these data and trying to integrate the data from Th-*MYCN* mice and human neuroblastomas.

### Summary/Conclusions:

Single-cell transcriptomic analysis captured variations in neuroblastoma fates observed in early ages of Th-*MYCN* mice. Early-stage subpopulation of *MYCN*<sup>+</sup> neuroblastoma cells can be discriminated by

the differential expressions of several genes. These data obtained from Th-*MYCN* mice and ongoing analysis of human neuroblastoma at single-cell resolution will possibly provide fundamental cellular and molecular bases of fate determination, i.e. spontaneous regression, in neuroblastoma.

## **P125 Catecholamine metabolism in neuroblastoma differs from pheochromocytoma due to a reduced amount of neurosecretory vesicles and lack of PNMT expression**

*Mühlethaler-Mottet, A.<sup>2</sup>, Beck Popovic, M.<sup>3</sup>, Balmas Bourlout, K.<sup>4</sup>, Grand-Guillaume, J.<sup>5</sup>, Schoumans, J.<sup>6</sup>, La Rosa, S.<sup>7</sup>, Uccella, S.<sup>8</sup>, Grouzmann, E.<sup>9</sup>, Abid, K.<sup>10</sup>*

Lausanne University Hospital and University of Lausanne, Woman-Mother-Child Department, Lausanne, Switzerland <sup>2</sup>, Lausanne University Hospital and University of Lausanne, Woman-Mother-Child Department, Lausanne, Switzerland<sup>3</sup>, Lausanne University Hospital and University of Lausanne, Woman-Mother-Child Department, Lausanne, Switzerland<sup>4</sup>, Lausanne University Hospital and University of Lausanne, Service of Clinical Pharmacology, Lausanne, Lausanne University Hospital and University of Lausanne<sup>5</sup>, Lausanne University Hospital and University of Lausanne, Hematology Service, Lausanne, Switzerland<sup>6</sup>, Lausanne University Hospital and University of Lausanne, Clinical Pathology, Lausanne, Switzerland<sup>7</sup>, University of Insubria, Department of Medicine and Surgery, Varese, Italy<sup>8</sup>, Lausanne University Hospital and University of Lausanne, Service of Clinical Pharmacology, Lausanne, Switzerland<sup>9</sup>, Lausanne University Hospital and University of Lausanne, Service of Clinical Pharmacology, Lausanne, Switzerland<sup>10</sup>

### Background/Introduction:

Metabolism of catecholamines (CATs, dopamine (DA), norepinephrine (NE) and epinephrine (E)), and metanephrines (MNs; methoxytyramine (MT), normetanephrine (NMN), and metanephrine (MN)) remains enigmatic in patients affected by neuroblastoma (NB). NMN and especially MT are used as biomarkers of NB in plasma and urine. MNs are suspected to directly arise from the tumor, as extensively described for pheochromocytoma and paraganglioma (PHEO/PGL) two other neuroendocrine tumors secreting MNs.

### Aims:

To study and compare CAT metabolism and secretion in NB relative to PHEO/PGL.

### Methods/Materials:

NB patient-derived xenografts (PDX) were generated by subcutaneous injection of primary NB cells into athymic Swiss nude mice. CATs and MNs were measured by HPLC MS/MS in NB-PDX, NB, and PHEO/PGL tumor tissues, in primary and established cell lines *in vitro* as well as in plasma from mice and patients. Neuroendocrine markers were analyzed by immunoblotting, and the presence of neurosecretory vesicles within tumor cells was observed by electron microscopy.

### Results:

Due to difficulties in finding sufficient samples of NB biopsies, we first characterized CAT metabolism in six NB-PDX models as compared to NB patients. Plasma levels of MNs were comparable in NB-PDX-bearing mice and patients with NB. Also, the profile of individual MNs, characterized by a reduced level of MN relative to NMN and MT, previously identified in the plasma and urines of patients with NB and referred to as noradrenergic, was also conserved in the plasma of mouse with NB. Moreover, we observed that the intratumoral concentrations of MNs and CATs were similar in NB and NB-PDX tissues and displayed a noradrenergic profile as well. Phenylethanolamine N-methyltransferase (PNMT), the enzyme that converts NE to E, was undetectable by immunoblotting in primary NB tumors, NB-PDX and NB cell lines, which explains the noradrenergic CAT profile of NB.

Furthermore, comparison between plasma from patients with NB and PHEO revealed comparable levels of MNs, while CATs displayed a slight reduction (2.7x) in NB. Surprisingly, intratumoral concentrations of MNs and CATs in human primary NB and NB-PDX were extremely low contrasting with elevated values commonly observed in PHEO/PGL (~120 and ~3500 fold change, respectively). To understand the biological bases of this discrepancy, we analyzed the amount of neurosecretory granules and their associated markers. A low expression level of markers of neurosecretory vesicles, such as VMAT1/2, CHGRA/B, and SYP, was identified in NB relative to PHEO through investigation of

published transcriptomic datasets (R2: Genomics Analysis and Visualization Platform (<http://r2.amc.nl>)). This was confirmed for SYP protein expression by immunoblotting. Moreover, a very low number of neurosecretory granules was observed in NB-PDX relative to PHEO.

#### Summary/Conclusions:

Our results demonstrate that murine NB-PDX are relevant NB preclinical models reflecting the CATs and MNs metabolism of NB patients. The noradrenergic profile of CATs and MNs results from the lack of PNMT expression in NB. Moreover, conversely to PHEO/PGL where high amount of neurosecretory vesicles enable CATs storage, their low amount in NB cells do not protect CATs of being metabolized and lead to their rapid transformation in the cytoplasm into MNs that diffuse in blood.

## **P126 Levels of Neuroblastoma-specific mRNAs in children with stage 2/3 *MYCN*-Amplified NB are below the prognostic cut-points**

*Corrias, M.V.*<sup>2</sup>, *Parodi, S.*<sup>3</sup>, *Viprey, V.F.*<sup>4</sup>, *Tchirkov, A.*<sup>5</sup>, *Lammens, T.*<sup>6</sup>, *Vicha, A.*<sup>7</sup>, *Yanez, Y.*<sup>8</sup>, *Trager, C.*<sup>9</sup>, *Luksch, R.*<sup>10</sup>, *Valteau-Couanet, D.*<sup>11</sup>, *Laureys, G.*<sup>12</sup>, *Canete, A.*<sup>13</sup>, *Poetschger, U.*<sup>14</sup>, *Ladenstein, R.*<sup>15</sup>, *Burchill, S.A.*<sup>16</sup>

Istituto Giannina Gaslini, Department of Services and Laboratories, Genova, Italy <sup>2</sup>, Istituto Giannina Gaslini, Epidemiology and Biostatistics, Genova, Italy<sup>3</sup>, Leeds institute of Medical Research, Clinical trials unit, Leeds, United Kingdom<sup>4</sup>, CHU Clermont-Ferrand, Service de Cytogenetique, Clermont-Ferrands, CHU Clermont-Ferrand<sup>5</sup>, Ghent University Hospital, Ghent university hospital, O, Belgium<sup>6</sup>, 2nd Medical Faculty Charles University, Faculty Hospital Motol, Prague, Czech Republic<sup>7</sup>, Hospital Universitari i Politecnic La Fe, Oncologia Pediatrica, Valencia, Spain<sup>8</sup>, Karolinska Institute, Karolinska University Hospital, Stockholm, Sweden<sup>9</sup>, Fondazione IRCCS Istituto Nazionale Tumori, Oncologia Pediatrica, Milano, Italy<sup>10</sup>, Institute Gustave Roussy, Pediatric oncology, Villejuif, France<sup>11</sup>, Ghent University Hospital, University Hospital, Ghent, Belgium, <sup>12</sup>, Hospital Universitari i Politecnic La Fe, Oncologia pediatrica, Valencia, Spain <sup>13</sup>, CCRI/St Anna Children's Hospital, Oncology, Vienna, Austria <sup>14</sup>, CCRI/St Anna Children Hospital, Oncology, Vienna, Austria <sup>15</sup>, Leeds institute of Medical Research, Pediatric Cancer Research, Leeds, United Kingdom <sup>16</sup>

### Background/Introduction:

High levels of adrenergic mRNAs in bone marrow aspirates (BM) and peripheral blood (PB) samples from children<sup>1</sup> and infants/toddlers<sup>2</sup> with stage 4 disease are associated with a worse outcome.

### Aims:

To determine the frequency of NB-specific mRNAs detection in samples from the small subset of high-risk children with localized *MYCN*-amplified NB tumors, and ascertain whether the levels were predictive of outcome.

### Methods/Materials:

Out of 121 children with localized *MYCN*-amplified NB enrolled in the SIOPEN/HR-NBL-1 trial, we collected 86 BM and 67 PB samples from 91 patients at diagnosis. Samples were also collected at end of induction (54 BM and 47 PB), pre-maintenance (35 BM and 29 PB) and at end of treatment (37 BM and 37 PB). Samples collected in PAXgene blood RNA tubes™ were processed and analysed by RTqPCR according to standardized procedures.

### Results:

At diagnosis, 59/86 (69%) and 63/83 (76%) BM samples had detectable TH and PHOX2B mRNAs, respectively. In PB samples TH and PHOX2B mRNAs were detected in 48/67 (72%) and 50/65 (77%) samples respectively. At end of induction, detectable TH and PHOX2B mRNAs were found in 20/54 (37%) and 9/51 (18%) BM, 15/45 (33%) and 5/47 (11%) PB, respectively. Pre-maintenance, 6/35 (17%) and 1/34 (3%) BM, 8/29 (28%) and 2/29 (7%) PB had detectable TH and PHOX2B mRNAs, respectively; whereas at end of treatment, detectable TH and PHOX2B mRNAs were found in 9/37 (24%) and 3/35 (9%) BM, 6/37 (16%) and 3/36 (8%) PB, respectively.

However, when cut-points associated with outcome in children with metastatic NB<sup>1</sup> were applied, the results indicated that 0/86 BM and 1/67 PB at diagnosis, 5/54 BM and 0/45 PB at end of induction, 1/35 BM and 0/29 PB pre-maintenance, 0/37 BM and 0/37 PB at end of treatment had levels of TH mRNAs above the specific cut-points. For PHOX2B mRNA, 48/83 BM and 1/65 PB at diagnosis, 2/51 BM and 4/47 PB at end of induction, 0/34 BM and 1/29 PB pre-maintenance, and 0/35 BM and 3/36 PB at end of treatment were above the specific cut-points.

These findings indicated that in children with localized *MYCN*-amplified NB the levels of NB-specific mRNAs at each time points were mostly below the prognostic cut-points, with the exception of PHOX2B mRNAs in BM samples at diagnosis. Survival analysis performed by dividing patients with

levels of PHOX2B mRNAs above or below the BM cut-point failed to show association with EFS or OS, likely because levels in 24/48 (50%) positive samples were close to the established cut-point.

Summary/Conclusions:

In the small subset of high-risk children with localized *MYCN*-amplified NB tumors, the levels of TH and PHOX2B mRNAs in BM and PB samples are mostly below the specific cut-points associated to worse outcome of children and infants/toddlers with metastatic NB, confirming that low levels of these mRNAs associated indeed with a more favorable outcome.

<sup>1</sup> Viprey et al, J Clin Oncol, 32, 1074-83, 2014; <sup>2</sup>Corrias et al, Pediatric Blood Cancer 65(7):e27052, 2017

## **P127 Hypoxia predicts poor prognosis and associates with the ability of the tumors to evade immune surveillance and gain immortality in neuroblastoma**

*Cangelosi, D.<sup>2</sup>, Morini, M.<sup>3</sup>, Zanardi, N.<sup>4</sup>, Sementa, A.<sup>5</sup>, Muselli, M.<sup>6</sup>, Conte, M.<sup>7</sup>, Garaventa, A.<sup>8</sup>, Pfeffer, U.<sup>9</sup>, Bosco, M.<sup>10</sup>, Eva, A.<sup>11</sup>*

IRCCS Istituto Giannina Gaslini, Laboratory of Molecular Biology, Genova, Italy <sup>2</sup>, IRCCS Istituto Giannina Gaslini, Laboratory of Molecular Biology, Genova, Italy<sup>3</sup>, IRCCS Istituto Giannina Gaslini, Laboratory of Molecular Biology, Genova, Italy<sup>4</sup>, IRCCS Istituto Giannina Gaslini, Laboratory of Pathology, Genova, IRCCS Istituto Giannina Gaslini<sup>5</sup>, Italian National Research Council, Institute of Electronics, Computer and Telecommunication Engineering, Genova, Italy<sup>6</sup>, IRCCS Istituto Giannina Gaslini, Pediatric Oncology Unit, Genova, Italy<sup>7</sup>, IRCCS Istituto Giannina Gaslini, Pediatric Oncology Unit, Genova, Italy<sup>8</sup>, IRCCS Ospedale Policlinico San Martino, Integrated Oncology Therapies Department, Genova, Italy<sup>9</sup>, IRCCS Istituto Giannina Gaslini, Laboratory of Molecular Biology, Genova, Italy<sup>10</sup>, IRCCS Istituto Giannina Gaslini, Laboratory of Molecular Biology, Genova, Italy<sup>11</sup>

### Background/Introduction:

Neuroblastoma is one of the most deadly pediatric cancers despite treatments. Hypoxia, a condition of low oxygen tension occurring in aberrant vascularized tissue areas, has profound effects on tumor metastasization, metabolism, and resistance to radio-, immuno-, and chemo-therapy.

### Aims:

Our aims are to investigate the contribution of hypoxia to neuroblastoma prognosis in a large cohort of patients and to uncover the molecular mechanisms deregulated within the hypoxic microenvironment in neuroblastoma.

### Methods/Materials:

We collected the gene expression profile of 1620 neuroblastoma tumor specimens covering the entire spectrum of the disease included into three publicly available data sets. We used machine learning methods to build an accurate predictor of tumor hypoxia. We assessed the functional enrichment of gene sets by STRING-DB database. We used the Microenvironment Cell Populations-counter (MCP-counter) method to quantify the absolute abundance of eight immune and two stromal cell populations from the transcriptomic data set.

### Results:

We identified a small set of 7 genes (NB-hop) involved in the cellular response to hypoxia. We integrated the gene expression profiles of the 1620 patients into one data set using bioinformatic techniques to achieve a large-scale genomic data analysis. We filtered out 834 patients from the data set for improving reliability, independence, and homogeneity of the data. The expression profiles of 236 randomly selected tumors out of 786 (30%) served to build a classifier and the profiles of the remaining 550 tumors (70%) were used to test its prognostic value in a validation data set. NB-hop classifier predicted 414 out of 550 patients (75%) with favorable prognosis (F) and 136 out of 550 patients (25%) with unfavorable prognosis (UF) and was able to stratify patients into subgroups that had a significantly different overall survival (OS) and event-free survival (EFS) (OS: HR 5.2 95% confidence interval (CI) 7.3-16.3 and EFS: HR 3.3 95%CI 3.8-7.5, both  $p < 0.0001$ ). NB-hop classifier maintained a significant prognostic effect in the model adjusted for these clinical covariates in multivariate analysis (OS: HR 1.8 95%CI 1.2-2.6,  $p = 0.004$  and EFS: HR 1.7 95%CI 1.2-2.5,  $p = 0.001$ ).

The molecular mechanisms altered by hypoxia in neuroblastoma primary tumors remain still unknown to date. Hence, we compared the transcriptome of the two populations of patients whose prognosis was predicted according to NB-hop in the validation set and identified a 2377 differentially regulated genes (fold change  $\geq 1.5$  or fold change  $\leq -1.5$  and Benjamini-Hochberg  $p < 0.05$ ). Pathway analysis evidenced the significant deregulation of fundamental biological processes including the response to hypoxia, telomere maintenance, immune and inflammation response, and cell differentiation

(Benjamini-Hochberg  $p < 0.05$ ). MCPcounter analysis evidenced a global immunosuppressed microenvironment associated with NB-hop unfavorable respect to NB-hop favorable prognosis.

Summary/Conclusions:

Hypoxia is an unfavorable condition of the tumor microenvironment, which is prognostic for NB patient and might play a fundamental role on the ability of the most aggressive tumors to suppress immune response, to evade immune surveillance and to progress.



## P128 The landscape of circular RNAs in neuroblastoma

*Klironomos, F.<sup>2</sup>, Danßmann, C.<sup>3</sup>, Naderi, J.<sup>4</sup>, Winkler, A.<sup>5</sup>, Luz, U.<sup>6</sup>, Hundsdoerfer, P.<sup>7</sup>, Eggert, A.<sup>8</sup>, Toedling, J.<sup>9</sup>, Hertwig, F.<sup>10</sup>, Schulte, J.H.<sup>11</sup>, Fuchs, S.<sup>12</sup>*

Charité, Pediatric Oncology/Hematology, Berlin, Germany <sup>2</sup>, Charité, Pediatric Oncology/Hematology, Berlin, Germany<sup>3</sup>, Max Planck Institute for Molecular Genetics, Genome Regulation, Berlin, Germany<sup>4</sup>, Charité, Pediatric Oncology/Hematology, Berlin, Charité<sup>5</sup>, Charité, Pediatric Oncology/Hematology, Berlin, Germany<sup>6</sup>, Charité, Pediatric Oncology/Hematology, Berlin, Germany<sup>7</sup>, Charité, Pediatric Oncology/Hematology, Berlin, Germany<sup>8</sup>, Charité, Pediatric Oncology/Hematology, Berlin, Germany<sup>9</sup>, Charité, Pediatric Oncology/Hematology, Berlin, Germany<sup>10</sup>, Charité, Pediatric Oncology/Hematology, Berlin, Germany<sup>11</sup>, Charité, Pediatric Oncology/Hematology, Berlin, Germany, <sup>12</sup>

### Background/Introduction:

Circular RNAs (circRNAs) are co-transcriptional variants of cognate mRNAs produced by backsplicing events that result in a characteristic RNase-resistant loop structure. Not every gene produces circRNAs and their overall expression profile is highly tissue-specific. CircRNAs have diverse functional roles involving coding for proteins, acting as decoys or transporters of proteins and microRNAs, or acting as protein-scaffolds. Neuroblastoma arises from embryonic neural-crest cells and is stratified into distinct risk-groups reflecting disease prognosis. Here, we systematically investigated the circRNA expression profiles of 104 primary neuroblastomas stratified by risk-group and as a whole when compared to healthy human brain tissue and various cancers.

### Aims:

We aimed to comprehensively profile all neuroblastoma circRNAs and particularly those with functional relevance to the disease.

### Methods/Materials:

Whole transcriptome paired-end Illumina sequencing RNA libraries of 104 neuroblastomas were generated and analyzed.

### Results:

Originating from 2,290 genes, we identified 5,171 unique circRNAs whose acceptor and donor sites did not vary more than one nucleotide from annotated exon boundaries and were expressed in at least 25%, or had strong support in at least three, of the 104 neuroblastoma tumours. The median number of unique circRNAs across tumours was 2,730. More than half of the 2,290 genes produced only a single circRNA isoform and circRNA and cognate mRNA expression was overall uncorrelated. High-risk non-*MYCN*-amplified tumours showed a higher number of unique circRNAs expressed compared to the rest of the risk-groups and had overall higher circRNA/mRNA expression ratios. The lowest number of unique circRNAs expressed with also the lowest circRNA/mRNA expression ratios were observed in the high-risk *MYCN*-amplified tumours. In this risk-group, we found also the majority of human splicing factors to be upregulated and that 540 genes were differentially spliced. These findings were independently reproduced in a Tetracycline-inducible *MYCN*-overexpression system based on the SKNAS neuroblastoma cell line, suggesting that *MYCN* drives this global circRNA repression and differential splicing. We found enrichment of RNA-binding-protein (RBP) motifs in both the flanking introns and exon sequences of the identified circRNAs, suggesting that RBPs might play regulatory roles beyond the known aspects of circRNA biogenesis. Finally, we identified 26 neuroblastoma-specific circRNAs with higher expression in neuroblastoma compared to healthy human brain tissue and various cancers. The RNaseR-resistance of 10 of them was validated in neuroblastoma cell lines.

### Summary/Conclusions:

We globally profiled for the first time in the field of neuroblastoma research the expression of circRNAs in a cohort of 104 primary tumours. Our results indicate that *MYCN* plays a crucial role in the global

repression of circRNAs, whereas in non-*MYCN* amplified high-risk tumors circRNAs seem to proliferate. We also identified circRNAs that were significantly higher expressed in neuroblastoma when compared to healthy human brain tissue and various other cancers. We are currently in the process of assaying the functional roles of a selected subset of these neuroblastoma-specific circRNAs.

## **P129 Inhibition of Teneurin 4 Suppresses Growth and Induces Differentiation in Neuroblastoma**

*Andonova, T.*<sup>2</sup>, *Pepich, P.A.*<sup>3</sup>, *Olsen, T.K.O.*<sup>4</sup>, *Fransson, F.S.*<sup>5</sup>, *Martinsson, M.T.*<sup>6</sup>, *Johnsen, J.I.J.*<sup>7</sup>, *Kogner, P.K.*<sup>8</sup>, *Wickström, M.W.*<sup>9</sup>

Karolinska Institutet, Women's and Children's Health, Stockholm, Sweden<sup>2</sup>, Karolinska Institutet, Women's and Children's Health, Stockholm, Sweden<sup>3</sup>, Karolinska Institutet, Women's and Children's Health, Stockholm, Sweden<sup>4</sup>, University of Gothenburg, Department of Laboratory Medicine, Gothenburg, University of Gothenburg<sup>5</sup>, University of Gothenburg, Department of Laboratory Medicine, Gothenburg, Sweden<sup>6</sup>, Karolinska Institutet, Women's and Children's Health, Stockholm, Sweden<sup>7</sup>, Karolinska Institutet, Women's and Children's Health, Stockholm, Sweden<sup>8</sup>, Karolinska Institutet, Women's and Children's Health, Stockholm, Sweden<sup>9</sup>

### Background/Introduction:

Neuritogenesis is the process during embryonic development when neural crest cells migrate and differentiate into multiple cell types. Neuroblastoma originates from neural crest progenitor cells of the sympathoadrenal lineage. The teneurin proteins (TEN) are highly expressed during the development and differentiation of the central nervous system in the drosophila fly, mouse and chicken embryos. Genetic mutations of teneurins (*TENM*) in humans have shown to play a role in different diseases affecting the nervous system, such as eye anomalies, schizophrenia and tremor, suggesting a link between the nervous system and the teneurin family of proteins. Several studies have shown disruption of the teneurin family of proteins in tumors, where somatic mutations and structural aberrations of the teneurin proteins have been found in for example neuroblastoma. However, investigations of a deeper cellular and functional understanding of this family of proteins in cancer is missing.

### Aims:

The aim of this study is to investigate the teneurin family of proteins, and in particular teneurin 4, to determine the role and function of these proteins in neuroblastoma tumor development, progression and metastasis.

### Methods/Materials:

Tumor samples from 53 Swedish neuroblastoma patients were collected with matched germline DNA and were whole genome or exome sequenced. In addition, gene expressions were analyzed in the publicly available R2 database, correlating gene expressions with overall survival of patients. To further explore the role of the teneurins, functional studies in four neuroblastoma cell lines were conducted. We used silencing RNA (siRNA) to study the effects of teneurin 1 to 4 knock-down on proliferation by measuring real-time impedance. Furthermore, we also performed RNA sequencing and pathway analysis after siRNA mediated knock down of *TENM4*. Two different CRISPR/Cas9 cell line models of the cell line SK-N-BE(2)C have since been created; a *TENM4* knockout cell line, and an inducible *TENM4* knockdown by a tetracycline promoter that are currently used for further investigation.

### Results:

From the whole genome sequencing of the 53 neuroblastoma tumor samples, we found that five (9.5%) of the patient samples contained a somatic mutation or a structural aberration in the protein family. When investigated in publicly available and validated neuroblastoma cohorts of the R2 database, low expression levels of *TENM1*, *TENM2* and *TENM3* were associated with poor overall survival, while low *TENM4* expression correlated with high overall survival. Using transient siRNA against *TENM1*, *TENM2*, *TENM3* and *TENM4*, we observed that *TENM4* consistently and significantly inhibited proliferation in all cell lines knocked down, by at least 50% reduction in proliferation. However, knockdown of *TENM1*, *TENM2* and *TENM3* lead to varied results regarding effect on tumor growth in the four cell lines investigated. Interestingly, RNA sequencing of *TENM4* knockdown cells

showed an upregulation in important pathways in neural differentiation and neuritogenesis, while among other pathways mTOR signaling was significantly downregulated.

**Summary/Conclusions:**

Our data suggests that teneurin 4 plays a role in neuroblastoma growth and differentiation, and that manipulation could offer a possible therapy for neuroblastoma. Ongoing studies will further explore the specific functions of teneurins in neuroblastoma.

## **P130 Pentraxin 3 (PTX3) in the neuroblastoma microenvironment**

*Tümmler, C.*<sup>2</sup>, *Marken, M.*<sup>3</sup>, *Moens, U.*<sup>4</sup>, *Johnsen, J.I.*<sup>5</sup>, *Kogner, P.*<sup>6</sup>, *Baryawno, N.*<sup>7</sup>, *Wickström, M.*<sup>8</sup>, *Sveinbjørnsson, B.*<sup>9</sup>

Karolinska Institutet, Department of Women's and Children's Health, Childhood Cancer Research Unit, Stockholm, Sweden <sup>2</sup>, UiT The Arctic University of Norway, Department of Medical Biology, Molecular Inflammation Research Group, Tromsø, Norway<sup>3</sup>, UiT The Arctic University of Norway, Department of Medical Biology, Molecular Inflammation Research Group, Tromsø, Norway<sup>4</sup>, Karolinska Institutet, Department of Women's and Children's Health, Childhood Cancer Research Unit, Stockholm, Karolinska Institutet<sup>5</sup>, Karolinska Institutet, Department of Women's and Children's Health, Childhood Cancer Research Unit, Stockholm, Sweden<sup>6</sup>, Karolinska Institutet, Department of Women's and Children's Health, Childhood Cancer Research Unit, Stockholm, Sweden<sup>7</sup>, Karolinska Institutet, Department of Women's and Children's Health, Childhood Cancer Research Unit, Stockholm, Sweden<sup>8</sup>, UiT The Arctic University of Norway, Department of Medical Biology, Molecular Inflammation Research Group, Tromsø, Norway<sup>9</sup>

### **Background/Introduction:**

The tumor microenvironment (TME) is a complex interplay between tumor cells, supporting immune and stromal cells, and non-cellular factors. Cancer-related inflammation is an important modulator of the tumor microenvironment with potent tumor promoting and tumor suppressing capabilities. Persistent inflammatory processes can contribute to all stages of tumorigenesis and various inflammatory mediators and pathways have been identified as promising therapeutic targets. Pentraxin 3 (PTX3) is a humoral pattern recognition molecule with functions in innate immunity and inflammation. In cancer, a complex role of PTX3 is emerging as both tumor promoting and tumor suppressing functions have been described. Moreover, PTX3 has been identified as a prognostic biomarker in a variety of cancers. Despite advances in diagnosis and therapy, 50% of high-risk neuroblastoma (NB) patients are refractory to treatment or relapse within two years. Several inflammatory mediators and pathways have to date been identified that can contribute to NB tumorigenesis. However, further work is warranted for an in-depth understanding of the NB microenvironment, which may lead to the identification of novel therapeutic approaches and biomarkers.

### **Aims:**

To evaluate the function of PTX3 in the NB microenvironment and its' potential as a biomarker and therapeutic target.

### **Methods/Materials:**

Gene expression analysis was performed using the publically available R2:Genomics Analysis and Visualization Platform (<http://r2.amc.nl>). The expression and secretion of PTX3 in neuroblastoma cell lines was determined using RT-PCR, ICC, ELISA, and Western blot. Furthermore, ELISAs were used to investigate the effect of the pro-inflammatory cytokines TNF $\alpha$ , IL-1 $\beta$ , and IL-6 on the secretion of PTX3 from NB cell lines.

### **Results:**

Examination of two publically available gene expression datasets indicated a correlation between high expression of PTX3 and a reduced overall survival probability in NB. Screening of NB cell lines demonstrated that PTX3 mRNA was present in a variety of NB cell lines. Moreover, using Western blots, PTX3 was detected in the protein precipitates from cell supernatants of half the NB cell lines examined. In contrast, no significant levels of PTX3 protein were observed in the cell lysates. ELISAs confirmed the presence of PTX3 in the cell supernatants of certain NB cell lines. Furthermore, TNF $\alpha$  and IL-1 $\beta$  promoted the secretion of PTX3 from SK-N-AS and SK-N-FI cells. Of note, the highest levels of PTX3 were detected following stimulation with a combination of TNF $\alpha$  and IL-1 $\beta$ . In contrast, no effect on PTX3 secretion was observed in response to IL-6. While a rapid increase in PTX3 secretion from SK-N-AS cells, 3h post stimulation, was detected, SK-NFI

cells reacted more slowly and increased levels of PTX3 were observed after 24h of stimulation.

**Summary/Conclusions:**

Our findings demonstrate that inflammatory cytokines can promote the secretion of PTX3 from certain NB cell lines. Further studies will investigate the specific functions of PTX3 in the NB microenvironment and its potential as a biomarker and therapeutic target.

## **P131 Impact on genomics of extracellular matrix biotensegrity using 3D bioprinted and orthotopic xenograft mice models of neuroblastoma**

Lopez-Carrasco, A.<sup>2</sup>, Martín-Vañó, S.<sup>3</sup>, Burgos-Panadero, R.<sup>4</sup>, Monferrer, E.<sup>5</sup>, Noguera, I.<sup>6</sup>, Navarro, S.<sup>7</sup>, Samitier, J.<sup>8</sup>, Noguera, R.<sup>9</sup>

University of Valencia / INCLIVA / CIBERONC, Pathology Department, Medical School, Valencia, Spain <sup>2</sup>, University of Valencia / INCLIVA / CIBERONC, Department of Pathology, Medical School, Valencia, Spain<sup>3</sup>, University of Valencia / INCLIVA / CIBERONC, Department of Pathology, Medical School, Valencia, Spain<sup>4</sup>, University of Valencia / INCLIVA / CIBERONC, Department of Pathology, Medical School, Valencia, University of Valencia / INCLIVA / CIBERONC<sup>5</sup>, University of Valencia, Spain, Central Support Service for Experimental Research (SCSIE), Valencia, Spain<sup>6</sup>, University of Valencia / INCLIVA / CIBERONC, Department of Pathology, Medical School, Valencia, Spain<sup>7</sup>, University of Barcelona / Institute for Bioengineering of Catalonia (IBEC), Electronics and Biomedical Engineering Department, Barcelona, Spain<sup>8</sup>, University of Valencia / INCLIVA / CIBERONC, Department of Pathology, Medical School, Valencia, Spain<sup>9</sup>

### Background/Introduction:

Tumor cells are submitted to mechanical forces, tensional networks or biotensegrity, some of them related to the surrounding extracellular matrix (ECM). Our previous results about tumor microenvironment of neuroblastoma (NB) noted an increase of ECM stiffness in the most aggressive tumors, defining a group of ultra-high risk patients and an abundant amount of intra-pericellular vitronectin (VN) expression in genetically instable tumors. However, it is still unknown how ECM biotensegrity affects the genomics.

### Aims:

To find out the genomic consequences of growing two NB cell-lines in variable ECM stiffness scaffolding models.

### Methods/Materials:

NB cell-lines were cultured independently in three platforms: a) 2D traditional *in vitro* culture; b) 3D bioprinting with a variable *in vitro* growing time (2 to 10 weeks) composed of hydrogel with different stiffness depending on methacrylated alginate concentration (0 to 2%) plus tumor cells (SK-N-BE-(2) NB cells with *MYCN* amplification (MNA), or SH-SY5Y NB cells with *ALK* mutated, with and without SW10 Schwann cells); and c) intra-adrenal gland serial xenograft VN knock-out mice (RAG1<sup>-/-</sup>VN<sup>-/-</sup>) and control mice (RAG1<sup>-/-</sup>VN<sup>+/+</sup>). Serial tumor passages were carried out up to, at least, three generations of xenografted mice with unaltered high quantity of tumor VN-rich ECM. We have performed single nucleotide polymorphism array (aSNP, Affymetrix) analysis to identify whole genome copy number aberrations (CNAs).

### Results:

We identified variable patterns of genomic aberrations in the SK-N-BE-(2) cells, mainly affecting to chromosomes 1, 9 and 21, related to the used models and its time of *in vitro* growth/mice passages. The cells cultured in 2D, all the 3D hydrogels with less stiffness, short time stiffest hydrogels and initial passages control mice, had similar CNAs, unlike the cells cultured in long time stiffest hydrogels and RAG1<sup>-/-</sup>VN<sup>-/-</sup> mice that promoted selection of the same CNAs that were minority present in 2D culture. Special interest had been the KANK gene deletion located in 9p which protein encoded inhibits actin fiber formation and cell migration. No genomic differences in SH-SY5Y cell-line between any of the assayed conditions were found.

### Summary/Conclusions:

Evidence of clonal selection of SK-N-BE-(2) cells with the same CNAs could be related to mechanosensing of ECM stiffness. Large Genetic studies of other cell-lines and human tumors should be done to confirm the CNAs changes related to VN-rich ECM stiffness.





## **P132 Analysis of MYCN amplification, ALK alterations and RASSF1a methylation in cfDNA: Utility for the therapy of neuroblastoma patients**

*Combaret, V.*<sup>2</sup>, *Lapouble, E.*<sup>3</sup>, *Pierron, G.*<sup>4</sup>, *Iacono, I.*<sup>5</sup>, *Brejon, S.*<sup>6</sup>, *Clement, N.*<sup>7</sup>, *Bellini, A.*<sup>8</sup>, *Chicard, M.*<sup>9</sup>, *Michon, J.*<sup>10</sup>, *Valteau-Couanet, D.*<sup>11</sup>, *Defachelles, A.*<sup>12</sup>, *Plantaz, D.*<sup>13</sup>, *Thebaut, E.*<sup>14</sup>, *Jannier, S.*<sup>15</sup>, *Gambart, M.*<sup>16</sup>, *Coze, C.*<sup>17</sup>, *Corradini, N.*<sup>18</sup>, *Taque, S.*<sup>19</sup>, *Poiree, M.*<sup>20</sup>, *Auger, N.*<sup>21</sup>, *Sartelet, H.*<sup>22</sup>, *Delattre, O.*<sup>23</sup>, *Peuchmaur, M.*<sup>24</sup>, *Schleiermacher, G.*<sup>25</sup>

Centre Léon BERARD, DRTI, Lyon, France <sup>2</sup>, Institut CURIE, UGS, PSL Research University, Paris, France<sup>3</sup>, Institut CURIE, UGS, PSL Research University, Paris, France<sup>4</sup>, Centre Léon BERARD, DRTI, Lyon, Centre Léon BERARD<sup>5</sup>, Centre Léon BERARD, DRTI, Lyon, France<sup>6</sup>, Institut CURIE, SIREDO Oncology Center, INSERM U830, PSL Research University, Paris, France<sup>7</sup>, Institut CURIE, SIREDO Oncology Center, INSERM U830, PSL Research University, Paris, France<sup>8</sup>, Institut CURIE, SIREDO Oncology Center, INSERM U830, PSL Research University, Paris, France<sup>9</sup>, Institut CURIE, SIREDO Oncology Center, INSERM U830, PSL Research University, Paris, France<sup>10</sup>, GUSTAVE ROUSSY, Department of Pediatric Oncology, Villejuif, France<sup>11</sup>, CENTRE OSCAR LAMBRET, Department of Pediatric Oncology, Lille, France, <sup>12</sup>, UNIVERSITY HOSPITAL, Department of Pediatric Haematology-Oncology, Grenoble, France <sup>13</sup>, UNIVERSITY HOSPITAL, Department of Pediatric Haematology-Oncology, Nantes, France <sup>14</sup>, HOPITAL DE HAUTEPIERRE, Department of Pediatric Haematology-Oncology, Strasbourg, France <sup>15</sup>, Hôpital des Enfants, Unite d'Hemato-Oncologie, Toulouse, France <sup>16</sup>, Hôpital d'Enfants de La Timone, Université Aix-Marseille, APHM, Service d'Hématologie-Oncologie Pédiatrique,, Marseille, France <sup>17</sup>, Centre Léon BERARD, Institut of Pediatric Hematology-Oncology, Lyon, France <sup>18</sup>, UNIVERSITY HOSPITAL, Department of Pediatric Haematology-Oncology, Rennes, France <sup>19</sup>, UNIVERSITY HOSPITAL, Department of Pediatric Haematology-Oncology, Nice, France <sup>20</sup>, GUSTAVE ROUSSY, Department of Biopathology, Villejuif, France <sup>21</sup>, UNIVERSITY HOSPITAL, Department of Pathology, Grenoble, France <sup>22</sup>, Institut CURIE, SIREDO Oncology Center, INSERM U830, PSL Research University, Paris, France <sup>23</sup>, Hopital Robert DEBRE, APHP, University Paris DIDEROT, Department of Pathology, Paris, France <sup>24</sup>, Institut CURIE, SIREDO Oncology Center, INSERM U830, PSL Research University, Paris, France <sup>25</sup>

### **Background/Introduction:**

The aim of this study was to compare for the same patient the MYCN status obtained from circulating free DNA (cfDNA) analyzed at diagnosis to the one observed by analysis of the primary tumor and/or metastasis within a prospective study IC2007-09/PHRCNB07 (ClinicalTrials.org, NCT02864563) including all patients with neuroblastic tumors treated in the centers of the SFCE (Société Française des Cancers de l'Enfant). Moreover, using cfDNA, MYCN amplification was studied at different timepoints during treatment and follow-up and compared to other known biomarkers such as ALK mutation and/or amplification and RASSF1a methylation.

### **Methods:**

MYCN status from the tumor and/or metastasis samples was evaluated by FISH analysis and/or aCGH. MYCN amplification in cfDNA obtained after extraction from plasma samples was analyzed by qPCR from 2007 to 2014 and after this date by ddPCR. An amplification was defined by a ratio of gene of interest /reference gene >3. The other biomarkers were analyzed by ddPCR. The presence of more than 6 mutated or methylated events identified a mutation or a methylation.

### **Results:**

Among the 580 patients included in this study, cfDNA was studied at diagnosis for 348 patients. 70 showed presence of MYCN amplification, 275 showed absence of MYCN amplification, and a borderline result was observed in 3 cases.

Among the 275 cfDNA samples without MYCN amplification, concordance with the results obtained by tumor samples analysis was observed in 246 cases. For 26 cases, MYCN amplification was never analyzed in tumor samples because of very low tumor cell percentage (22 cases) or they were not received (4 cases). For 3 cases; there was a discrepancy between cfDNA and tumor sample result since a MYCN amplification was detected in tumors but not in cfDNA. Among the 70 cfDNA samples with MYCN amplification, concordance with tumor samples was observed in 66 cases (48 stage 4, 14 stage 3, 2 stage 2, 2 stage 4s). For 1 case, the tumor

sample was not received and consequently MYCN had never analyzed and for 3 cases the tumor samples were not contributive since the percentage of tumor cells was very low.

Using cfDNA, MYCN amplification was studied at different timepoints of the disease in 30 patients showing MYCN amplification. Furthermore RASSF1a methylation could be analysed sequentially in 10 patients and ALK mutation and/or amplification in 7 patients with known ALK alterations. All three biomarkers decreased under treatment and increased at relapse. For one patient showing MYCN amplification and ALK amplification, both biomarkers were detected for each analyzed timepoint between diagnosis and relapse.

#### Conclusion

The data obtained from this prospective study confirmed that MYCN amplification and ALK alterations could be detected at diagnosis in cfDNA, contributing to clinical decision-making in particular in cases when primary tumor analysis was not contributive. The analysis of these biomarkers and RASSF1a methylation at different timepoints of the disease could also help to define minimal residual disease and response to treatment. The analysis of RASSF1a methylation should be of interest especially for the patients without genomic alterations.

## P133 EXPLORATORY ANALYSIS OF GENOMIC INSTABILITY IN HIGH-RISK NEUROBLASTOMA

Fernández-Blanco, B.,<sup>1</sup> Noguera, R.<sup>2</sup>, Berbegall, A.P.<sup>3</sup>, Martín-Vañó, S.<sup>4</sup>, Castel, V.<sup>5</sup>, Navarro-Fos, S.<sup>6</sup>, Noguera, R.<sup>7</sup>

Medical School, University of Valencia-INCLIVA, Pathology Department, Valencia, Spain<sup>1</sup>, (1) Medical School, University of Valencia-INCLIVA (2) CIBERONC, Pathology Department, (1) Valencia (2) Madrid, Spain<sup>2</sup>, (1) Medical School, University of Valencia-INCLIVA (2) CIBERONC, Pathology Department, (1) Valencia (2) Madrid, Spain<sup>3</sup>, (1) Medical School, University of Valencia-INCLIVA (2) CIBERONC, Pathology Department, (1) Valencia (2) Madrid, Spain<sup>4</sup>, Investigation Institute La Fe, Clinical and Translational Oncology Research Group, Valencia, Investigation Institute La Fe<sup>5</sup>, (1) Medical School, University of Valencia-INCLIVA (2) CIBERONC, Pathology Department, (1) Valencia (2) Madrid, Spain<sup>6</sup>, (1) Medical School, University of Valencia-INCLIVA (2) CIBERONC, Pathology Department, (1) Valencia (2) Madrid, Spain<sup>7</sup>

### Background/Introduction:

Survival of high-risk neuroblastoma (HR-NB) patients remains poor despite multimodal treatment. Identification of ultra-high-risk (UHR) patients (if death occurred before 18 months from diagnosis) is pivotal to offer them therapeutic alternatives and to gain insight into the biology of NB. For some types of cancer driven by copy number aberrations (CNAs), it has been demonstrated that the length of the altered genome encompassed by CNAs, that is the CNA burden, is related to biological features and prognostic. The impact of the CNA burden and other measurements of segmental chromosomal aberrations (SCAs) to define genomic instability (GI) in HR-NB remains unknown.

### Aims:

To explore the GI derived from SCAs in primary HR-NB tumors in relation to biological features and survival in order to gain insight into the identification of UHR patients.

### Methods/Materials:

We reviewed primary tumor profiles previously analyzed by SNP array for clinical diagnosis from 127 HR-NB patients. For each sample we annotated the length of all SCAs (>2mb) and calculated 3 GI indexes: i) the CNA burden (%gains<sup>length</sup>+%losses<sup>length</sup>), ii) the Copy Number Load (CNL), based on the predominance of gained or lost chromosomal material (%gains<sup>length</sup>-%losses<sup>length</sup>) and iii) the Net Genomic Load (NGL), considering the number of copies gained or lost for each SCA (%gains<sup>amount</sup>-%losses<sup>amount</sup>). Tumors were classified according to a negative CNL (nCNL) or positive CNL (pCNL) and decreased or increased NGL. Statistical analyses were performed to determine the relation of GI indexes to age, stage, histology, ploidy, *MYCN* status (69 non-amplified, 43 homogeneous amplified and 15 heterogeneous amplified), 11q loss, number and type of SCAs, overall survival (OS) and event-free survival (EFS).

### Results:

The CNA burden and the NGL were not related to survival. Combinations of CNL genomic type and *MYCN* status allowed us to identify patients with worse prognostic. We found that, within the nCNL group (71/127), the subgroups of patients with inferior outcome (5-year OS<20%, OS time<5 years) were 24 patients with homogeneous *MYCN* amplification and 17 patients with non-amplified tumors plus high nCNL values (>2.2%, above the mean). However, we did not find any difference in the survival within the pCNL group (56/127). For patients with heterogeneous amplification, the pCNL group (8/15) was related to a worse OS than the nCNL (5-year OS=22% vs 67%). Overall, a classification based on the CNL genomic type distinguished that the 39% of our cohort had an especially poor prognostic. Differences between CNL genomic types were related to number of SCAs, loss of 1p, 4p and 14q, and gain of 1q. We highlight that the frequency of 1p loss in non-amplified nCNL tumors was higher than expected (48% vs 14% for pCNL tumors).

### Summary/Conclusions:

We propose that CNL can be useful to better characterize UHR-NB patients. Ongoing studies are needed to validate the results in a higher cohort.

## **P134 A comprehensive genetic analysis of paired diagnosis and relapse neuroblastoma cell lines**

*Allinson, L.M.*<sup>2</sup>, *Gabriel, A.S.*<sup>3</sup>, *Beckett, E.*<sup>4</sup>, *Underhill, A.*<sup>5</sup>, *Chen, L.*<sup>6</sup>, *Goodman, A.*<sup>7</sup>, *Harding, F.*<sup>8</sup>, *Cuthbert, G.*<sup>9</sup>, *Bown, N.*<sup>10</sup>, *Tweddle, D.A.*<sup>11</sup>

Newcastle University, Translational and Clinical Research Institute, Newcastle Upon Tyne, United Kingdom<sup>2</sup>, Newcastle University, Translational and Clinical Research Institute, Newcastle Upon Tyne, United Kingdom<sup>3</sup>, Newcastle University, Translational and Clinical Research Institute, Newcastle Upon Tyne, United Kingdom<sup>4</sup>, Newcastle University, Translational and Clinical Research Institute, Newcastle Upon Tyne, Newcastle University<sup>5</sup>, Newcastle University, Translational and Clinical Research Institute, Newcastle Upon Tyne, United Kingdom<sup>6</sup>, Northern Genetic service, IGM, Newcastle Upon Tyne, United Kingdom<sup>7</sup>, Northern Genetic service, IGM, Newcastle Upon Tyne, United Kingdom<sup>8</sup>, Northern Genetic service, IGM, Newcastle Upon Tyne, United Kingdom<sup>9</sup>, Northern Genetic service, IGM, Newcastle Upon Tyne, United Kingdom<sup>10</sup>, Newcastle University, Translational and Clinical Research Institute, Newcastle Upon Tyne, United Kingdom<sup>11</sup>

### Background/Introduction:

Around 50-60% of all high risk neuroblastoma patients relapse, resulting in incurable disease. An improved understanding of relapsed neuroblastoma biology is required to develop new treatments. Copy number aberrations and other single nucleotide variant (SNV) genetic abnormalities are linked to poor patient outcome. Known genetic abnormalities associated with relapsed neuroblastoma include an increased frequency of anaplastic lymphoma kinase (ALK), RAS/MAPK and p53 pathway gene mutations.

### Aims:

To perform a comprehensive genetic analysis of paired neuroblastoma cell lines using whole exome sequencing (WES), high-density single nucleotide polymorphism (SNP) array data and targeted sequencing to identify genetic abnormalities involved in neuroblastoma at diagnosis and relapse.

### Methods/Materials:

DNA from *MYCN* amplified NBLW, NLBW-R, PER-106, PER-107, PER-108, SKNBE1n (BE1n) and SKNBE2c (BE2c) paired neuroblastoma cell lines established at diagnosis, relapse or further relapse from three different patients were analysed by whole exome sequencing (WES) (Illumina Truseq Rapid Exome Library Prep Kit) to a read depth of 100x. Sequence reads were analysed using a GATK pipeline. SNP arrays were generated using Illumina CytoSNP-850k arrays and data analysed using Nexus copy number software. Targeted next generation sequencing was performed using a panel of 38 genes frequently mutated in neuroblastoma on NBLW, NLBW and BE2c cell line DNA with an Illumina Truseq custom amplicon kit and sequenced on an Illumina NextSeq 550.

### Results:

An increase in copy number abnormalities was observed in all relapsed cell lines compared to the matched diagnostic one (median = 5). Segmental chromosomal aberrations -1p, +1q and +17q often seen in neuroblastoma were observed in all of the cell lines assessed with the exception of -1p in NBLW cells. Aberrations -4q and +20q developed in two or more cell lines at relapse. A loss of +2q and +3q was also observed between the BE1n and BE2c cell lines. No additional amplicons except for *MYCN* were observed at diagnosis or relapse. WES identified an increased mutational burden at relapse in two out of the three paired cell lines (median increase = 25.5 mutations). Genetic mutations identified by WES and targeted sequencing included those previously reported for *ALK* (NBLW and NLBW-R), *TP53* (BE2c), *NF1* (NBLW), *MDM4* (NBLW), *PTPRD* (NBLW-R) and *TERT* (BE2c). Those mutations detected by targeted sequencing but not WES were *MDM4*, *PTPRD*, *TERT* and *NF1* (BE2c only). Interestingly an *ALK* mutation was detected in the diagnostic BE1n cell line (F1174S), but was lost in the matched relapse cell line, BE2c. Previously unreported mutations in *CREBBP* and *ERBB3* in BE2c cells, as well as *CHEK2* in PER-106 cells were also identified in this study.

#### Summary/Conclusions:

Paired neuroblastoma cell lines established from patients at diagnosis and relapse treated with combination chemotherapies currently used in the clinic are valuable preclinical models for studying disease progression. Identification of novel variants at relapse as well as loss of pathogenic variants from clonal selection will improve our understanding of neuroblastoma evolution and aids selection of cell lines for future research testing novel targeted therapies.

## **P135 Role of GOLPH3 and TPX2 in neuroblastoma DNA damage response and cell resistance to chemotherapy**

Pezzolo, A.<sup>2</sup>, Podestà, M.<sup>3</sup>, Garaventa, A.<sup>4</sup>, Ognibene, M.<sup>5</sup>

IRCCS Istituto Giannina Gaslini, Laboratorio Cellule Staminali Post Natali e Terapie Cellulari, Genova, Italy <sup>2</sup>, IRCCS Istituto Giannina Gaslini, Laboratorio Cellule Staminali Post Natali e Terapie Cellulari, Genova, Italy<sup>3</sup>, IRCCS Istituto Giannina Gaslini, Divisione di Oncologia, Genova, Italy<sup>4</sup>, IRCCS Istituto Giannina Gaslini, Laboratorio Cellule Staminali Post Natali e Terapie Cellulari, Genova, IRCCS Istituto Giannina Gaslini<sup>5</sup>

### Background/Introduction:

Golgi phosphoprotein 3 (GOLPH3) has been reported to be involved in the development and in DNA damage response of various human cancers. Golgi dispersal is a common feature of DNA damage response in mammalian cells. It has been demonstrated that DNA damage triggers dramatic reorganization of the Golgi shape, resulting in its dispersal throughout the cytoplasm via Golgi protein GOLPH3. Several studies have paid attention to the relationship between TPX2 and DNA damage response. TPX2 is a protein involved in spindle apparatus assembly and specifically associated to cell microtubules. *TPX2* gene is part of the signature of chromosomal instability from specific genes whose expression was consistently correlated with clinical outcome in multiple human cancers, and it has been identified as a driving oncogene in different kinds of neoplasm.

### Aims:

Because little was known regarding the effects of DNA damage on the Golgi, and about the Golgi shape in neuroblastic tumors, we focused on the underlying molecular mechanism of GOLPH3 in neuroblastoma cells. Understanding how neuroblastoma cells react to DNA damage is essential in order to recognize the systems used to escape from elimination.

### Methods/Materials:

To investigate the mechanism of resistance of neuroblastoma cells to elimination induced by DNA damaging agents, we used two inducible neuroblastoma cell models: IMR-32 cell line with amplification of *MYCN* oncogene, and SH-SY5Y cell line with *MYCN* single copy but with high level of c-MYC expression. We induced DNA damage in these neuroblastoma cell lines with curcumin. To investigate the relationship between GOLPH3 expression and DNA damage after treatment with curcumin for 48 hrs, we analyzed the expression of GOLPH3 and of other proteins such as MYO18A,  $\gamma$ H2AX, TPX2, *MYCN*, MYC, MAP-kinases, Akt, PARP, Beclin-1 and LC3-II in treated and untreated neuroblastoma cell lines by western blotting assay or immunofluorescence staining. Then, we examined GOLPH3, H2AX, and TPX2 protein expression by immunofluorescence in primary neuroblastoma human tissue coming from pre-chemotherapy and post-chemotherapy surgical resection. We evaluated the association of *TPX2* gene expression with neuroblastoma patient outcome using the public R2 Platform.

### Results:

Exposure of neuroblastoma cells to curcumin induced: 1) up-regulation of GOLPH3+ cells; 2) augmentation of double-strand-DNA breaks; 3) Golgi fragmentation and dispersal throughout the cytoplasm; 4) increase of apoptosis and autophagy; 5) increased expression of TPX2 oncoprotein, able to repair DNA damage. Primary neuroblastoma samples analysis confirmed these observations.

### Summary/Conclusions:

Over-expression of GOLPH3 and TPX2 caused by DNA damage induced by curcumin seems to confer a survival advantage to neuroblastoma cells. We have observed that cells drastically stop growing or die when incubated with curcumin, but, if surviving, they rapidly recover after curcumin depletion, starting growing again very rapidly, in spite of a so detrimental treatment. The GOLPH3 activation together with Golgi dispersal throughout the cytoplasm were co-localized with autophagy

markers expression, that could be the process through which these cells better resist and survive. Our findings suggest that GOLPH3 and TPX2 expression levels may represent the clinical markers of neuroblastoma patients' responsiveness to DNA damaging therapies, and of possible resistance to them.



## **P136 Analyzing circulating exosomal DNA in high-risk neuroblastoma**

*Morini, M.*<sup>2</sup>, *Fortunati, E.*<sup>3</sup>, *Beltrami, C.*<sup>4</sup>, *Lavezzari, D.*<sup>5</sup>, *Ognibene, M.*<sup>6</sup>, *Garaventa, A.*<sup>7</sup>, *Eva, A.*<sup>8</sup>, *Delledonne, M.*<sup>9</sup>, *Pezzolo, A.*<sup>10</sup>

IRCCS Istituto Giannina Gaslini, Laboratory of Molecular Biology, Genova, Italy <sup>2</sup>, Università di Verona, Italy, Dipartimento di Biotecnologie, Verona, Italy<sup>3</sup>, Università di Verona, Italy, Dipartimento di Biotecnologie, Verona, Italy<sup>4</sup>, Università di Verona, Italy, Dipartimento di Biotecnologie, Verona, Università di Verona, Italy<sup>5</sup>, IRCCS Istituto Giannina Gaslini, Laboratorio Cellule Staminali Post Natali e Terapie Cellulari, Genova, Italy<sup>6</sup>, IRCCS Istituto Giannina Gaslini, Divisione di Oncologia, Genova, Italy<sup>7</sup>, IRCCS Istituto Giannina Gaslini, UOSD Biologia Molecolare, Genova, Italy<sup>8</sup>, Università di Verona, Italy, Dipartimento di Biotecnologie, Verona, Italy<sup>9</sup>, IRCCS Istituto Giannina Gaslini, Laboratorio Cellule Staminali Post Natali e Terapie Cellulari, Genova, Italy<sup>10</sup>

### **Background/Introduction:**

Tumors release into the blood the exosomes, small vesicles that reflect the state of tumor cells and contain double-stranded DNA (exo-DNA) that can be used as a biomarker of disease, and for the detection of resistant clones leading to relapse occurrence. However, the ability of the exo-DNA to reflect mutational status of the parental tumor cells in neuroblastoma patients is largely unknown.

### **Aims:**

We focused on exo-DNA from neuroblastoma exosomes, and its potential as specimen for screening somatic mutations present in the parental tumor cells. Characterization of mutations of the exo-DNA from high-risk neuroblastoma patients at diagnosis, during treatment and possibly at relapse could serve as a promising noninvasive biomarker for clinical genomic treatment. Exo-DNA analysis will allow us: 1) to identify the genetic mutations associated with high-risk neuroblastoma, and 2) to choose the most suitable therapy in consideration of the dozens of FDA-approved oncology drugs that base the indications on the genomic profile of the tumor cells.

### **Methods/Materials:**

We isolated exosomes from plasma samples of 23 high-risk neuroblastoma patients including onset and relapse phases. Plasma was stored at -80 ° C or used immediately for exosomes isolation. Exosomes were isolated with the Exo-RNeasy serum/plasma midi kit (QIAGEN). Exosomes were lysed with Triton X-100 (4%), SDS (10%) and Buffer AL, and exo-DNA was extracted using the QIAamp MinElute ccfDNA kit (QIAGEN). Exo-DNA quantity and quality were assessed with 1x dsDNA HS assay on Qubit 4.0 fluorometer and with high sensitivity DNA assay on Agilent Bioanalyzer, respectively. Illumina libraries were prepared with KAPA Hyper Prep and captured with Twist Human Core Exome + RefSeq 2. Whole exome sequencing was performed using an Illumina NovaSeq 6000 system: 300x theoretical coverage, in 150PE (33-52 M fragments per sample). All sequenced fragments were mapped to the reference genome GRCh38 using BWA-mem. After duplicates removal, Mutect2 was used for somatic variant calling using the peripheral blood sample as germline control. Annotation and prioritization of the identified variants was performed using Varseq (GoldenHelix).

### **Results:**

The novel finding is consistent with the presence of double-stranded DNA in the exosomes from neuroblastoma cells. The results of whole-exome sequencing demonstrated that the exo-DNA shared the same mutations in the most frequently mutated genes present in the parental neuroblastoma cells. Our results revealed that the entire exome is covered by the exo-DNA in an unbiased manner.

### **Summary/Conclusions:**

We showed that the plasma derived exo-DNA in high-risk neuroblastoma patients was highly consistent with the paired tumor genome profile, and covered all chromosomes. Our findings provide the first evidence of the presence of circulating exosomes that reflect the mutation status of

neuroblastoma cells. The circulating neuroblastoma exo-DNA can be used as a noninvasive somatic mutation screens for the diagnosis when tissue biopsy is not feasible, for the detection of resistant clones leading to relapse occurrence, and for the set-up of a personalized treatment.

## **P137 Identification of a minimal region of loss on chromosome 6q27 associated with poor survival of high-risk neuroblastoma patients**

*Pezzolo, A.<sup>2</sup>, Morini, M.<sup>3</sup>, Podestà, M.<sup>4</sup>, Garaventa, A.<sup>5</sup>, Ognibene, M.<sup>6</sup>*

IRCCS Istituto Giannina Gaslini, Laboratorio Cellule Staminali Post Natali e Terapie Cellulari, Genova, Italy <sup>2</sup>, IRCCS Istituto Giannina Gaslini, UOSD Biologia Molecolare, Genova, Italy<sup>3</sup>, IRCCS Istituto Giannina Gaslini, Laboratorio Cellule Staminali Post Natali e Terapie Cellulari, Genova, Italy<sup>4</sup>, IRCCS Istituto Giannina Gaslini, Divisione di Oncologia, Genova, IRCCS Istituto Giannina Gaslini<sup>5</sup>, IRCCS Istituto Giannina Gaslini, Laboratorio Cellule Staminali Post Natali e Terapie Cellulari, Genova, Italy<sup>6</sup>

### Background/Introduction:

Patients with high-risk neuroblastoma initially respond to therapy, but afterwards up to 60% of these become resistant and disease recurred. Neuroblastoma is the example of how specific chromosome rearrangements can influence the trend of the disease. Currently, whole-genome analysis revealed large genomic DNA copy-number alterations (loss of chromosomes 1p, 3p, 4p, 11q and gain of 1q, 2p, 17q) that are of prognostic significance for newly diagnosed neuroblastoma patients. Unfortunately, it does not exist one or more specific chromosome defect associated with relapse or refractory high-risk neuroblastoma.

### Aims:

Previous studies have delineated a 7 Mb region at distal 6q27 loss in high-risk neuroblastoma patients with extremely poor survival outcome. A complete genomic analysis of the distal 6q region would help identifying the putative neuroblastoma tumor suppressor gene(s).

### Methods/Materials:

We recovered 15 primary untreated high-risk neuroblastoma patients diagnosed by the Italian Association of Italian Pediatric Hematology and Oncology (AIEOP). These patients harbored tumors with chromosome 6q loss. We determined both size and genetic content of these losses by high resolution array-CGH using Human Genome CGH Microarray 4x180K Kit (Agilent Technologies, CA, USA). We inquired for any association between *T*, *SFT2D1*, *RP56KA2*, *FGFR10P* and *UNC93A* gene expression and high-risk neuroblastoma patients survival using two large neuroblastoma tumor datasets with available survival endpoints via the R2 Genomic Analysis and Visualization Platform (<http://r2.amc.nl/>; SEQC n=498 and TARGET-Asgharzadeh n=249).

### Results:

Array-CGH disclosed multiple segmental chromosome gains or losses in the genomic profile of 15 neuroblastoma samples analyzed. Partial chromosome losses were most often detected for chromosome arms 1p, 3p, 4p, 11q, and partial chromosome gains for 1q, 2p, 7q, 17q, these abnormalities were associated with grim patients survival outcome. All neuroblastoma samples harbored 6q loss: 7 cases showed distal loss, 6 had interstitial loss, and 2 carried both distal and interstitial losses. Of interest, we defined a minimal common region of genomic loss in distal 6q by a sample displaying a small loss spanning an area of 4.68 Mb. The 6q27 region was deleted from oligomer A\_14\_P103168 (166.203.071 bp) (first deleted) to oligomer A\_14\_P120743 (170.890.108 bp) (last deleted). Among the cases carried distal 6q loss one sample showed a gain adjacent to the 6q loss. In particular, this sample carried in 6q27 region a focal gain from oligomer A\_14\_P200779 (168303033 bp) (first gained) to oligomer A\_14\_P120743 (170890108 bp) (last gained). So, by subtraction we can define a critical Smallest Region of Overlap (SRO) on chromosome 6q27 spanning an area of 2.09 Mb. The minimal common region of loss harbored the five genes *T*, *SFT2D1*, *RPS6KA2*, *FGFR10P*, and *UNC93A*. We found that low *SFT2D1*, *RPS6KA2* and *FGFR10P* gene expression predicted poor outcome in high-risk neuroblastoma patients.

### Summary/Conclusions:

We hypothesize that the genes contained in the commonly loss region are dosage-sensitive, and that the haploinsufficiency of one or more of these genes is associated with extremely poor survival outcome of high-risk neuroblastoma.

## **P138 Aurora-A regulates phosphorylation of Histone 3 S10 in S phase to prevent Transcription-Replication conflicts.**

*Roeschert, I.*<sup>2</sup>, *Büchel, G.*<sup>3</sup>, *Gatti, M.*<sup>4</sup>, *Altmeyer, M.*<sup>5</sup>, *Eilers, M.*<sup>6</sup>

University of Würzburg, Department Biochemistry and Molecular Biology, Würzburg, Germany <sup>2</sup>,  
University of Würzburg, Department of Biochemistry and Molecular Biology, Würzburg, Germany<sup>3</sup>,  
University of Zurich, Department of Molecular Mechanisms of Disease, Zurich, Switzerland<sup>4</sup>,  
University of Zurich, Department of Molecular Mechanisms of Disease, Zurich, University of Zurich<sup>5</sup>,  
University of Würzburg, Department of Biochemistry and Molecular Biology, Würzburg, Germany<sup>6</sup>

### Background/Introduction:

Members of the Aurora kinase family have important function in cell division and in the maintenance of genome stability. The functions of Aurora kinases are particularly well understood in mitosis (Marumoto et al., 2005). Aurora-A is known to stabilize *MYCN* in neuroblastoma cells and thereby preventing proteasomal degradation. Aurora-A is known to stabilize *MYCN* in neuroblastoma cells and thereby preventing proteasomal degradation (Otto et al., 2009). *MYCN*, which belongs to the proto-oncogene *MYC* family of transcription factors, is often amplified in neuroblastoma. *MYCN* amplification represent a marker of poor prognosis and less overall survival in neuroblastoma patients.

Recently, our laboratory could show that *MYCN* and Aurora-A mainly interact during S phase of the cell cycle (Büchel et al., 2017) but the function of this complex remains open.

:

### Results:

For our approach cells were synchronized in S phase and fractionated. Hereby, we identified that *MYCN* as well as Aurora-A are mainly bound to chromatin. We were able to show that the association of Aurora-A with chromatin is cell cycle dependent and its association in S phase is *MYCN* dependent.

It is known that Aurora-A is a kinase for Histone 3 Ser10 (H3S10) phosphorylation (Hsu et al., 2000). We identified that H3S10 is phosphorylated by Aurora-A only in S and to a lesser degree in G2 phase. Chromatin compaction marked by phosphorylation of H3S10 counteracts the formation of transcription-dependent R-loops (Castellano-Pozo et al., 2013). Consistently, inhibition of Aurora-A, by using MLN8237, led to a large increase in formation of RNA/DNA-hybrids.

Aurora-A prevents global stalling of elongating RNA Polymerase II (RNAPII) at the pause site and at exon/intron boundaries and promotes mRNA splicing. Additionally, we show that an extensive number of splicing related phosphosites are indirectly phosphorylated by Aurora-A in S phase. Our data suggest that inhibition of Aurora-A increases transcription-replication conflicts.

Those conflicts lead to the activation of the Ataxia telangiectasia and Rad3 related (ATR) kinase and stalling of RNAPII impede replication fork progression. Simultaneous blockade of Aurora-A and ATR kinases induces double-strand break formation and apoptosis.

### Summary/Conclusions:

We were able to show that Aurora-A is bound to chromatin in S phase where it is required to phosphorylate H3S10 to prevent transcription-replication conflicts.

**P139 DNA methylation profiling of High Risk Neuroblastoma: potential epigenetic biomarkers for treatment response assessment.**

*Garrido-García, A.<sup>2</sup>, Pérez-Jaume, S.<sup>3</sup>, García-Gerique, L.<sup>4</sup>, García, M.<sup>5</sup>, Lemos, I.<sup>6</sup>, Rodríguez, E.<sup>7</sup>, Muñoz, O.<sup>8</sup>, Suñol, M.<sup>9</sup>, Carén, H.<sup>10</sup>, Henrich, K.O.<sup>11</sup>, Westermann, F.<sup>12</sup>, Mora, J.<sup>13</sup>, Gómez-González, S.<sup>14</sup>, Lavarino, C.<sup>15</sup>*

Fundació Sant Joan de Déu, Institut de Recerca Sant Joan de Déu, Developmental Tumor Biology Laboratory, Barcelona, Spain <sup>2</sup>, Fundació Sant Joan de Déu, Institut de Recerca Sant Joan de Déu, Developmental Tumor Biology Laboratory, Barcelona, Spain<sup>3</sup>, Fundació Sant Joan de Déu, Institut de Recerca Sant Joan de Déu, Developmental Tumor Biology Laboratory, Barcelona, Spain<sup>4</sup>, Fundació Sant Joan de Déu, Institut de Recerca Sant Joan de Déu, Developmental Tumor Biology Laboratory, Barcelona, Spain<sup>5</sup>, Fundació Sant Joan de Déu, Institut de Recerca Sant Joan de Déu, Developmental Tumor Biology Laboratory, Barcelona, Spain<sup>6</sup>, Hospital Sant Joan de Déu, Department of Pathology, Barcelona, Spain<sup>7</sup>, Fundació Sant Joan de Déu, Institut de Recerca Sant Joan de Déu, Developmental Tumor Biology Laboratory, Barcelona, Spain<sup>8</sup>, Hospital Sant Joan de Déu, Department of Pathology, Barcelona, Spain<sup>9</sup>, Sahlgrenska Cancer Center, Institute of Biomedicine, Sahlgrenska Academy, Department of Pathology, Gothenburg, Sweden<sup>10</sup>, German Cancer Research Center (DKFZ), Neuroblastoma Genomics, Heidelberg, Germany<sup>11</sup>, German Cancer Research Center (DKFZ), Neuroblastoma Genomics, Heidelberg, Germany, <sup>12</sup>, Hospital Sant Joan de Déu, Department of Pediatric Oncology, Barcelona, Spain <sup>13</sup>, Fundació Sant Joan de Déu, Institut de Recerca Sant Joan de Déu, Developmental Tumor Biology Laboratory, Barcelona, Spain <sup>14</sup>, Fundació Sant Joan de Déu, Institut de Recerca Sant Joan de Déu, Developmental Tumor Biology Laboratory, Barcelona, Spain <sup>15</sup>

**Background/Introduction:**

High risk NB (HR-NB) represents a heterogeneous group of tumors, whereby patients can display response to treatment and long-term outcome or develop early progressive, chemoresistant disease with poor outcome (ultra-high risk; UHR-NB). However, most patients with HR-NB are treated uniformly without further stratification. Genetics underlying this aggressive subgroup is still greatly unknown and risk-stratification within HR-NB is challenging.

**Aims:**

We explored DNA methylation (DNAm) changes present in HR-NB tumors at diagnosis, and identify (epi)genetic alterations potentially related with prognosis of patients and response to treatment. Thereby, identify risk-stratification biomarkers and potential therapeutic targets.

**Methods/Materials:**

We analyzed previously published DNAm (Infinium HumanMethylation450 BeadChip) and gene expression (GE) datasets of 100 and 428 HR-NB samples, respectively. The study was restricted to patients aged >18 months at diagnosis with metastatic disease or/and *MYCN* amplified NB tumors. Median follow-up was of 5 years. DNAm data was annotated according to gene location, CpG islands and chromatin state categories. Discovery of potential biomarkers was performed using Cox regression models. Survival curves were estimated by Kaplan-Meier method and compared among groups by log-rank test. Pathway analysis was performed using KEGG Pathway Database. Bisulfite sequencing, pyrosequencing, quantitative RT-PCR and immunohistochemistry were used for validation purposes.

**Results:**

Unsupervised analysis of DNAm data of HR-NB tumors revealed two distinct profiles with differences in clinical evolution. Survival analysis identified CpG sites strongly associated with outcome, that permitted to differentiate a subgroup of rapidly progressing, chemo-refractory tumors, identified as UHR-NB (median overall survival (OS) 33.23 months), and a subgroup with long-term survival (median OS 63.55 months). There was no statistical evidence of *MYCN* amplification enrichment

associated to any of the two subgroups. Functional genomic analysis of the HR-NB subgroups unveiled CpG hypermethylation enrichment in UHR-NB tumors both at 5'-UTR and 3'-UTR regions and associated to CpG islands. These DNA methylation changes were located in regions flanking transcription start sites, polycomb repressed and enhancer chromatin state regions. Differentially methylated genes were associated with developmental pathways and metabolism processes.

Next, we performed a correlation analysis between differentially methylated and expressed genes comparing the two HR-NB subgroups using matching patient data. This analysis revealed differences in pathways related to metabolism and purine biosynthesis. Results were validated using an independent cohort of 329 NB samples.

#### Summary/Conclusions:

Our findings show that the heterogeneous clinical behavior of HR-NB is associated with specific DNA methylation changes significantly associated with clinical evolution of tumors. We have identified a reduced set of methylation-based biomarkers that can identify UHR-NB tumors and thus enhance risk-stratification. This has enabled us to identify aberrantly activated biological pathways that could be relevant for risk-adapted therapeutic strategies.

## **P140 A novel *MYCN*-specific antigene oligonucleotide inhibits the broad N-Myc tumorigenic cell deregulation in neuroblastoma**

*Tonelli, R.*<sup>2</sup>, *Montemurro, L.*<sup>3</sup>, *Raieli, S.*<sup>4</sup>, *Angelucci, S.*<sup>5</sup>, *Bartolucci, D.*<sup>6</sup>, *Amadesi, C.*<sup>7</sup>, *Lampis, S.*<sup>8</sup>, *Scardovi, A.L.*<sup>9</sup>, *Pession, A.*<sup>10</sup>, *Hrelia, P.*<sup>11</sup>

University of Bologna, Department of Pharmacy and Biotechnologies, Bologna, Italy <sup>2</sup>, University of Bologna, Interdepartmental Center for Cancer Research, Bologna, Italy<sup>3</sup>, Biogenera, Bioinformatic department, Ozzano Dell'Emilia Bo, Italy<sup>4</sup>, University of Bologna, Interdepartmental Center for Cancer Research, Bologna, University of Bologna<sup>5</sup>, University of Bologna, Interdepartmental Center for Cancer Research, Bologna, Italy<sup>6</sup>, Biogenera, Biology department, Ozzano Dell'Emilia Bo, Italy<sup>7</sup>, Biogenera, Biology department, Ozzano Dell'Emilia Bo, Italy<sup>8</sup>, Biogenera, Regulatory affairs, Ozzano Dell'Emilia Bo, Italy<sup>9</sup>, University of Bologna, Interdepartmental Center for Cancer Research, Bologna, Italy<sup>10</sup>, University of Bologna, Department of Pharmacy and Biotechnologies, Bologna, Italy<sup>11</sup>

### Background/Introduction:

Roughly half of high-risk neuroblastoma (NB) is characterized by *MYCN*-amplification. N-Myc promotes tumor progression by inducing cell growth and inhibiting differentiation. Moreover, *MYCN*-overexpression is leading to a broad gene expression reprogramming, spanning from metabolism to chromosome re-organization. Indeed, not all the aspects of *MYCN* neuroblastoma relationship are well understood. While N-Myc is a known driver of the disease, it remains a target for which no therapeutic drug exists.

### Aims:

we aimed to investigate how N-Myc overexpression was altering the neuroblastoma cell biology and whether the specific inhibition of *MYCN* can affect it and subsequently inhibits tumor progression.

### Methods/Materials:

we developed a bioinformatic pipeline to analyze gene expression data from neuroblastoma patients in order to identify specific *MYCN* induced alterations. Here, we evaluated a novel *MYCN*-specific antigene PNA oligonucleotide (BGA002) in *MYCN*-amplified (MNA) or *MYCN*-expressing NB and investigated the mechanism of its anti-tumor activity. We confirm by *in vivo* BGA002 administration block of tumor progression by *MYCN* inhibition.

### Results:

we identified *MYCN* induced biological alterations in neuroblastoma and their mechanistic role in tumor progression. Indeed, we showed a previous unknown mechanism on how N-Myc is reprogramming mitochondria by repressing autophagy and by controlling reactive oxygen species (ROS). Furthermore, we also showed how N-Myc is acting on metabolism and differentiation through different mechanisms. *In vivo* BGA002 administration in a *MYCN*-amplified neuroblastoma xenograft mouse model led to tumor growth inhibition and improved survival.

### Summary/Conclusions:

we are presenting undescribed mechanisms about N-Myc impact on neuroblastoma biology deregulation. Thus, we are showing how a specific inhibitor of *MYCN* expression is affecting this pathogenic program leading to blocking tumor progression.



## **P141 Targeting thymidylate biosynthesis pathway in *MYCN*-amplified neuroblastoma cells**

*Kiyonari, S.*<sup>2</sup>, *Kadomatsu, K.*<sup>3</sup>

Nagoya University, Graduate School of Medicine, Nagoya, Japan <sup>2</sup>, Nagoya University, Graduate School of Medicine, Nagoya, Japan<sup>3</sup>

### Background/Introduction:

*MYCN* gene amplification is consistently associated with poor prognosis in patients with neuroblastoma. Because transcriptional factors (e.g., N-Myc and c-Myc) are thought to be “undruggable” targets, alternative approaches are required to develop new treatments. Recently, synthetic lethal (SL) approaches are emerging as a promising strategy for cancer therapy. For instance, poly ADP-ribose polymerase inhibitor “Olaparib” has been approved for treating *BRCA*-deficient ovarian cancer, which can induce cancer cell-specific cell death.

### Aims:

Based on the SL approach, we sought to identify new SL genes in *MYCN*-amplified neuroblastoma cells, in which elevated levels of DNA damage and dysregulated cell division are observed.

### Methods/Materials:

We performed a genome-wide shRNA library screening by using a commercial library that consists of over 80,000 shRNA constructs targeting about 16,000 human genes. Briefly, IMR-32 cells (*MYCN*-amplified) and SH-SY5Y cells (*MYCN* single-copy) were transduced with lentiviruses carrying the shRNA sequences. The relative abundance of shRNA constructs in each cell line was then quantified by next-generation sequencing. shRNA sequences with a false discovery rate <0.1 from an exact test analysis in edgeR software were selected.

### Results:

In addition to already known synthetic lethal genes, about 130 genes were identified as new candidates. Based on experimental validations using siRNA or chemical inhibitors, we identified some mitotic kinases and enzymes involved in *de novo* thymidylate (dTTP) biosynthesis pathways (e.g., dUTPase and deoxythymidine kinase) as new SL target molecules in *MYCN*-amplified neuroblastoma cells. Compounds targeting the dTTP biosynthesis pathways, such as fluoropyrimidines and antifolates, are considered to be one of the major class of anticancer drug, however, their usefulness for neuroblastoma patients have not been intensively studied. Of note, it was reported that methotrexate, an antifolate drug, can specifically suppress the cell growth of *MYCN*-amplified neuroblastoma cells (Lau DT et al., *Oncotarget*, 2015), and thus we investigated the efficacies of other antifolates, raltitrexed (RTX) and pemetrexed, on neuroblastoma cell lines. Our cell growth-inhibitory assay revealed that RTX showed superior inhibitory activity against *MYCN*-amplified cell lines. Because this can be rescued by adding thymidine to the culture medium, a salvage pathway to produce dTTP was active in neuroblastoma cells and the shortage of dTTP was a sole cause of the observed suppression. Although RTX treatments induced high levels of single-stranded DNA induced DNA damage response in IMR-32 cells, this was not clearly observed in SH-SY5Y cells. Interestingly, the expression level of replication protein A2 (encoded by *RPA2* at 1p35.3), a DNA binding protein to protect the exposed single-strand DNA at damaged site, was low in IMR-32 cells. It is widely known that the loss at chromosome 1p is often correlated with *MYCN* amplification, and therefore RTX might be more effective for the treatment of such patients.

### Summary/Conclusions:

Pre-existing antifolate drugs targeting dTTP biosynthesis pathways would be useful for the treatment of high-risk neuroblastoma.



## **P142 Exosomics of Neuroblastoma: the role of exosomal proteins in risk stratification and induction-chemotherapy response**

*Morini, M.<sup>2</sup>, Raggi, F.<sup>3</sup>, Cangelosi, D.<sup>4</sup>, Petretto, A.<sup>5</sup>, Bartolucci, M.<sup>6</sup>, Segalerba, D.<sup>7</sup>, Castriconi, R.<sup>8</sup>, Mazzocco, K.<sup>9</sup>, Sementa, A.R.<sup>10</sup>, Luksch, R.<sup>11</sup>, Garaventa, A.<sup>12</sup>, Conte, M.<sup>13</sup>, Bottino, C.<sup>14</sup>, Varesio, L.<sup>15</sup>, Bosco, M.C.<sup>16</sup>, Eva, A.<sup>17</sup>*

IRCCS Istituto G. Gaslini, Laboratory of Molecular Biology, Genova, Italy <sup>2</sup>, IRCCS Istituto G. Gaslini, Laboratory of Molecular Biology, Genova, Italy<sup>3</sup>, IRCCS Istituto G. Gaslini, Laboratory of Molecular Biology, Genova, Italy<sup>4</sup>, IRCCS Istituto G. Gaslini, Core Facilities, Proteomics Laboratory, Genova, Italy<sup>5</sup>, IRCCS Istituto G. Gaslini, Core Facilities, Proteomics Laboratory, Genova, Italy<sup>6</sup>, IRCCS Istituto G. Gaslini, Laboratory of Molecular Biology, Genova, Italy<sup>7</sup>, Università di Genova, Centro di Eccellenza per la Ricerca Biomedica (CEBR), Genova, Italy<sup>8</sup>, IRCCS Istituto G. Gaslini, UOC Anatomia Patologica, Genova, Italy<sup>9</sup>, IRCCS Istituto G. Gaslini, UOC Anatomia Patologica, Genova, Italy<sup>10</sup>, IRCCS Istituto Nazionale dei Tumori, Dipartimento di Ematologia e Onco-ematologia Pediatrica, Milano, Italy<sup>11</sup>, IRCCS Istituto G. Gaslini, Oncologia Pediatrica, Genova, Italy, <sup>12</sup>, IRCCS Istituto G. Gaslini, Oncologia Pediatrica, Genova, Italy <sup>13</sup>, Università di Genova, Dipartimento di Medicina Sperimentale, Genova, Italy <sup>14</sup>, IRCCS Istituto G. Gaslini, Laboratory of Molecular Biology, Genova, Italy <sup>15</sup>, IRCCS Istituto G. Gaslini, Laboratory of Molecular Biology, Genova, Italy <sup>16</sup>, IRCCS Istituto G. Gaslini, Laboratory of Molecular Biology, Genova, Italy <sup>17</sup>

### **Background/Introduction:**

High-risk neuroblastoma (HR-NB) patients receive aggressive treatment that includes intensive chemotherapy, radiotherapy, surgery, autologous blood stem cell transplantation and immunotherapy with anti-GD<sup>2</sup> antibody. However, biological parameters enabling a dynamic early prediction of inadequate response, which would justify upfront different therapy, are lacking. The need of novel diagnostic molecular tools in oncology has led to increasing interest in liquid biopsies as a source of biomarkers, as they provide a minimally invasive method. Body fluids are a source of exosomes, nanosized extracellular vesicles that can drive tumor growth and chemoresistance. We previously identified exosomal microRNAs (exo-miRNA) indicative of HR-NB patient sensitivity/resistance to chemotherapy. As exosomes from cancer patients carry proteins that reflect the surface and cytoplasm content of parental cancer cells, including immunosuppressive molecules known to be associated with cancer progression and/or response to therapy, we extended our study to the exosomal proteins (exo-proteins) to assess whether they can predict induction-chemotherapy response and patient eligibility to immunotherapy.

### **Aims:**

The purpose of the study is to identify exo-proteins that (i) are associated with the tumor phenotype, in order to improve risk stratification, (ii) can be indicative of induction chemotherapy response and, therefore, be integrated with the exo-miRNA profile, and (iii) can serve as biomarkers of patient eligibility to immunotherapy.

### **Methods/Materials:**

We isolated exosomes from plasma specimens of HR-NB patients before and after induction chemotherapy (n=48), stage 1 (n=8), stage 2 (n=7) and stage 4S (n=8) NB patients, and 24 controls. Exo-protein expression was evaluated by liquid chromatography-mass spectrometry (LC-MS).

### **Results:**

We identified exo-proteins associated with tumorigenesis that were specifically expressed in NB plasma specimens at diagnosis and not in control samples. Moreover, we identified exo-proteins differentially expressed in plasma from stage 1 and 2 patients compared to HR patients: such proteins could contribute to the development of aggressive metastatic NB tumors. The principal component analysis (PCA) showed that the group of stage 4S patients was clearly different from both localized and HR-NB cases. We also identified exo-proteins differentially expressed after induction

chemotherapy in HR-NB patients, suggesting that exo-protein modulation could be indicative of different response to treatment. By integrating these data with our previous results obtained on exo-miRNA, we found a specific cellular pathway associated with p53/Ras as predictive model to stratify patients according to treatment response. This analysis was paralleled by the phenotyping of relevant exosomal surface molecules using a panel of fluorochrome-conjugated antibodies against 37 selected surface markers to define immunotherapeutic targets. We identified exosomes positive for GD2 and B7-H3, two critical NB markers.

#### Summary/Conclusions:

Our studies demonstrate that exo-proteins can be employed to improve patient stratification and can give deeper insights into the mechanisms mediating the development of aggressive metastatic NB tumors. The results show that exo-proteins, together with exo-miRNA, can be markers of HR-NB patients response to induction chemotherapy and pave the way for the application of such molecules to define targeted therapeutic approaches in NB. To this purpose, we are currently extending the study of the exosome surface proteins to identify new immune-checkpoint molecules for patient eligibility to immunotherapy.

## **P143 Identification of TIMP3 as a regulator for metastasis in neuroblastoma**

Wu, P.Y.<sup>2</sup>, Kargren, M.<sup>3</sup>, Liao, Y.<sup>4</sup>, Hsu, W.<sup>5</sup>, Lee, H.<sup>6</sup>

National Central University, Department of Life science, Taoyuan City, Taiwan <sup>2</sup>, National Taiwan University, Department of Life Science, Taipei City, Taiwan<sup>3</sup>, Academia Sinica, Institute of Cellular and Organismic Biology, Taipei City, Taiwan<sup>4</sup>, National Taiwan University Hospital, Department of Surgery, Taipei City, National Taiwan University Hospital<sup>5</sup>, National Taiwan University, Department of Life Science, Taipei City, Taiwan<sup>6</sup>

### Background/Introduction:

Neuroblastoma (NB) is the most common extra-cranial solid tumor in children. Despite improvements in available treatment options, the prognosis for high-risk neuroblastoma patients remains unfavorable, with metastatic spread being one of the reasons behind it. New therapeutics for patients with advanced NB are urgently needed. *MYCN* is an oncogene whose amplification is associated with high-risk NB. Additionally, EGFR and ErbB2 were both shown to be highly expressed in NB cells, and have been implicated in metastasis of various tumors. Therefore, targeting *MYCN* or EGFR and ErbB2 may have the therapeutic potential for the treatment of NB metastasis.

### Aims:

In this study, we tested the ability of JQ1, a Bromodomain and Extra-Terminal (BET) inhibitor, and CL-387,785, an EGFR/ErbB2 dual inhibitor, to block metastatic properties of NB cells. In addition, the underlying mechanism was verified.

### Methods/Materials:

The tumor metastasis-related cell behaviors were analyzed by wound healing, transwell assays and colony formation assays. A QPCR-based tumor metastasis array was performed to identify the differentially expressed genes (DEGs) in the NB cells treated with JQ1 and CL-387,785. The clinical significance of selected DEG was further analyzed by the web-based R2 platform and a Taiwanese cohort of neuroblastoma patients.

### Results:

Both JQ1 and CL-387,785 significantly reduce migration and invasion of NB cells. Consistently, both compounds can inhibit anchorage-independent growth of NB cells. We further identified tissue inhibitor of metalloproteinases 3 (TIMP3) as one of the significantly upregulated genes in NB cells treated with JQ1 or CL-387,785, indicating that TIMP3 may involve in the mechanism of NB cell metastasis. Lastly, both clinical datasets demonstrate that high expression of TIMP3 positively correlated with favorable prognosis of patients and the differentiation histology of the NB tumors.

### Summary/Conclusions:

Our findings provide the proof-of-principle evidence that JQ1 and CL-387,785 could be further developed to become the target therapies for high-risk neuroblastoma and also verify the prognostic merit of TIMP3 in neuroblastoma.

## **P144 Mitochondrial unfolded protein response (UPR<sup>mt</sup>) and spatial localization of the UPR<sup>mt</sup> proteins in neuroblastoma treated with 2-DG, poly(I:C) or cisplatin**

*Lin, H.Y.<sup>2</sup>, Chuang, H.C.<sup>3</sup>, Wang, P.W.<sup>4</sup>, Hsu, W.M.<sup>5</sup>, Chuang, J.H.<sup>6</sup>*

Kaohsiung Chang Gung Memorial Hospital, Center for Mitochondrial Research and Medicine, Kaohsiung, Taiwan <sup>2</sup>, Kaohsiung Chang Gung Memorial Hospital, Department of Otolaryngology, Kaohsiung, Taiwan<sup>3</sup>, Kaohsiung Chang Gung Memorial Hospital, Department of Internal Medicine, Kaohsiung, Taiwan<sup>4</sup>, National Taiwan University Hospital, Department of Surgery, Taipei, National Taiwan University Hospital<sup>5</sup>, Kaohsiung Chang Gung Memorial Hospital, Department of Pediatric Surgery, Kaohsiung, Taiwan<sup>6</sup>

### Background/Introduction:

Glycolysis and other metabolic event are deeply involved in tumor growth including neuroblastoma (NB), which is also highly dependent on the proper function of mitochondria. Mitochondrial unfolded protein response (UPR<sup>mt</sup>) is known to maintain proteostasis of the mitochondria through chaperones and proteases.

### Aims:

We conducted the study to unveil if UPR<sup>mt</sup> and spatial organization of UPR<sup>mt</sup> proteins in response to 2DG, poly(I:C) and cisplatin is involved in NB cell life and death.

### Methods/Materials:

Two NB cell lines without (SK-N-AS) and with (SK-N-DZ) *MYCN* amplification were treated with 2DG, poly(I:C) and cisplatin in different doses, combinations for different time points. Mitochondrial chaperones (HSP10, HSP60) and proteases (CLPP, AGF3L2 and LONP1) as well as their regulators (CHOP, ATF4, ATF5) were studied *in vitro* and the spatial localization of HSP60 was studied in animal xenografts.

### Results:

Treating SK-N-AS and SK-N-DZ cells with increasing dose of 2DG from 2 to 20 mM, resulted in up-regulation of CHOP, ATF4, ATF5 and AGF3L2, while at the same time significant and progressive decrease of CLPP, but no change in HSP10. In SK-N-DZ, LONP1 is also up-regulated. On the contrary, treatment of SK-N-AS poly(I:C) from 5-20 mg/ml, showing dose-dependent increase of HSP10, while there is no significant change in CLPP. Treating SK-N-AS xenograft with 2DG, cisplatin and poly(I:C) resulting in significant expression of HSP60 in NB cells. The same is true for SK-N-DZ xenografts treated with cisplatin, and the distribution of HSP60 is mainly peri-vascular.

### Summary/Conclusions:

The results suggested that there is cell-type specific response of UPR<sup>mt</sup> proteins to 2DG, CDDP and poly(I:C). The distribution of UPR<sup>mt</sup> proteins in animal xenografts deserves future study to unveil their clinical significance.

## **P145 Early molecular characterization and monitoring of neuroblastoma by circulating tumor DNA analysis**

*Dolz Giménez, S.<sup>2</sup>, Gargallo Tatay, P.<sup>3</sup>, Castel Sánchez, V.<sup>4</sup>, Font de Mora, J.<sup>5</sup>, Yañez Peralta, Y.<sup>6</sup>, Coronado Mondragón, E.<sup>7</sup>, Méndez Peiró, A.<sup>8</sup>, Cañete Nieto, A.<sup>9</sup>*

IIS/Hospital La Fe, Cell and Molecular Biology of Cancer Research Laboratory. Pediatric Oncology., Valencia, Spain <sup>2</sup>, IIS/Hospital La Fe, Pediatric Oncology, Valencia, Spain<sup>3</sup>, IIS/Hospital La Fe, Pediatric Oncology, Valencia, Spain<sup>4</sup>, IIS/Hospital La Fe, Cell and Molecular Biology of Cancer Research Laboratory. Pediatric Oncology., Valencia, IIS/Hospital La Fe<sup>5</sup>, IIS/Hospital La Fe, Cell and Molecular Biology of Cancer Research Laboratory. Pediatric Oncology., Valencia, Spain<sup>6</sup>, IIS/Hospital La Fe, Cell and Molecular Biology of Cancer Research Laboratory. Pediatric Oncology., Valencia, Spain<sup>7</sup>, IIS/Hospital La Fe, Cell and Molecular Biology of Cancer Research Laboratory. Pediatric Oncology., Valencia, Spain<sup>8</sup>, IIS/Hospital La Fe, Pediatric Oncology, Valencia, Spain<sup>9</sup>

### Background/Introduction:

Molecular aspects of neuroblastoma establish a wide clinical variability. Given the oncogenic role of *MYCN*, *ALK* or genes involving the hemizygous deletion in chromosome 11q (del11q), characterization and monitoring of these molecular biomarkers are crucial for neuroblastoma patients. Recent data indicate that implementing molecular characterization of tumors by circulating tumor DNA (ctDNA) analysis, will improve disease assessment. Certainly, plasma ctDNA liquid biopsy (LB) testing using high sensitivity molecular assays would allow early detection of tumor growth through a non-invasive peripheral blood extraction. In fact, the invasiveness of surgical biopsies complicates disease monitoring and cannot allow molecular characterization for extremely difficult access or poor quality of surgical samples.

### Aims:

This work aims to perform a methodological strategy for neuroblastoma molecular characterization and patient monitoring using plasma LB.

### Methods/Materials:

The study included 26 pediatric patients with neuroblastoma that were diagnosed or referred to the Pediatric Oncology Unit of Hospital La Fe (58% at diagnosis and 42% from relapse), from 2017 to 2019.

Peripheral blood samples were collected into EDTA tubes and separated into plasma and blood cells within 24 h (1,900 g 10 min 4 °C; 16,000 g 10 min °C). Circulating cell-free DNA (ccfDNA) from plasma and germinal DNA from blood cells were isolated with *QIAamp Circulating Nucleic Acid* and *QIAamp DNA mini* kits (Qiagen), respectively. Tumor DNA (tDNA) from matched tumor samples was extracted with *QIAamp DNA Investigator kit* (Qiagen). The DNA size distribution and quantification were performed in a Bioanalyzer TapeStation (Agilent Technologies) or Qubit (Invitrogen).

DNA samples were sequenced in a Illumina NGS platform (NextSeq500), using a NGS custom panel and *SeqCap EZ HyperCap kit* (Roche). The panel allowed coding (with flanking intronic) regions and copy number variation (CNV) analysis in 408 genes related to pediatric cancer.

*ALK* oncogenic mutations, *MYCN* or *ALK* amplifications and del11q were monitored by ddPCR in ccfDNA, using a QX200™ Droplet Digital™ PCR platform and specific assays (Bio-rad).

### Results:

In all cases, somatic genetic variants identified in tDNA were also detected in matched ctDNA samples by NGS panel testing. Also, CNV in matched ctDNA were detected when there was more than 20% ctDNA in ccfDNA. In two patients, both somatic variants and CNV were detected in ctDNA but unnoticed in matched tDNA (highlighting the oncogenic *ALK*:p.R1275Q variant in one patient and *MYCN* amplification in the other).

Monitoring was performed in 45% (9/20) patients with *MYCN* amplification, 15% (3/20) with *ALK* amplification, 40% (8/20) with del11q and 30% (6/20) with oncogenic somatic *ALK* mutations (83% *ALK*:p.R1275Q and 17% p.F1245C). A total of 62 serial ccfDNA samples were analyzed by ddPCR during patient follow-up. The results were plotted in follow-up graphs, showing clonal evolution of tumors carrying the specific oncogenic alteration. The results agreed with the disease status and predicted recurrences in all cases.

#### Summary/Conclusions:

The methodological strategy performed to analyze plasma LB by our custom NGS panel and specific ddPCR assays proved to be a potential non-invasive alternative to characterize neuroblastoma tumors and monitor the disease during follow-up, showing new oncogenic biomarkers not seen in tumor biopsies.



## **P146 Integrated molecular analysis from SIOOPEN HR-NBL1 trial identifies an association between multi-exon deletions in ATRX and a poor response to induction chemotherapy**

*George, S.L.<sup>2</sup>, Tall, J.<sup>3</sup>, Bellini, A.<sup>4</sup>, Ambros, I.<sup>5</sup>, Ambros, P.<sup>6</sup>, Noguera, R.<sup>7</sup>, Bown, N.<sup>8</sup>, Vicha, A.<sup>9</sup>, Taschner-Mandl, S.<sup>10</sup>, Marques, B.<sup>11</sup>, Mühlethaler-Mottet, A.<sup>12</sup>, Hughes, D.<sup>13</sup>, Jamal, S.<sup>14</sup>, Hubank, M.<sup>15</sup>, Tweddle, D.A.<sup>16</sup>, Poetschger, U.<sup>17</sup>, Glogova, E.<sup>18</sup>, Combaret, V.<sup>19</sup>, Valteau-Couanet, D.<sup>20</sup>, Ladenstein, R.<sup>21</sup>, Schleiermacher, G.<sup>22</sup>, Chesler, L.<sup>23</sup>*

The Institute of Cancer Research, Paediatric Tumour Biology, London, United Kingdom <sup>2</sup>, The Institute of Cancer Research, Paediatric Tumour Biology, London, United Kingdom<sup>3</sup>, Institut Curie, Translational Pediatric Oncology Team, Paris, France<sup>4</sup>, Childrens Cancer Research Institute, Tumour Biology, Vienna, Childrens Cancer Research Institute<sup>5</sup>, Childrens Cancer Research Institute, Tumour Biology, Vienna, Austria<sup>6</sup>, University of Valencia, Department of Pathology, Valencia, Spain<sup>7</sup>, Northern Genetics Service, Cytogenetics, Newcastle, United Kingdom<sup>8</sup>, Charles University, Department of Paediatric Haematology and Oncology, Prague, Czech Republic<sup>9</sup>, Childrens Cancer Research Institute, Tumour Biology, Vienna, Austria<sup>10</sup>, Instituto Nacional de Saúde Doutor Ricardo Jorge, Genetics, Lisbon, Portugal<sup>11</sup>, Lausanne University Hospital and University of Lausanne, Woman-Mother-Child Department, Lausanne, Switzerland, <sup>12</sup>, The Institute of Cancer Research, Clinical Studies, London, United Kingdom <sup>13</sup>, The Institute of Cancer Research, Clinical Studies, London, United Kingdom <sup>14</sup>, The Institute of Cancer Research, Clinical Studies, London, United Kingdom <sup>15</sup>, Newcastle University, Faculty of Medical Sciences, Newcastle, United Kingdom <sup>16</sup>, Childrens Cancer Research Institute, Studies & Statistics for Integrated Research and Projects, Vienna, Austria <sup>17</sup>, Childrens Cancer Research Institute, Studies & Statistics for Integrated Research and Projects, Vienna, Austria <sup>18</sup>, Centre Leon Berard, Genetics, Lyon, France <sup>19</sup>, Gustave Roussy, Child and Adolescent Cancer, Paris, France <sup>20</sup>, Childrens Cancer Research Institute, Studies & Statistics for Integrated Research and Projects, Vienna, Austria <sup>21</sup>, Institut Curie, Translational Pediatric Oncology Team, Paris, France <sup>22</sup>, The Institute of Cancer Research, Paediatric Tumour Biology, London, United Kingdom <sup>23</sup>

### **Background/Introduction:**

With intensification of therapy, patients with *MYCN* amplified neuroblastoma now have similar survival to patients with non-*MYCN* amplified high-risk disease. However, age at diagnosis remains an important prognostic factor and increasing age at diagnosis is strongly associated with worse survival.

Older age at diagnosis is associated with specific biological features including 11q loss of heterozygosity (LoH), *ATRX* alterations and alternative lengthening of telomeres (ALT). 11q LoH, *ATRX* alterations, and ALT have all individually been associated with adverse outcomes although there is significant overlap amongst these genetic features and the relative influence of each factor on clinical phenotype is unclear.

### **Aims:**

To determine *ATRX*, 11q and ALT status in patients with non-*MYCN* amplified neuroblastoma, treated on the SIOOPEN HR-NBL1 clinical trial and link molecular findings to the clinical phenotype.

### **Methods/Materials:**

Eligibility criteria were *MYCN* non-amplified neuroblastoma, aged >18 months, and treated on the SIOOPEN HR-NBL1 trial. ALT status was evaluated by c-circle assay. Tumour DNA was sequenced using a 91-gene custom paediatric solid tumour hybrid capture next generation sequencing (NGS) panel. The NGS panel detects single nucleotide variants small insertions/deletions, copy number and structural variants. 11q status was determined by existing array-CGH/SNP-array data from SIOOPEN biology laboratories, NGS panel, or where sufficient DNA available, by low coverage whole genome sequencing.

### **Results:**

36/64 tumours fulfilling the eligibility criteria were ALT positive by c-circle assay. However, c-circle stability experiments showed that c-circles rapidly degrade if DNA is left at ambient temperature for 1 week, highlighting the probability of false negatives in these archival samples. In cases confirmed as ALT positive, the most commonly altered gene was *ATRX* (13), followed by *SMARCA4* (4) and *ALK* (3).

The most common *ATRX* variant detected was a multi-exon deletion (13), followed by missense alterations (5). One tumour had a frame-shift *ATRX* alteration and another a splice site variant. There was no significant difference in EFS according to *ATRX* status, although median time to 1<sup>st</sup> event was significantly shorter in the *ATRX* deleted group (189 vs 465 days,  $p=0.0192$ ). Consistent with this, patients with *ATRX* multi-exon deletions had much lower overall response rates to induction chemotherapy (46% in patients with *ATRX* deletions, 83% in patients with other *ATRX* mutations, 85% *ATRX* wild-type group). Overall response rate to induction chemotherapy in c-circle positive patients in the absence of an *ATRX* deletion was 84%.

Information on both 11q and *ATRX* status was available in 58 patients. 80% of patients with *ATRX* alterations had concurrent 11q loss. Patients with 11q loss who were *ATRX* wild-type ( $n=22$ ) had an induction response rate of 90%.

#### Summary/Conclusions:

Preliminary SIOOPEN HR-NBL1 trial data identifies a strong association between *ATRX* deletions and a poor induction chemotherapy response, however patients with 11q LoH and/or ALT have an excellent response to induction therapy in the absence of a concurrent *ATRX* deletion.

This data will be integrated into a larger on-going SIOOPEN biology study. Over 400 patients enrolled on the SIOOPEN-HR-NBL-1 trial have now had tumour DNA sequenced by NGS panel, which will be linked with telomere status and clinical outcome data.

## **P147 Clinical and genomic differences according to the metastatic burden in stage 4 high-risk neuroblastoma**

Seo, E.<sup>2</sup>, Lee, E.<sup>3</sup>, Cho, H.E.E.<sup>4</sup>, Hee Young, H.<sup>5</sup>, Yoo, K.<sup>6</sup>, Lee, J.<sup>7</sup>, Sung, K.I.<sup>8</sup>, Koo, H.<sup>9</sup>

Samsung Medical Center, Pediatrics, Seoul, South Korea <sup>2</sup>, Samsung Medical Center, Samsung Genome Institute, Seoul, South Korea<sup>3</sup>, Samsung Medical Center, Pediatrics, Seoul, South Korea<sup>4</sup>, Samsung Medical Center, Pediatrics, Seoul, Samsung Medical Center<sup>5</sup>, Samsung Medical Center, Pediatrics, Seoul, South Korea<sup>6</sup>, Samsung Medical Center, Pediatrics, Seoul, South Korea<sup>7</sup>, Samsung Medical Center, Pediatrics, Seoul, South Korea<sup>8</sup>, Samsung Medical Center, Pediatrics, Seoul, South Korea<sup>9</sup>

### Background/Introduction:

Stage 4 high-risk neuroblastoma is a remarkably heterogeneous disease with varying outcomes. Previous analyses of several cohorts have reported that metastatic burden and/or patterns have prognostic significance in stage 4 neuroblastoma patients. However, we need further detailed information on clinical behavior and genetic aberrations in tumors with different metastatic spread to explain different treatment outcomes and to inform additional risk stratification.

### Aims:

The aim of this study was to identify subgroups of stage IV high-risk neuroblastoma based on pattern and burden of metastatic spread and compare their clinical and genomic differences.

### Methods/Materials

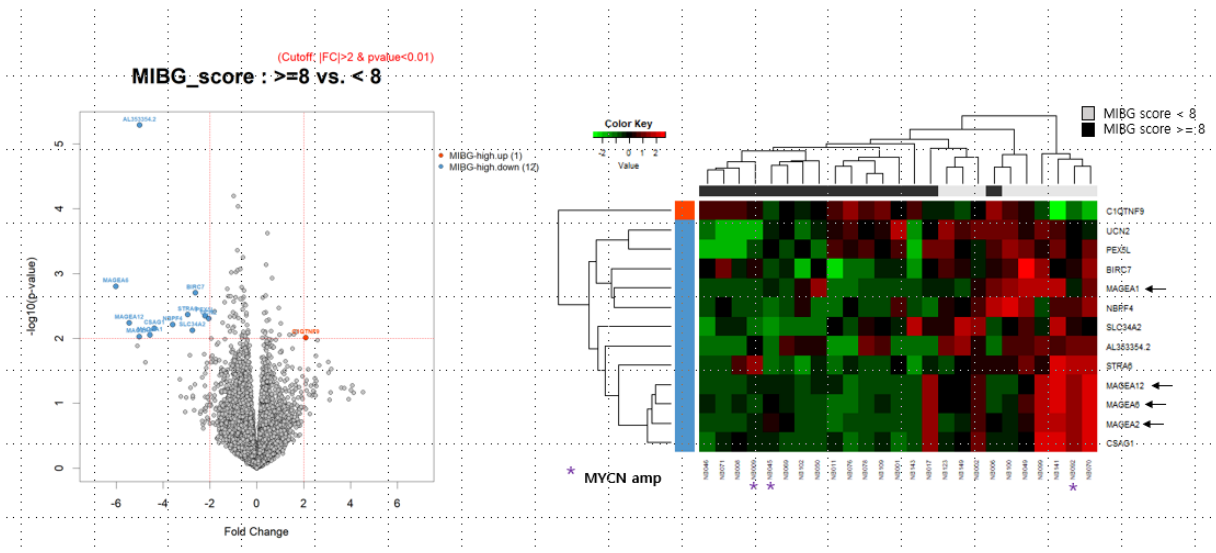
Patients with stage 4 mIBG-avid neuroblastoma (age $\geq$ 18 months) were categorized by Curie score ( $8 <$  vs.  $\geq 8$ ) at diagnosis and their clinical and genomic differences were compared (n=117). The optimum cutoff value of Curie score was determined by Fine and Gray competing-risks analysis with respect to progression free survival. We reviewed their medical records and, also performed whole transcriptome analysis with RNA-Seq to explore gene expression differences.

### Results

Patients with a higher Curie score ( $\geq 8$ ) at diagnosis had a significantly worse progression-free survival (PFS) than those with a lower score ( $< 8$ ) (3-y PFS:  $59.9\% \pm 6.6\%$  vs.  $84.4 \pm 6.6\%$ , respectively;  $P = 0.004$ ). Patient with a higher Curie score at diagnosis were more likely to have a smaller primary tumor size ( $244 \pm 304 \text{ cm}^3$  vs.  $475 \pm 461 \text{ cm}^3$ ,  $P = 0.003$ ), a non-MNA tumors ( $P = 0.032$ ) and elevated serum ferritin and urine VMA levels. ( $P < 0.001$ ,  $P = 0.001$ , respectively). And patients with a higher Curie score also had a higher score of metastatic site index ( $P < 0.001$ ) which is also representing metastatic burden. Meanwhile, better reduction in MIBG score (%) after 3 cycles of chemotherapy was observed in patients with a higher Curie score ( $64.7\% \pm 4.3$  vs.  $36.7\% \pm 5.3$ ,  $P = 0.015$ ). However, there was no difference in primary tumor volume reduction after 3 cycles of chemotherapy. In genomic study, we identified 13 differentially expressed genes in patients with a higher Curie score as compared to the patients with a lower Curie score ( $p$ -value  $< 0.01$ ,  $|FC| > 2$ ). Among those genes, *MAGEA1*, *MAGEA2*, *MAGEA6*, *MAGEA12*, *CSGA1* and *C1QTNF9* were expressed differentially with the highest significance between two groups.

### Summary/Conclusions:

We found differences in clinical behavior and gene expression according to the extent of metastatic neuroblastoma which carries prognostic significance. We believe that our findings contribute to the understanding of stage 4 high-risk neuroblastoma and provide ideas for further studies.



Caption 1: Figure 1. Whole transcriptome analysis

## **P148 P-glycoprotein overexpression is a resistance mechanism for induction chemotherapy in high-risk neuroblastoma**

*Atkinson, C.J.*<sup>2</sup>, *Tactacan, C.M.*<sup>3</sup>, *Kamili, A.*<sup>4</sup>, *Rosser, S.*<sup>5</sup>, *Saletta, F.*<sup>6</sup>, *Gana, C.C.*<sup>7</sup>, *Eden, G.L.*<sup>8</sup>, *Mayoh, C.*<sup>9</sup>, *Jayatilleke, N.*<sup>10</sup>, *Lock, R.B.*<sup>11</sup>, *Norris, M.D.*<sup>12</sup>, *Haber, M.*<sup>13</sup>, *Gifford, A.J.*<sup>14</sup>, *Nath, C.*<sup>15</sup>, *Trahair, T.N.*<sup>16</sup>, *Fletcher, J.I.*<sup>17</sup>

Children's Cancer Institute, Experimental Therapeutics, Randwick, Australia <sup>2</sup>, Children's Cancer Institute, Personalized medicine, Randwick, Australia<sup>3</sup>, Children's Cancer Institute, Personalized Medicine, Randwick, Australia<sup>4</sup>, The Children's Hospital at Westmead, Pharmacokinetics Laboratory, Department of Biochemistry and Oncology, Westmead, The Children's Hospital at Westmead<sup>5</sup>, The Children's Hospital at Westmead, Children's Cancer Research Unit, Kids Research Institute, Westmead, Australia<sup>6</sup>, Children's Cancer Institute, Experimental Therapeutics, Randwick, Australia<sup>7</sup>, Children's Cancer Institute, Experimental Therapeutics, Randwick, Australia<sup>8</sup>, Children's Cancer Institute, Bioinformatics, Randwick, Australia<sup>9</sup>, Children's Cancer Institute, Bioinformatics, Randwick, Australia<sup>10</sup>, Children's Cancer Institute, Leukemia Biology, Randwick, Australia<sup>11</sup>, Children's Cancer Institute, Molecular Oncology, Randwick, Australia, <sup>12</sup>, Children's Cancer Institute, Experimental Therapeutics, Randwick, Australia <sup>13</sup>, Children's Cancer Institute, Department of Anatomical Pathology, Randwick, Australia <sup>14</sup>, The Children's Hospital at Westmead, Pharmacokinetics laboratory, Department of Biochemistry and Oncology, Westmead, Australia <sup>15</sup>, Sydney Children's Hospital, Kid's Cancer Centre, Randwick, Australia <sup>16</sup>, Children's Cancer Institute, Experimental Therapeutics, Randwick, Australia <sup>17</sup>

### Background/Introduction:

High-risk neuroblastoma (HR-NB) patients undergo intensive therapeutic intervention which is heavily reliant on combination chemotherapy. Despite this, these patients have a survival rate of only ~50%, largely due to intrinsic or acquired drug resistance. P-glycoprotein (P-gp; *ABCB1*) is a multidrug transporter known to efflux multiple conventional agents used in HR-NB induction therapy including doxorubicin, vincristine and etoposide and has been reported as a resistance mechanism for the ALK inhibitor crizotinib. We observed surprisingly high P-gp expression in HR-NB cell lines and patient-derived xenograft (PDX) models, so investigated whether P-gp expression was relevant for response to therapy in this disease.

### Aims:

To determine: i) what drives high P-gp expression in HR-NB and if P-gp levels influence the effectiveness of chemotherapy; ii) whether P-gp inhibition can increase the therapeutic window for standard-of-care chemotherapies and targeted agents.

### Methods/Materials:

To determine the prevalence of high *ABCB1*/P-gp expression we performed RNA-sequencing, immunohistochemistry and western blot on panels of neuroblastoma tumour samples, PDX models and cell lines. We also interrogated *ABCB1* expression in larger cohorts using the R2 database (<https://hgserver1.amc.nl/cgi-bin/r2/main.cgi>) and are investigating *ABCB1* regulation using publicly available CHIP-Seq and RNA-Seq data. To assess the influence of P-gp in resistance to standard of care and targeted therapies, cytotoxicity assays were performed on high P-gp expressing HR-NB cell lines with or without the P-gp inhibitor tariquidar. Following *in vitro* assessment with western blot, proliferation and cytotoxicity assays, P-gp knockdown (shRNA), *ABCB1*-null (CRISPR/Cas9) or control neuroblastoma cells were engrafted into NOD-SCID Gamma (NSG) mice then treated with vincristine (P-gp substrate) or cisplatin (non-substrate). To determine feasibility of combining tariquidar with vincristine *in vivo*, we examined the influence of tariquidar on vincristine pharmacokinetics using volumetric absorptive microsampling and liquid chromatography-tandem mass spectrometry.

### Results:

RNA-sequencing, immunohistochemistry, western blot and R2 database interrogation showed high *ABCB1* P-gp expression is frequently observed in HR-NB and is more common than in most other cancers ( $p < 0.0001$ ). High relative expression at diagnosis is associated with poorer patient outcome (NCI TARGET,  $n=217$ ,  $p=0.04$ ), consistent with its multidrug transporter function. *in vitro* tariquidar and P-gp knockdown both sensitize high P-gp expressing neuroblastoma cells to vincristine, doxorubicin and etoposide, but not the ALK inhibitors crizotinib, ceritinib and alectinib. P-gp knockdown sensitized neuroblastoma xenografts to vincristine, extending survival, suggesting P-gp levels are sufficient to confer resistance to standard-of-care therapies. An *ABCB1* null neuroblastoma cell line was sensitized to P-gp substrates *in vitro* however *ABCB1*-null xenografts were not sensitized to vincristine, suggesting that additional resistance mechanisms can rapidly develop. In the presence of tariquidar, plasma vincristine exposure was approximately doubled in mice. We are currently assessing whether tariquidar can provide an increased therapeutic window in xenograft models.

#### Summary/Conclusions:

Elevated P-gp expression is common in HR-NB and can be sufficient to confer resistance to standard-of-care chemotherapies in model systems. Our findings suggest that tumour P-gp levels might be used to guide treatment options for individual patients to avoid ineffective treatments. The potential of P-gp inhibitors as adjuncts to conventional chemotherapy for HR-NB should be further investigated.

## **P149 Multiplexed quantification of four DNA targets in a single reaction using droplet digital PCR**

*Peitz, C.*<sup>2</sup>, *Sprüssel, A.*<sup>3</sup>, *Grimaldi, M.*<sup>4</sup>, *Linke, R.*<sup>5</sup>, *Astrahantseff, K.*<sup>6</sup>, *Tiburtius, D.*<sup>7</sup>, *Schmelz, K.*<sup>8</sup>, *Messerschmidt, C.*<sup>9</sup>, *Beule, D.*<sup>10</sup>, *Keilholz, U.*<sup>11</sup>, *Eggert, A.*<sup>12</sup>, *Deubzer, H.E.*<sup>13</sup>, *Lodrini, M.*<sup>14</sup>

Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin, Germany<sup>2</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin, Germany<sup>3</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin, Germany<sup>4</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin, Germany<sup>5</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin, Germany<sup>6</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin, Germany<sup>7</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin, Germany<sup>8</sup>, Berlin Institute of Health (BIH), Core Unit Bioinformatics – CUBI, Berlin, Germany<sup>9</sup>, Berlin Institute of Health (BIH), Core Unit Bioinformatics – CUBI, Berlin, Germany<sup>10</sup>, Charité - Universitätsmedizin Berlin, Charité Comprehensive Cancer Center, Berlin, Germany<sup>11</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin, Germany<sup>12</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin, Germany<sup>13</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin, Germany<sup>14</sup>

### Background/Introduction:

Major challenges of tumor-derived cell-free DNA (cfDNA) analysis purified from blood samples are small sample volumes and low cfDNA concentrations. Droplet digital PCR (ddPCR) is a suitable technology to analyze low levels of cfDNA.

### Aims:

We intended to establish two quadruplexed ddPCR assays that (i) reliably quantify *MYCN* and *ALK* copy numbers in a single reaction together with two reference genes and (ii) accurately estimate *ALK*<sup>F1174L</sup> and *ALKR*<sup>1275Q</sup> mutant allele fractions using cfDNA or genomic DNA (gDNA) as input.

### Methods/Materials:

We optimized separation of positive and negative droplets to detect two targets in each ddPCR fluorescence channel by adjusting probe and primer concentrations for each target molecule. Accuracy and sensitivity was assessed in both assays using a panel of 10 neuroblastoma cell lines and blood plasma samples paired with corresponding primary tumor tissue from 9 neuroblastoma patients.

### Results:

Assay conditions were first established for a triplexed reaction, detecting 2 reference genes (*NAGK* and *AFF3*) in channel 2 and *MYCN* or *ALK* in channel 1. Droplet clusters were accurately separated for *AFF3* and *NAGK* in channel 2 as well as *ALK* or *MYCN* in channel 1. Primer/probe concentrations for simultaneously detecting *ALK* and *MYCN* in channel 1 were next established. Following optimization, QuantaSoft Analysis Pro software accurately distinguished 16 different droplet clusters that included negative, single-, double-, triple- and quadruple-positive droplets. The quadruplexed reaction maintained the same linearity as duplexed and triplexed reactions within the range of 0.5 to 100 ng DNA of input DNA. *MYCN* and *ALK* copy number status was re-analyzed in 8 neuroblastoma cell lines to demonstrate that quadruplexed ddPCR clearly distinguished between amplification, gain and diploid status. We next applied quadruplexed ddPCR-based *MYCN* and *ALK* copy number assessment to blood plasma samples paired with corresponding primary neuroblastoma tissue from 3 patients. Simultaneous *MYCN* and *ALK* copy number quantification was feasible using cfDNA purified from plasma and tumor-derived genomic DNA as input. We also established a quadruplexed ddPCR protocol detecting *ALK*<sup>F1174L</sup> together with the respective *ALK*<sup>wildtype</sup> sequence in channel 1 and

$ALK^{R1275Q}$  and with its corresponding  $ALK^{wildtype}$  sequence in channel 2. Detection of both *ALK* hotspot mutations maintained linearity within the range of 0.5 to 130 ng of input DNA. Sensitivity thresholds were set for the quadruplexed assay, and resembled those for the respective duplexed assays. Data from 6 neuroblastoma cell lines and matched blood plasma samples paired with corresponding primary neuroblastoma tissue from 6 patients confirmed that quadruplexed ddPCR assays accurately distinguished among  $ALK^{F1174L}$ ,  $ALK^{R1275Q}$  and  $ALK^{wildtype}$  status and correctly estimated allelic fractions of both mutations using up to 20 ng of input DNA.

#### Summary/Conclusions:

We present two robust quadruplexed ddPCR protocols to assess *MYCN* and *ALK* copy numbers as well as  $ALK^{F1174L}$  and  $ALK^{R1275Q}$  mutant allele fractions in cfDNA or gDNA that are applicable in the routine clinical setting.



## **P150 High resolution copy number analysis of UK high risk neuroblastomas**

*Gabriel, A.S.*<sup>2</sup>, *Whiteman, S.*<sup>3</sup>, *Goodman, A.*<sup>4</sup>, *Harding, F.*<sup>5</sup>, *Eves, M.*<sup>6</sup>, *Basta, N. O.*<sup>7</sup>, *Elliott, M.*<sup>8</sup>, *McNally, R.J.Q.*<sup>9</sup>, *Cuthbert, G.*<sup>10</sup>, *Bown, N.*<sup>11</sup>, *Tweddle, D.A.*<sup>12</sup>

Newcastle upon Tyne hospitals NHS Trust, Translational & Clinical Research Institute, Newcastle Upon Tyne, United Kingdom<sup>2</sup>, Newcastle upon Tyne hospitals NHS Trust, Translational & Clinical Research Institute, Newcastle Upon Tyne, United Kingdom<sup>3</sup>, The Newcastle Upon Tyne Hospitals NHS Foundation Trust, Northern Genetics Service, Newcastle Upon Tyne, United Kingdom<sup>4</sup>, The Newcastle Upon Tyne Hospitals NHS Foundation Trust, Northern Genetics Service, Newcastle Upon Tyne, The Newcastle Upon Tyne Hospitals NHS Foundation Trust<sup>5</sup>, The Newcastle Upon Tyne Hospitals NHS Foundation Trust, Northern Genetics Service, Newcastle Upon Tyne, United Kingdom<sup>6</sup>, Newcastle upon Tyne hospitals NHS Trust, Population and Health Sciences, Newcastle Upon Tyne, United Kingdom<sup>7</sup>, Leeds Teaching Hospital NHS Trust, Paediatric Oncology, Leeds, United Kingdom<sup>8</sup>, Newcastle upon Tyne hospitals NHS Trust, Population and Health Sciences, Newcastle Upon Tyne, United Kingdom<sup>9</sup>, The Newcastle Upon Tyne Hospitals NHS Foundation Trust, Northern Genetics Service, Newcastle Upon Tyne, United Kingdom<sup>10</sup>, The Newcastle Upon Tyne Hospitals NHS Foundation Trust, Northern Genetics Service, Newcastle Upon Tyne, United Kingdom<sup>11</sup>, Newcastle upon Tyne hospitals NHS Trust, Translational & Clinical Research Institute, Newcastle Upon Tyne, United Kingdom,<sup>12</sup>

### Background/Introduction:

Neuroblastoma (NB) is the commonest childhood extracranial solid tumour with around 100 new cases per year in the UK. In Europe high risk NB (HRNB) comprises patients > 1 year of age with metastatic disease, or < 1 year with metastatic disease and *MYCN* amplification or localised (stage 2 or 3) disease with *MYCN* amplification.

### Aims:

To perform a comprehensive genetic analysis of HRNB using high-density single nucleotide polymorphism (SNP) array data to identify other genetic abnormalities that may be targeted therapeutically or used to develop a future HRNB risk score.

### Methods/Materials:

SNP arrays from NB DNA from UK and Ireland patients on the European HRNB trial (HR-NBL1/SIOPEN) were generated through the national NB reference laboratory at the Northern Genetics Service (Illumina CytoSNP-850k array) (n=195), and AROS (Affymetrix CytoScan HD array) (n=6). SNP array data was analysed using BioDiscovery Nexus copy number software (BioDiscovery, USA). Chi-squared analyses were performed to assess associations using IBM SPSS Statistics Version 25.

### Results:

Thirty-two DNA samples were removed because their genomic profile was flat (low tumour content) and three were removed for poor quality (n=166). *MYCN* amplification (MNA) was identified in 79 samples (48%). Chromosome 1p deletion was associated with MNA (p<0.001) and chromosome 11q deletion was associated with non-MNA (p<0.001) as previously reported. MNA tumours had significantly fewer copy number abnormalities (CNA) (mean=7.1) compared to non-*MYCN*-amplified tumours (mean=11.7) (p<0.001). 17q gain was the most common SCA, (141 cases, (84.9%)). Intragenic deletions of the *ATRX* gene were identified in 7 cases (4.2%) all in non-*MYCN*-amplified tumours. This study identified 102 amplified regions (amplicons) in addition to the *MYCN* amplicon, with the most frequent amplified genes in tumours being *ALK* (n=9, all MNA), *CDK4* (n=5, 2 MNA) and *MDM2* (n=4, 2 MNA), all of which can currently be targeted therapeutically. *TERT* amplification was identified in three cases (1.8%); two cases with MNA. Focal gain of *TERT* was found in a further five MNA tumours (3.0%). *PTPRD* heterozygous deletion was found in eight cases (4.8%). In two cases *MYCN* and *ALK* amplification were the only CNA found at diagnosis. Novel potentially targetable amplicons including oncogenes such the insulin receptor substrate 1 (*IRS-1*) (n=1) and Baculoviral

IAP repeat-containing protein 1 *BIRC-1, Survivin* ( $n=1$ ) were also identified. Analysis of structural defects identified the catastrophic event of chromothripsis (chromosome shattering and incorrect re-joining) in 5 tumours (3%), (one with stage 2 *MYCN*-amplified disease and four with stage 4 disease).

Summary/Conclusions:

MNA is present in NB with fewer CNA than non-MNA HRNB and in two cases occurred with only one other CNA. Other targetable amplified genes occur in up to 12% of high risk NB and have been reported to lead to an inferior prognosis. The frequency of chromothripsis is low and its clinical significance uncertain. Ongoing work as part of an international SIOPEN Biology Group HRNBL-1 trial collaborative study will combine national CNA datasets with known clinical and genetic risk factors to generate a risk score which can be tested in future clinical trials of high risk NB.

## **P151 Deriving predictive biomarkers for epigenetic drug response in neuroblastoma by supervised machine learning**

*Toedling, J.<sup>2</sup>, Klironomos, F.<sup>3</sup>, Witthauer, M.J.<sup>4</sup>, Szymansky, A.<sup>5</sup>, Hertwig, F.<sup>6</sup>, Eggert, A.<sup>7</sup>, Janoueix-Lerosey, I.<sup>8</sup>, Boeva, V.<sup>9</sup>, Schulte, J.H.<sup>10</sup>*

Charite - Universitaetsmedizin Berlin, Department of Pediatrics, Division of Oncology and Hematology, Berlin, Germany <sup>2</sup>, Charite - Universitaetsmedizin Berlin, Department of Pediatrics, Division of Oncology and Hematology, Berlin, Germany<sup>3</sup>, Charite - Universitaetsmedizin Berlin, Department of Pediatrics, Division of Oncology and Hematology, Berlin, Germany<sup>4</sup>, Charite - Universitaetsmedizin Berlin, Department of Pediatrics, Division of Oncology and Hematology, Berlin, Charite - Universitaetsmedizin Berlin<sup>5</sup>, Charite - Universitaetsmedizin Berlin, Department of Pediatrics, Division of Oncology and Hematology, Berlin, Germany<sup>6</sup>, Charite - Universitaetsmedizin Berlin, Department of Pediatrics, Division of Oncology and Hematology, Berlin, Germany<sup>7</sup>, Institut Curie, Inserm U830, Genetics and biology of cancers, Paris, France<sup>8</sup>, ETH Zürich, Department of Computer Science, Zürich, Switzerland<sup>9</sup>, Charite - Universitaetsmedizin Berlin, Department of Pediatrics, Division of Oncology and Hematology, Berlin, Germany<sup>10</sup>

### Background/Introduction:

Children with high-risk neuroblastoma suffer from the lack of treatment options, and since relapse tumours are almost always resistant to chemotherapy, the disease outcome often is fatal. Recently, several drugs which either directly target neuroblastoma oncoproteins, such as ALK, or indirectly target *MYCN* and other drivers via epigenetic regulation have been introduced as promising treatment options for multiple cancer indications and are being evaluated in early-phase clinical trials. We and others have demonstrated preclinical efficacy of BET bromodomain protein inhibitors (BETi) and ALK inhibitors in neuroblastoma, and several inhibitors have already entered into clinical trials for adult tumours and paediatric indications.

### Aims:

With respect to biomarkers, mechanistic insights and potential combination therapies of these inhibitors, clinical trials currently run ahead of preclinical development. Our goal is to facilitate future clinical trials by deriving biomarkers for and insights into the activity of promising candidate drugs.

### Methods/Materials:

We screen a representative panel of 21 neuroblastoma cell lines for their response to three BET inhibitors and two ALK inhibitors which have a high potential to enter clinical trials for neuroblastoma. Based on cell-viability measurements upon treating the cells with a wide range of drug concentrations, the dose-response curve for each drug and each cell line combination is derived. Leveraging the histone-modification ChIP-Seq data and RNA-Seq gene expression data available for these cell lines, we then employ an iterative machine-learning algorithm, based on Random Forests, to identify genes and epigenetic marks which robustly predict treatment outcome for each class of inhibitor drugs. Thus found candidate predictive biomarkers are further evaluated in independent data sets and by small-scale wet-lab perturbation experiments.

### Results:

For BET inhibitors, the cell lines were reproducibly stratified into good responders, poor responders and non-responders. A small set of genomic and epigenomic markers was found to robustly and accurately predict neuroblastoma response to BET inhibitors. These predictive features, which include features strongly linked to BET protein biology, are currently being validated in the lab and in independent data sets. For ALK inhibitors, the drug-efficacy tests have been completed and features that are predictive for drug response are currently being elucidated using the machine-learning algorithm.

### Summary/Conclusions:

Validated predictive biomarkers can serve to define inclusion and exclusion criteria as well as to establish cohort sizes for future clinical trials. Moreover, certain predictive biomarkers may provide additional insights into potential resistance mechanisms and suggestions for combination therapies for treating high-risk neuroblastoma.

## **P152 A genome-wide study of an unfavourable subgroup of intermediate-risk (Group 8) neuroblastoma: A SIOPEX Biology and LINES group study**

*Gabriel, A.S.<sup>2</sup>, Beckett, E.<sup>3</sup>, Goodman, A.<sup>4</sup>, Harding, F.<sup>5</sup>, Cuthbert, G.<sup>6</sup>, Jeison, M.<sup>7</sup>, Noguera, R.<sup>8</sup>, Berbegall, A.P.<sup>9</sup>, Schoumans, J.<sup>10</sup>, Tchinda, J.<sup>11</sup>, Muehlethaler-Mottet, A.<sup>12</sup>, Pezzolo, A.<sup>13</sup>, Mazzocco, K.<sup>14</sup>, Beiske, K.<sup>15</sup>, Cañete, A.<sup>16</sup>, Bown, N.<sup>17</sup>, Herd, F.<sup>18</sup>, Wheeler, K.<sup>19</sup>, Di Cataldo, A.<sup>20</sup>, Papadakis, V.<sup>21</sup>, Segura, M.F.<sup>22</sup>, Schleiermacher, G.<sup>23</sup>, Tweddle, D.A.<sup>24</sup>*

Newcastle upon Tyne hospitals NHS Trust, Translational & Clinical Research Institute, Newcastle Upon Tyne, United Kingdom <sup>2</sup>, Newcastle University, Translational & Clinical Research Institute, Newcastle Upon Tyne, United Kingdom<sup>3</sup>, The Newcastle Upon Tyne Hospitals NHS Foundation Trust, Northern Genetics Service, Newcastle Upon Tyne, United Kingdom<sup>4</sup>, The Newcastle Upon Tyne Hospitals NHS Foundation Trust, Northern Genetics Service, Newcastle Upon Tyne, The Newcastle Upon Tyne Hospitals NHS Foundation Trust<sup>5</sup>, The Newcastle Upon Tyne Hospitals NHS Foundation Trust, Northern Genetics Service, Newcastle Upon Tyne, United Kingdom<sup>6</sup>, Schneider Children's Medical Center of Israel, The Rina Zaizov Pediatric Hematology Oncology Dept, Petah Tikva, Israel<sup>7</sup>, University of Valencia-INCLIVA-Ciberonc, Department of Pathology, Valencia, Spain<sup>8</sup>, University of Valencia-INCLIVA-Ciberonc, Department of Pathology, Valencia, Spain<sup>9</sup>, Lausanne University Hospital and University of Lausanne, Switzerland, Hematology Service,, Lausanne, Switzerland<sup>10</sup>, University Children's Hospital Zurich, Zurich, Switzerland, Oncology Laboratory,, Zurich, Switzerland<sup>11</sup>, Lausanne University Hospital and University of Lausanne, Switzerland, Woman-Mother-Child Department, Lausanne, Switzerland, <sup>12</sup>, IRCCS Istituto Giannina Gaslini, Dipartimento Ricerca Traslazionale, Genova, Italy <sup>13</sup>, IRCCS Istituto Giannina Gaslini, Dipartimento Ricerca Traslazionale, Genova, Italy <sup>14</sup>, University of Oslo, Department of Pathology, Oslo University Hospital and Institute of Clinical Medi, Oslo, Norway <sup>15</sup>, Hospital Universitario y Politecnico La Fe , Valencia, Clinical and Translational Research in Cancer. Pediatric Oncology Unit, Valencia, Spain <sup>16</sup>, The Newcastle Upon Tyne Hospitals NHS Foundation Trust, Northern Genetics Service, Newcastle Upon Tyne, United Kingdom <sup>17</sup>, NHS Grampian, Royal Aberdeen Children's Hospital, Aberdeen, Aberdeen, United Kingdom <sup>18</sup>, Oxford University, Oxford Children's Hospital, Oxford, United Kingdom <sup>19</sup>, University Hospital, Catania, Department of Pediatric Hematology-Oncology,, Catania, Italy <sup>20</sup>, Children's Hospital, Agia Sofia, Athens, Greece <sup>21</sup>, Instituto de Investigación Sanitaria La Fe, Valencia, Laboratory of Translational Research in Childhood Cancer, Barcelona, Spain <sup>22</sup>, Institut Curie, Département d'Oncologie Pédiatrique, Paris, France <sup>23</sup>, Newcastle University, Translational & Clinical Research Institute, Newcastle Upon Tyne, United Kingdom <sup>24</sup>

### **Background/Introduction:**

Children with intermediate-risk neuroblastoma (20% of all NB) have an 80% 5-year survival but this is a heterogeneous group including a subgroup aged over 18 months at diagnosis with localised, unresectable disease (L2), without amplification of the *MYCN* oncogene and unfavourable histology according to the International Neuroblastoma Pathology Classification (INPC), which have a poorer outcome Within the SIOPEX current low and intermediate neuroblastoma (LINES) trial these patients termed Group 8 are treated with conventional chemotherapy, surgery, local radiotherapy and 13 cis retinoic acid while other protocols treat them, as high risk.

### **Aims:**

To investigate the genetics of Group 8 (G8) tumours and to determine if a subgroup with a poorer prognosis can be identified which in the future could be re-classified as high risk and treated accordingly.

### **Methods/Materials:**

Copy number analysis of 48 G8 diagnostic tumours from 7 countries participating in the LINES trial and the UK was performed from single-nucleotide polymorphism array (SNPa), array-CGH, MLPA, and FISH to detect numerical (NCA) and segmental chromosomal aberrations (SCA). Raw SNPa (Illumina CytoSNP-850k array or Affymetrix CytoScan HD array) copy number data was obtained and

analysed by Nexus software (BioDiscovery,CA, USA) in 10 cases. Processed data was analysed for the remaining cases. Whole-exome sequencing (WES) (Illumina Truseq Rapid Exome Library Prep Kit) to a read depth of 100x run on an Illumina NextSeq 500 or NovaSeq 6000 flow cell with sequence reads analysed using a GATK pipeline, and SNP arrays were conducted on 8 paired UK G8 cases for analysis of SCAs and mutations acquired at relapse ( 1 case 3 relapses). The cohort had a mean age at diagnosis of 51.0 months (range 18.3-204.5).

#### Results:

33/48 (68.8%) profiles contained 'typical' SCAs found in neuroblastoma. 13/48 profiles (27.1%) were NCA only, 2/48 (4.1%) profiles only had atypical SCAs. A mean of 2.47 SCAs (range 1-21) were observed with the following SCAs, in order of frequency: 17q gain (43.8%; n=21); 11q loss (41.7%; n=20); 2p gain (27.1%; n=13); 1q gain (20.8%; n=10); 1p loss (14.6%; n=7); 4p loss (14.6%; n=7) and 3p loss (10.4%; n=5). Gene amplifications were identified in 2 patients, both with SCA profiles. One case had 3 amplicons on chromosome 8: 8p(36,53-37,11Mb); 8q(94,84-95,15Mb) and 8q(128,52-128,77Mb) including *MYC*. One case had *TERT* amplification with additional 16q(72,83-73,30Mb) amplicon containing *ZFX3*. In 7/8 paired cases there was a change in copy number profile between diagnosis and relapse. In 5 cases an increase in SCAs at relapse and in 2 cases a decrease. *ALK* gene mutations were found in 4/11 (36%) post chemotherapy or relapsed tumours by WES and Sanger sequencing with one case switching from an F1174L mutation post chemotherapy to an R1275Q mutation at first relapse, before returning to the F1174L mutation in 2 subsequent relapse samples.

#### Summary/Conclusions:

This ongoing SIOPEN Biology study reports a high frequency of SCA and novel amplicons in G8 tumours. WES is underway together with an investigation of telomere maintenance mechanisms to link with clinical outcome and help guide future allocation to treatment groups.

## **P153 INDIA NEUROBLASTOMA REGISTRATION AND BIOLOGY STUDY (INPOG-NB-18-01): IMPROVING OUTCOME FOR PATIENTS WITH NEUROBLASTOMA IN A LOWER-MIDDLE INCOME COUNTRY**

*Kanwar, V.S.<sup>2</sup>, Bhattacharya, A.<sup>3</sup>, Arora, R.S.<sup>4</sup>, Howard, S.C.<sup>5</sup>, Shah, P.S.<sup>6</sup>*

CIMS Hospital, Pediatric hematology-oncology, Ahmedabad, India <sup>2</sup>, Tata Medical Center, Pediatric hematology-oncology, Kolkata, India<sup>3</sup>, Max Healthcare Saket, Pediatric hematology-oncology, New Delhi, India<sup>4</sup>, University of Tennessee Health Science Center, Assoc. Dean for Research College of Nursing, Memphis, University of Tennessee Health Science Center<sup>5</sup>, Dartmouth Hitchcock Medical Center, Medical oncology, Lebanon, Nh, USA<sup>6</sup>

### Background/Introduction:

Neuroblastoma (NB) is a common childhood cancer, but India has an annual incidence rate one-third that of expected (4.3/million children 0-14 yrs age per year), with 75-90% of patients presenting at an advanced stage. It is unclear whether this represents selection bias. Outcome data for patients with NB who undergo therapy, or have associated complications, are lacking in the absence of a national neuroblastoma registry. Risk stratification of NB in India and clinical management is affected by the lack of genetic testing for N-MYC amplification and other validated markers, and India lacks an effort to systematically analyse NB using next generation sequencing (NGS), such as utilized by the MAPPYACTS study in France and the NANT consortium in the USA, to allow for translational research. This prospective trial is an effort to generate all of these data.

### Aims:

The aims of this study are two-fold. An online registry will be used to collect data on the incidence and stage of patients with NB in India and their clinical outcomes, including complications such as opsoclonus-myoclonus syndrome and spinal cord compression. Genetic testing on NB tumors using NGS will help risk stratify patients for clinical management, as well as for soon-to-be-opened InPOG NB clinical trials, and document the genomic landscape of NB in India, creating a biobank of NB tumour DNA to allow meaningful translational research.

### Methods/Materials:

The study was designed by a pediatric oncology committee within India, peer-reviewed within and outside India, and registered with the Indian Pediatric Oncology Group (InPOG). A custom designed online database housed in the Resonance platform, will allow collection of clinical and staging data of patients with NB (<http://www.Resonanceoncology.org>). Formalin-fixed paraffin embedded tumor slides (10 unstained, 2 H&E) are couriered to Neuberger Supratech in Ahmedabad where NGS is performed with the OncoPrint Childhood Cancer Assay. N-MYC amplification and ploidy status are returned to institutions within 3 weeks. The report includes fluorescent in situ hybridization (FISH) for 1p and 11q deletions, to provide meaningful data for risk stratification. The total cost of analysis is approximately USD \$25 per patient. Variant genomic data on all NB samples is stored at Neuberger in cBioportal for further analysis. The goal is to enroll 300 patients with NB over 3 years, and 20 institutions in India have expressed interest. Funding support was gratefully received from Cankids...kidscan, (<http://www.cankidsindia.org>), a childhood cancer support organization.

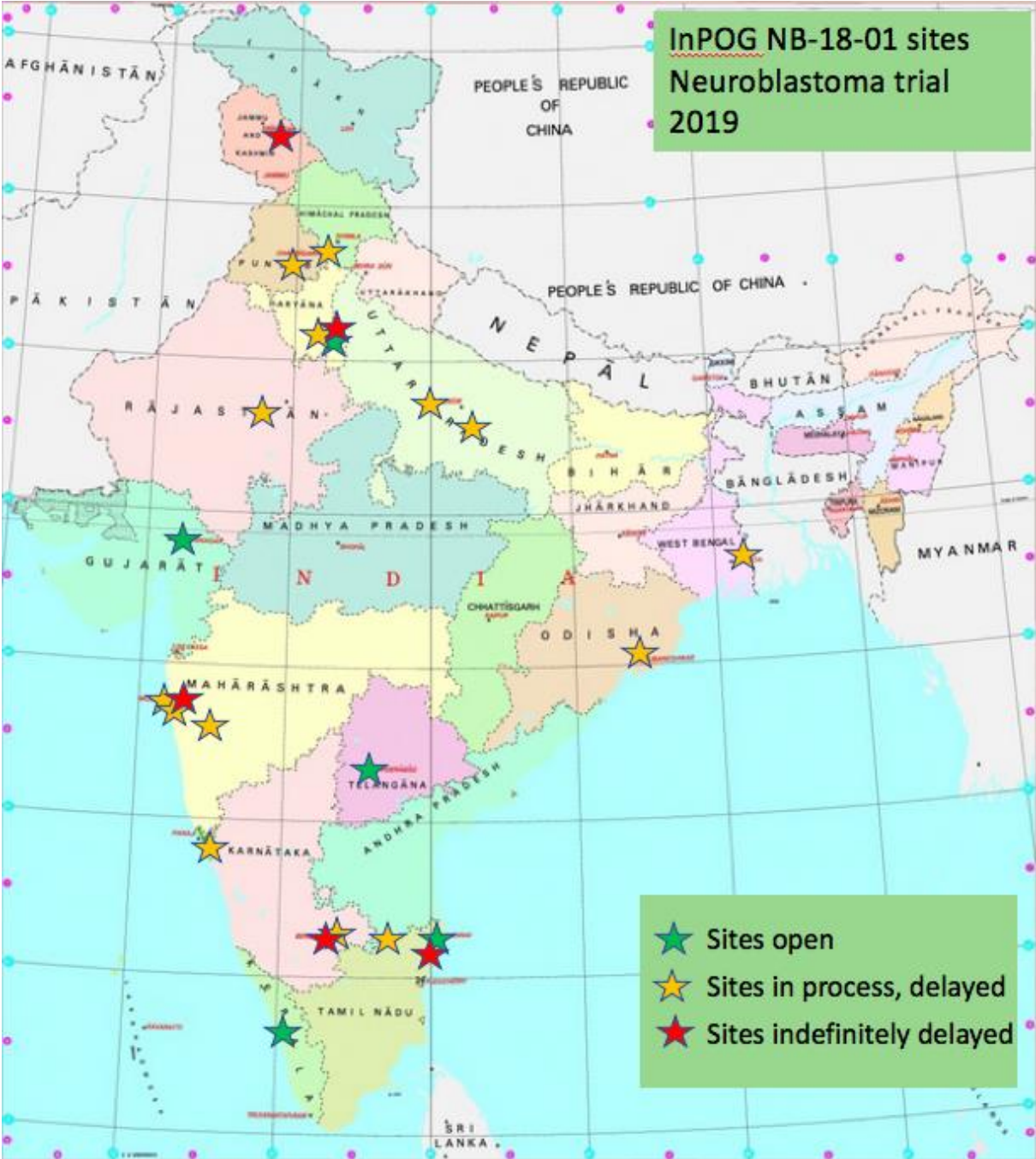
### Results:

Five institutions already have ethics committee approval and have begun enrollment, and despite delays in opening the study due to lack of investigator protected time, sufficient research staff, or a centralized ethics review board, we expect to meet our accrual target by 2023.

### Summary/Conclusions:

This study demonstrates it is possible to bring together organizations and individuals in a lower-middle income country such as India, to create a clinical trial network to register patients with NB to better

define outcomes, and to analyze NB tumor biology to allow improved risk stratification, with an overall goal of improving NB outcomes.



Caption 1: InPOG NB-18-01 trial sites India

**P154 Bim protein-protein interactions provide predictive biomarkers for Bcl2-family inhibitor responses in neuroblastoma**



*Zapata, C.*<sup>2</sup>, *Liu, K.*<sup>3</sup>, *Goutnik, M.*<sup>4</sup>, *Bodducherla, R.*<sup>5</sup>, *Coku, J.*<sup>6</sup>, *Vemu, R.*<sup>7</sup>, *Vu, A.*<sup>8</sup>, *Reynolds, C. P.*<sup>9</sup>, *Goldsmith, K.*<sup>10</sup>, *Hogarty, M.*<sup>11</sup>

Children's Hospital of Philadelphia, Pediatric Oncology, Philadelphia, USA<sup>2</sup>, Children's Hospital of Philadelphia/University of Pennsylvania, Pediatric Oncology, Philadelphia, USA<sup>3</sup>, Children's Hospital of Philadelphia/University of Pennsylvania, Pediatric Oncology, Philadelphia, USA<sup>4</sup>, Children's Hospital of Philadelphia/University of Pennsylvania, Pediatric Oncology, Philadelphia, Children's Hospital of Philadelphia/University of Pennsylvania<sup>5</sup>, Children's Hospital of Philadelphia/University of Pennsylvania, Pediatric Oncology, Philadelphia, USA<sup>6</sup>, Children's Hospital of Philadelphia/University of Pennsylvania, Pediatric Oncology, Philadelphia, USA<sup>7</sup>, Children's Hospital of Philadelphia/University of Pennsylvania, Pediatric Oncology, Philadelphia, USA<sup>8</sup>, Texas Tech University Health Sciences Center, Pediatric Oncology, Lubbock, USA<sup>9</sup>, Children's Healthcare of Atlanta/Emory University, Pediatric Oncology, Atlanta, USA<sup>10</sup>, Children's Hospital of Philadelphia/University of Pennsylvania, Pediatric Oncology, Philadelphia, USA<sup>11</sup>

#### Background/Introduction:

Chemotherapy induced cell death is governed by the Bcl2-protein family. Effective therapies activate death-domain proteins (like Bim) that engage Bak or Bax, causing cell death. In cancers, these death-domain proteins are often sequestered by survival proteins like Bcl2 or Mcl1 instead. This preserves viability but makes such cells continually dependent on this function. Drugs that competitively displace death-proteins from these survival proteins can restore apoptosis. Such Bcl2-family inhibitors show great promise to impact cancer outcomes. Venetoclax, a Bcl2 inhibitor, is FDA-approved and many other drugs in this class are advancing in the clinic. However, no predictive biomarkers exist to guide their development.

#### Aims:

We sought to identify the mechanisms underlying neuroblastoma survival dependencies, and develop predictive biomarkers to guide the clinical use of emerging Bcl2-family inhibitors.

#### Methods/Materials:

Neuroblastoma cell lines and PDXs were studied, including paired samples from the same patients at different times in therapy and/or different anatomic sites. Tumor models were studied using: mitochondrial profiling (probing tumor-isolated mitochondria with diverse stress proteins) to infer their survival dependency; cytotoxicity assays after exposure to ABT199 (BCL2-inhibitor, aka BCL2i), ABT737 (BCL2/BCLXi), A1155463 (BCLXi), and S63845 (MCL1i) alone and in combination; and colP to identify key Bcl2-family protein:protein interactions (PPIs) that correlate with inhibitor sensitivity.

#### Results:

We identified two patterns of mitochondrial profiles: the first predicts a dependency on Bcl2, and all such tumors are sensitive to BCL2i's in vitro (IC<sub>50</sub><1uM) and in vivo, but not other inhibitors. In each, we found Bim bound by Bcl2 using colP (not Mcl1). The mechanism of BCL2i activity in "Bim:Bcl2" tumors is displacement of Bim, freeing it to activate Bak/Bax. The second mitochondrial profile predicts an Mcl1 dependency, and in each Bim was bound by Mcl1 (not Bcl2). Surprisingly, "Bim:Mcl1" tumors are insensitive to MCL1i's (IC<sub>50</sub>>2uM). Our colPs show Bim is displaced from Mcl1 by the drug, but re-sequestered by Bclx, and these tumors are exquisitely sensitive to inhibition of both Mcl1 and Bclx (IC<sub>50</sub><50nM), fully predicted by Bim PPIs. Seven sets of patient-matched tumors from distinct sites and times had identical Bim PPIs, supporting this as a stable actionable tumor feature. Neither *MYCN* nor *ALK* mutation status correlated with Bcl2 or Mcl1 survival dependency, yet all tumors harboring *MAPK* mutations (*PTPN11*, *RAS*, *RAF*, *NF1*) were in the Bim:Mcl1 class. Genetically-activating *RAS* (via *NF1* knock-down) drives Bim from Bcl2 to Mcl1, with the predicted consequences on inhibitor sensitivity.

#### Summary/Conclusions:

We show that neuroblastomas sequester Bim to stay alive, and drugs that selectively displace Bim provide a lethal stress. Our work decodes these survival interactions to provide numerous opportunities to develop predictive biomarkers, and to optimize the clinical efficacy of these agents, particularly as our Bim PPI-defined survival dependency appears stable and cell intrinsic. Our work also defines a hierarchical Mcl1-Bclx dependency and links *MAPK* activation to this class, and are testing *MAPK* inhibitors for their potential to synergize with Bclx inhibition, as predicted by our data.

## **P155 CircARID1A is an oncogenic circular RNA and regulates neuroblastoma differentiation states**

*Fuchs, S.*<sup>2</sup>, *Klironomos, F.*<sup>3</sup>, *Danssmann, C.*<sup>4</sup>, *Naderi, J.*<sup>5</sup>, *Winkler, A.*<sup>6</sup>, *Luz, U.*<sup>7</sup>, *Hundsdoerfer, P.*<sup>8</sup>, *Eggert, A.*<sup>9</sup>, *Toedling, J.*<sup>10</sup>, *Hertwig, F.*<sup>11</sup>, *Schulte, J.H.*<sup>12</sup>

Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology, Berlin, Germany <sup>2</sup>, Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology, Berlin, Germany<sup>3</sup>, Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology, Berlin, Germany<sup>4</sup>, Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology, Berlin, Germany<sup>5</sup>, Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology, Berlin, Germany<sup>6</sup>, Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology, Berlin, Germany<sup>7</sup>, Helios Klinikum Berlin-Buch, Department of Pediatric Oncology, Berlin, Germany<sup>8</sup>, Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology, Berlin, Germany<sup>9</sup>, Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology, Berlin, Germany<sup>10</sup>, Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology, Berlin, Germany<sup>11</sup>, Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology, Berlin, Germany, <sup>12</sup>

### Background/Introduction:

Circular RNAs (circRNAs), a noncoding RNA class originating from alternative splicing, are highly abundant in neural tissues and were shown to regulate gene expression e.g. by interacting with microRNAs and RNA-binding proteins. Neuroblastoma is an embryonal neoplasia, which arises from undifferentiated neural-crest cells. Recent data suggest that neuroblastoma is composed of a mesenchymal and an adrenergic differentiation state. Previously, we have sequenced whole transcriptomes of 104 primary neuroblastoma and identified 26 neuroblastoma-specific circRNAs, including a circRNA derived from the *ARID1A* tumor suppressor gene.

### Aims:

Here, we aimed to elucidate the role of circARID1A in neuroblastoma biology and the functional mechanism behind it.

### Methods/Materials:

The abundance of circARID1A was investigated in a neuroblastoma cell line panel by specific qRT-PCR and Northern blot. Overexpression and knockdown models were established to explore the impact of circARID1A gain or loss on cell viability, proliferation, apoptosis and differentiation. To further investigate the molecular changes upon circARID1A knockdown, RNA sequencing was performed. The subcellular localization of circARID1A was assessed by RNA FISH. An RNA pulldown assay and bioinformatics motif analysis were performed to identify interaction partners of circARID1A.

### Results:

CircARID1A was validated and quantified in 10 neuroblastoma cell lines of different genetic background. It was found to be less abundant than the linear *ARID1A* mRNA, but had a longer half-life after blocking transcription with actinomycin D. Moreover, circARID1A proved resistant against exonuclease degradation, thus confirming circularity of the molecule. Cell fractionation and RNA FISH revealed a cytoplasmatic localization of circARID1A. We detected on average 2-5 transcripts per cell, whereas for the linear *ARID1A* mRNA the number was at least 2-3 fold higher. Specific circARID1A knockdown resulted in reduced proliferation, cell numbers and viability, and prompted apoptosis. The outgrowth of neurite-like extensions and up-regulation of differentiation markers were observed. Neither knockdown nor overexpression of circARID1A affected *ARID1A* mRNA and protein levels. RNA sequencing after knockdown confirmed the specificity of the knockdown, as circARID1A was the only circRNA significantly down-regulated and the linear *ARID1A* mRNA expression was unchanged. Moreover, among the genes, which showed significant expression changes upon circARID1A knockdown, there was an upregulation of adrenergic (e.g. *DLK1*, *ALK*) and a suppression of mesenchymal (e.g. *PRRX1*, *SEMA3C*) marker genes. We computationally identified several binding

sites of microRNAs and RNA-binding proteins enriched in the circARID1A sequence. An RNA pulldown assay strongly and specifically enriched circARID1A in lysates of the neuroblastoma cell line IMR-5. Mass spectrometry and RNA sequencing are currently being performed to identify RNA and protein interaction partners of circARID1A in order to validate *in silico* predictions.

#### Summary/Conclusions:

We demonstrated that circARID1A, a circRNA that is derived from the *ARID1A* tumor suppressor gene is specifically upregulated in neuroblastoma tumor tissues and acts in a tumor-promoting manner in neuroblastoma cell lines, independent of linear mRNA transcripts. We showed that a knockdown of circARID1A induces differentiation and expression of adrenergic markers, indicating a potential role of circARID1A in maintaining the mesenchymal neuroblastoma state. This study highlights an important role of circRNAs in neuroblastoma biology and may establish this RNA class as future therapeutic targets.

## **P156 Detection of Alternative Lengthening of Telomeres Phenotype via C-Circle Assay in EDTA and Heparinized Plasma**

*Burrow, T.<sup>2</sup>, Barr, E.<sup>3</sup>, Macha, S.<sup>4</sup>, Reynolds, C.P.<sup>5</sup>*

Texas Tech University Health Sciences Center, Pediatrics, Translational Neuroscience and Pharmacology, Lubbock, USA <sup>2</sup>, Texas Tech University Health Sciences Center, Pediatrics, Lubbock, USA<sup>3</sup>, Texas Tech University Health Sciences Center, Pediatrics, Cell Biology and Biochemistry, Lubbock, USA<sup>4</sup>, Texas Tech University Health Sciences Center, Cancer Center, Pediatrics, Lubbock, Texas Tech University Health Sciences Center<sup>5</sup>

### **Background/Introduction:**

The alternative lengthening of telomeres (ALT) mechanism is present in approximately 20% of high-risk neuroblastoma tumors, conferring an indolent, but often lethal prognosis. C-circles, self-primed circular telomeric DNA repeats, are a sensitive and specific biomarker for ALT+ cancers. The C-circle assay (CCA) identifies a subset of neuroblastoma patients with a uniformly poor clinical outcome. Methods for identifying ALT+ neuroblastoma patients will be useful for identifying patients for participation in clinical trials of drugs with high activity against ALT+ neuroblastoma. Plasma-derived cell-free DNA (cfDNA) has been shown to be a potentially useful biomarker in many cancers, including neuroblastoma. We hypothesized that C-circles could be detected in cfDNA collected in both EDTA and heparinized tubes to identify ALT+ neuroblastoma patients.

### **Aims:**

### **Methods/Materials:**

Plasma was collected in EDTA and lithium heparin BD Vacutainer tubes, and cfDNA was extracted using the QIAamp Circulating Nucleic Acid Kit. The CCA employs phi29 DNA polymerase rolling cycle amplification (RCA), which selectively enriches the telomeric C-circles. Subsequently, real-time PCR amplification of telomere content was normalized to the VAV2 single copy gene and compared to non-RCA controls.

### **Results:**

ALT+ DNA was seeded into 2 ml of fresh plasma using serial dilutions (1000, 100, 10, and 1 ng/ml). C-circles were readily detected down to 10 ng/ml in plasma using EDTA as an anticoagulant. The CCA failed in heparinized samples, likely, as heparin is a polymerase inhibitor. C-circles, however, could be detected in heparinized samples that were pre-treated with ecteola cellulose (50-55mg) to remove heparin. Use of Bacteroides Heparinase I (24 units) did not overcome heparin inhibition of CCA. Results are shown in Table 1. The CCA was positive in bone marrow plasma with metastatic neuroblastoma that grew a CCA+ neuroblastoma cell line and in peripheral blood plasma from a patient with an *ATRX*-mutant neuroblastoma.

### **Summary/Conclusions:**

The CCA was positive with as low as 10 ng/ml of ALT+ DNA in plasma cfDNA obtained in EDTA and ecteola cellulose pre-treated heparinized samples. Heparin depletion diminished sensitivity of the assay and required larger CCA reaction volumes. Detection of C-circles in plasma provides an alternative to obtaining tumor tissue in order to identify ALT+ neuroblastoma patients. Use of EDTA as an anticoagulant is optimal, but heparin depletion enables use of the CCA in heparinized plasma, though with diminished sensitivity.

<b>Serial Dilution (ng/ml)</b>	<b>EDTA 10ul CCA</b>	<b>Heparinized 10ul CCA</b>	<b>Heparinized Cellulose 10ul CCA</b>	<b>Heparinized Cellulose 25ul CCA</b>	<b>Heparinized Heparinase 10ul CCA</b>
1000	+	-	+	+	-
100	+	N/A	+	+	N/A
10	+	N/A	-	+	N/A
1	-	N/A	-	-	N/A

Caption 1: TABLE 1: C-Circle Detection of cfDNA in EDTA and Heparinized Plasma

## **P157 MYCN negatively regulates starvation-induced autophagy through upregulating miR19**

*An, Z.*<sup>2</sup>, *Chanthery, Y.*<sup>3</sup>, *Menard, M.*<sup>4</sup>, *McHenry, L.*<sup>5</sup>, *Nasholm, N.*<sup>6</sup>, *Wong-Michalak, S.*<sup>7</sup>, *Winchell, M.*<sup>8</sup>, *Weiss, W.*<sup>9</sup>

UCSF, Neurology, San Francisco, USA <sup>2</sup>, UCSF, Neurology, San Francisco, USA<sup>3</sup>, UCSF, Neurology, San Francisco, USA<sup>4</sup>, UCSF, Neurology, San Francisco, UCSF<sup>5</sup>, UCSF, Neurology, San Francisco, USA<sup>6</sup>, UCSF, Neurology, San Francisco, USA<sup>7</sup>, UCSF, Neurology, San Francisco, USA<sup>8</sup>, UCSF, Neurology, San Francisco, USA<sup>9</sup>

### Background/Introduction:

*MYCN* is known to regulate expression of many microRNAs. However, how *MYCN* regulates cell growth and survival through microRNAs remains unclear. Autophagy is a key pathway for cell survival under starvation and other stressors. Here we reported that *MYCN* negatively regulates expression of a large group of autophagy genes and of starvation-induced autophagy, through miR-17 family member miR-19.

### Aims:

Aim 1: To dissect the function of *MYCN* in regulating autophagy.

Aim 2: To understand how miR-19 links *MYCN* and autophagy.

Aim 3: To design new therapeutics targeting the *MYCN*-miR-19-autophagy cascade.

### Methods/Materials:

SHEP-Control, SHEP-*MYCN*, Kelly, IMR32, SMS-KCN-R, SY5Y, CHLA20 and SKNSH cells were used in this study. Trypan blue, cell titer glo and flow cytometry assays were performed to measure cell growth and survival. Western blotting and qRT-PCR were performed to determine protein and mRNA levels. Neuroblastoma patient survival and mRNA expression levels in tumors were obtained from R2. For mouse experiments, *TH-MYCN* mice were treated twice a week, for 2 weeks with low dose anti-miR19 (10mg/kg) or vehicle. C57BL/6 mice bearing orthotopic *MYCN*-driven xenografts were treated every 3 days with low doses anti-miR19 LNA (1.5mg/kg) or vehicle for 5 doses.

### Results:

Compared to SHEP-Control cells, SHEP-*MYCN* cells were more sensitive to starvation-induced apoptosis, more resistant to starvation-induced autophagy, and hypersensitive to inhibition of autophagy regulator ULK1. The results were confirmed in additional *MYCN*-amplified and non-amplified neuroblastoma cell lines. In neuroblastoma patient samples in the R2 database, expression levels of a large group of autophagy genes correlated inversely with those of *MYCN* and positively with survival. Moreover, cells with amplification or overexpression of *MYCN* expressed significantly lower levels of autophagy proteins. We found binding sequences for miR-17 family microRNAs miR-19 on the 3'-UTRs of those autophagy genes. In addition, miR-19 was transcriptionally upregulated by *MYCN*. Anti-miR-19 treatment prolonged survival of *TH-MYCN* mice and C57BL/6 mice bearing orthotopic *MYCN*-driven xenografts, and induced autophagy in the tumors.

### Summary/Conclusions:

Our findings suggest that autophagy, commonly held as a survival pathway, plays a surprising and critical role in inhibiting the growth and survival of *MYCN*-amplified neuroblastoma. Our study also clarifies a role for *MYCN* in regulating autophagy, and suggests both miR19 and the autophagy pathway as therapeutic pathways/targets for patients with *MYCN* amplified high-risk neuroblastoma.

## **P158 Inhibition of Exportin-1 as part of combinatorial treatment for patients with high-risk neuroblastoma**

*Galinski, B.*<sup>2</sup>, *Luxemburg, M.*<sup>3</sup>, *McKnight, J.*<sup>4</sup>, *Landesman, Y.*<sup>5</sup>, *Weiser, D.*<sup>6</sup>

Albert Einstein College of Medicine, Pediatrics, Bronx, USA <sup>2</sup>, Albert Einstein College of Medicine, Pediatrics, Bronx, USA<sup>3</sup>, Albert Einstein College of Medicine, Pediatrics, Bronx, USA<sup>4</sup>, Karyopharm Therapeutics, Research & Translational Medicine, Newton, Karyopharm Therapeutics<sup>5</sup>, Albert Einstein College of Medicine, Pediatrics, Bronx, USA<sup>6</sup>

### Background/Introduction:

In many adult cancers, upregulation of the nuclear-to-cytoplasmic transport protein Exportin-1 (XPO1) correlates with poor outcome and responsiveness to selinexor, an FDA-approved XPO1 inhibitor. Similar data are emerging in childhood cancers, for which selinexor is being evaluated in early phase clinical studies. We hypothesize that selinexor and the proteasome inhibitor, bortezomib, act synergistically to decrease NF- $\kappa$ B-mediated proliferation by halting excessive exportation and proteasome degradation of I $\kappa$ B, an XPO1 cargo protein.

### Aims:

Our aims are to identify patients most likely to benefit from selinexor combination therapy and define the cellular mechanism responsible for this effect.

### Methods/Materials:

We performed ion intensity-based label-free semiquantitative proteomic profiling on primary tumor material from fifty patients with uniformly treated high-risk NB, half of whom were long term survivors and half of whom died <18 months from diagnosis from disease progression. We cross-referenced differentially abundant proteins with gene expression profiling data available through the R2 database. A panel of neuroblastoma cell lines was screened for XPO1 expression using Western blotting and sensitivity to selinexor and bortezomib as single agents and in combination using cellular viability assays. Localization and quantification of I $\kappa$ B after vehicle or selinexor treatment was done by confocal imaging, using immunofluorescent labeling and Volocity software. Flow cytometry was used to define treatment effect on cell cycle arrest and apoptosis using BrdU and Annexin-V labeling, respectively. siRNA-mediated knockdown of I $\kappa$ B was used to investigate the relationship between presence of I $\kappa$ B and drug effectiveness. The downstream effect on NF- $\kappa$ B activity was measured with a luciferase assay.

### Results:

High tumor protein and gene expression of XPO1 correlates with poor outcome. Neuroblastoma cell lines demonstrate variably high XPO1 expression compared to normal tissue, as well sensitivity to selinexor (IC<sub>50</sub> range 4-300nM). When selinexor is used in combination with bortezomib the effect on cell proliferation is synergistic. Flow cytometry demonstrates that selinexor contributes to G1-0 arrest while combination treatment results in significantly enhanced apoptosis. I $\kappa$ B is retained in the nuclear compartment after treatment with selinexor, and siRNA against I $\kappa$ B diminishes the effect of single and combination treatment. Measurement of NF- $\kappa$ B transcriptional activity is in progress.

### Summary/Conclusions:

This work demonstrates that XPO1 expression is a biomarker of highly aggressive neuroblastoma and may define patients most likely to be responsive to treatment with selinexor-based combinatorial therapies. Synergistic treatment effect with selinexor and bortezomib is mediated, in part, through I $\kappa$ B and independent of *MYCN* copy number and *TP53* status.



## **P159 Loss of function of ATRX associates with an inflammatory phenotype in neuroblastoma isogenic cell lines and xenografts**

*Lorenzi, F.*<sup>2</sup>, *George, S.*<sup>3</sup>, *Gao, Q.*<sup>4</sup>, *Martins da Costa, B.*<sup>5</sup>, *Tall, J.*<sup>6</sup>, *Chesler, L.*<sup>7</sup>

The Institute of Cancer Research, Paediatric Tumour Biology, London, United Kingdom<sup>2</sup>, The Institute of Cancer Research, Paediatric Tumour Biology, London, United Kingdom<sup>3</sup>, The Institute of Cancer Research, Bioinformatics Core Facility, London, United Kingdom<sup>4</sup>, The Institute of Cancer Research, Paediatric Tumour Biology, London, The Institute of Cancer Research<sup>5</sup>, The Institute of Cancer Research, Paediatric Tumour Biology, London, United Kingdom<sup>6</sup>, The Institute of Cancer Research, Paediatric Tumour Biology, London, United Kingdom<sup>7</sup>

### Background/Introduction:

Neuroblastoma patients carrying loss of function (LoF) mutations of *ATRX* present with slowly-growing disease that often does not respond to conventional chemotherapy. *ATRX* mutant neuroblastoma cells exploit a strategy called alternative lengthening of telomeres (ALT) to regularly extend the telomeric regions of their chromosomes thus escaping senescence and/or apoptosis through mechanisms largely uncharacterised. Elucidating the mechanisms that are responsible for growth and maintenance of *ATRX* mutant neuroblastoma is urgently needed to identify new therapeutic strategies for this subgroup of high-risk patients.

### Aims:

To improve our understanding about the effects of *ATRX* LoF in neuroblastoma in order to identify new therapeutic strategies and improve survival of these patients who frequently relapse following conventional chemotherapy.

### Methods/Materials:

CRISPR-Cas9 genetic engineering was used to induce frame-shift alterations in *ATRX* in SK-N-SH and NBL-S cell lines (non-*MYCN* amplified, non-ALT activated). Single cells transiently transfected were flow-sorted and a total of ten *ATRX* mutant cell lines were obtained. Loss of *ATRX* expression was validated by western blot and qRT-PCR. Activation of ALT, found in neuroblastoma carrying LoF mutations of *ATRX*, was analysed in the isogenic cell lines by c-circle assay.

Next, RNA sequencing and bioinformatic analysis of the isogenic cell lines was performed to identify common deregulated pathways that were shared among all *ATRX* mutant cell lines. Resulting deregulated pathways were validated *in vitro* and further confirmed *in vivo* by immunohistochemistry in both xenografts generated from the isogenic cell lines and in patient derived xenograft models.

Lastly, publicly available RNA sequencing data of primary neuroblastomas were utilised to further confirm our results.

### Results:

Three *ATRX* knock-out SK-N-SH and seven *ATRX* knock-out NBL-S cell lines were obtained by using CRISPR-Cas9, following inactivation of TP53. LoF of *ATRX* is sufficient to activate ALT in NBL-S isogenic cell lines but not in SK-N-SH isogenic cells.

By RNA sequencing and gene set enrichment analysis (GSEA) of *ATRX* mutant cell lines, we found that *ATRX* LoF associates with up-regulation of inflammatory response pathways in isogenic cell lines derived from both SK-N-SH and NBL-S. In xenograft models of the isogenic cell lines and patient-derived material, inactivation of *ATRX* mediates an increased macrophage infiltration in immunocompromised mice.

Interestingly, bioinformatic analysis of publicly available human RNA sequencing data from primary neuroblastoma samples showed up-regulation of inflammation-related pathways in patients with low or null expression of *ATRX* in direct concordance with our findings.

#### Summary/Conclusions:

Our results suggest that LoF of ATRX may mediate an inflammatory phenotype in neuroblastoma by mechanisms currently under investigation. Understanding the role of the immune system in *ATRX* mutant neuroblastoma represents an important opportunity to take into consideration novel combinational therapies.

## **P160 PRMT5 Inhibition Reduced Neuroblastoma Metastasis Independent of MYC Status**

*Wu, J.*<sup>2</sup>, *Huang, L.*<sup>3</sup>, *Zhang, X.*<sup>4</sup>, *Verdejo-Torres, O.*<sup>5</sup>, *Wigglesworth, K.*<sup>6</sup>, *Huang, T.*<sup>7</sup>, *Sallis, B.*<sup>8</sup>, *Rozen, E.*<sup>9</sup>, *Lee, M.*<sup>10</sup>, *Shohet, J.*<sup>11</sup>

UMass medical school, Pediatrics, Worcester, USA <sup>2</sup>, UMass medical school, Pediatrics, Worcester, USA<sup>3</sup>, UMass medical school, Program in Bioinformatics and Integrative Biology, Worcester, USA<sup>4</sup>, UMass medical school, Pediatrics, Worcester, UMass medical school<sup>5</sup>, UMass medical school, Pediatrics, Worcester, USA<sup>6</sup>, UMass medical school, Pediatrics, Worcester, USA<sup>7</sup>, UMass medical school, Graduate School, Worcester, USA<sup>8</sup>, UMass medical school, Pediatrics, Worcester, USA<sup>9</sup>, Nemours Children's Health System, N/A, Wilmington, De, USA<sup>10</sup>, UMass medical school, Pediatrics, Worcester, USA<sup>11</sup>

### Background/Introduction:

Protein arginine methyltransferase 5 (PRMT5), a type II arginine methyltransferase, is the primary methyltransferase generating symmetric dimethylarginine (SDMA). PRMT5 regulates gene expression via dimethylation of arginine residues on histones H4R3, H3R8, and H2AR3 as well as non-histone proteins. Deregulated expression of PRMT5 is found in many cancers and high levels of PRMT5 is linked to cancer progression and poor prognosis. Of note, several PRMT5 inhibitors are currently under evaluation in early phase clinical trials (NCT02783300, NCT03573310). Evaluation of PRMT5 in neuroblastoma multiple patient cohorts reveals both a strong correlation with advanced disease ( $p=2.2e-16$ ) and with worse overall survival ( $p<1.7e-15$ ). Based on these observations, we evaluated the impact of PRMT5 inhibition using *in vitro* and *in vivo* models of neuroblastoma.

### Aims:

1) Test the efficacy of PRMT5 inhibitor in NB cell lines; 2) Validate PRMT5 inhibitor in a mouse xenograft model; 3) Investigate the mechanisms how PRMT5 regulates NB metastasis

### Methods/Materials:

1) analyzing PRMT5 expression using R2 and TARGET databases evaluated; 2) testing PRMT5 inhibitor and PRMT5 knockdown by shRNA in three NB cell lines in cell proliferation and apoptosis assays; 3) testing PRMT5 inhibitor in a mouse renal capsule xenograft model in two NB cell lines; 4) RNA-seq analysis and 5) targeted proteomic approach to interrogate the major signaling proteins in these two NB cell lines treated with either vehicle or PRMT5 inhibitor.

### Results:

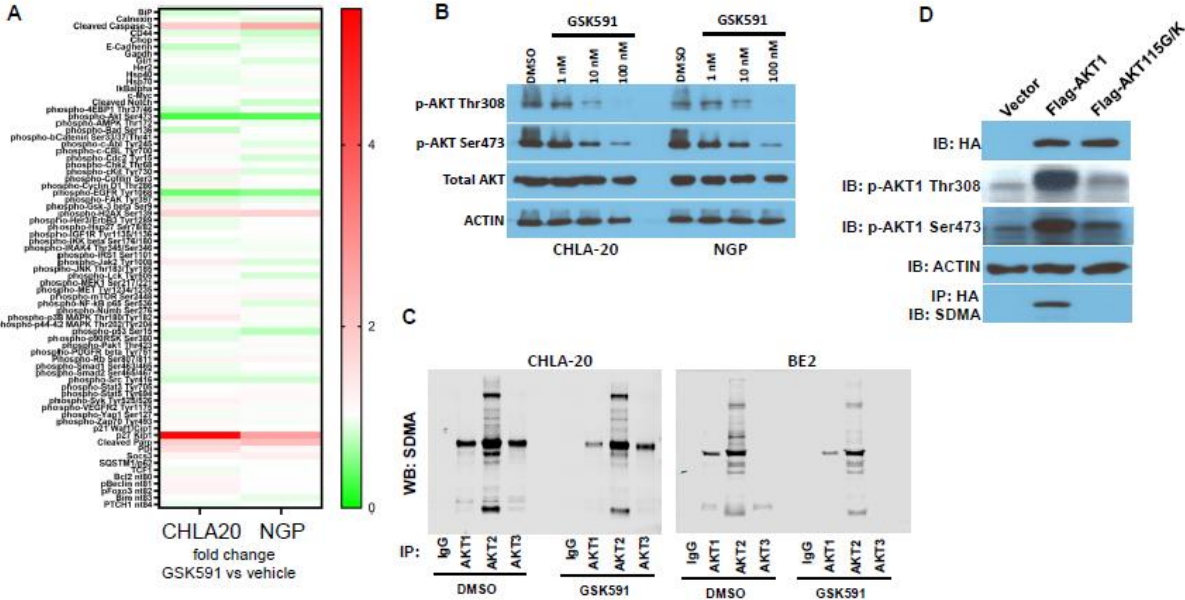
PRMT5 inhibition by a small molecule inhibitor (GSK3203591, a sister compound of GSK3326595 for *in vitro* study) or shRNA targeting PRMT5 reduced cell proliferation and induced apoptosis in both *MYCN* non-amplified (CHLA20) and amplified neuroblastoma cell lines (NGP, SK-N-BE2). In orthotopic xenograft models, GSK3326595 treatment attenuated primary tumor growth and strikingly reduced liver metastases as determined by bioluminescent and fluorescent imaging of livers from mice treated with vehicle or GSK3326595 in CHLA20 and NGP. Transcriptional profiling by RNA-seq in these two cell lines treated with vehicle or GSK3203591 revealed that PRMT5 transcriptionally regulates EGFR. Neuroblastoma cells treated with GSK3203591 failed to activate tyrosine phosphorylation in response to EGF stimulation. Targeted signaling pathway analysis by proteomics in CHLA20 and NGP revealed that PRMT5 inhibition drastically decreased phosphor-AKT levels. We demonstrated that PRMT5 methylated AKT1 at arginine 15 residue and this methylation event is required for AKT activation. Further, we found that EGFR and AKT signaling pathways converge on regulating the expression of master transcription factors TWIST and SNAIL that are responsible for the epithelial-mesenchymal transition and tumor metastasis.

### Summary/Conclusions:

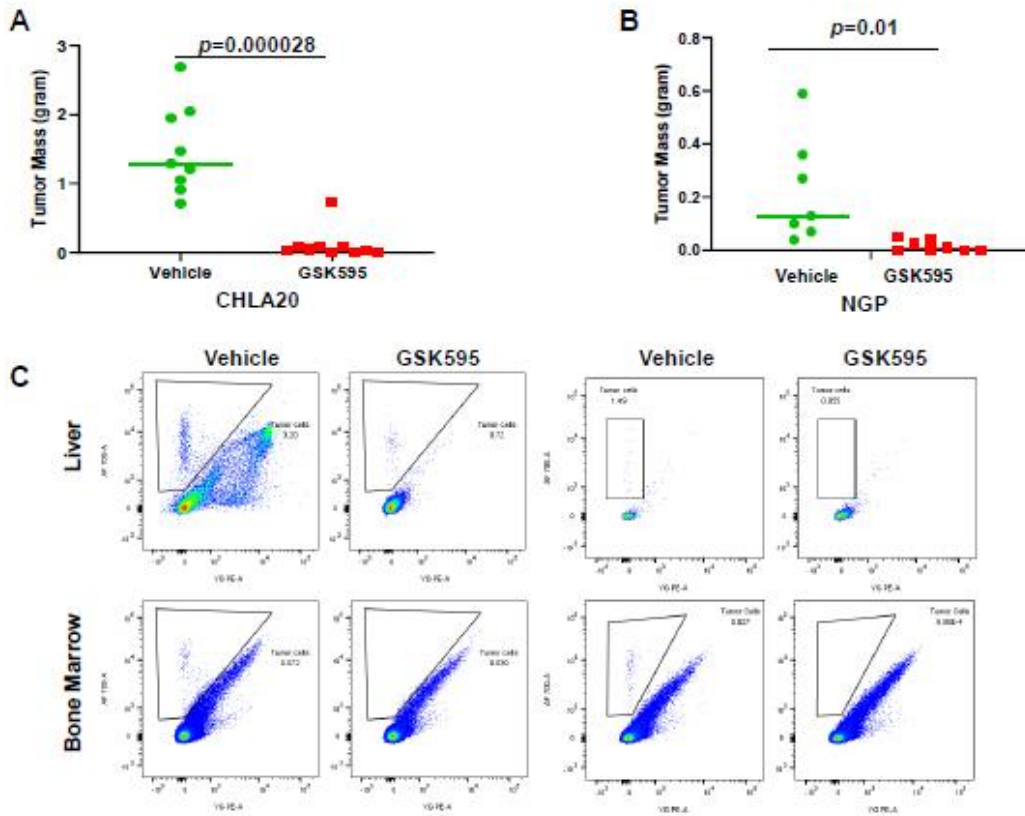
These results illuminate a critical role for PRMT5 regulating EGFR and AKT activation in neuroblastoma. We are pursuing further to define the role of PRMT5 in neuroblastoma pathogenesis

with genetic approach in the neurocrest cells from which the neuroblastoma arises. Together our findings, as well as the ongoing clinical testing of PRMT5 inhibitors in other solid tumors, support further preclinical evaluation of anti-PRMT5 strategies in neuroblastoma for potential translation into the clinic.

Figure 4. PRMT5 modulates AKT activation via methylation



**Figure 2. GSK595 Treatment Reduced Primary Tumor Growth and Metastasis**



**Figure 1. PRMT5 Inhibition Reduced Cell Proliferation & Induces Apoptosis**

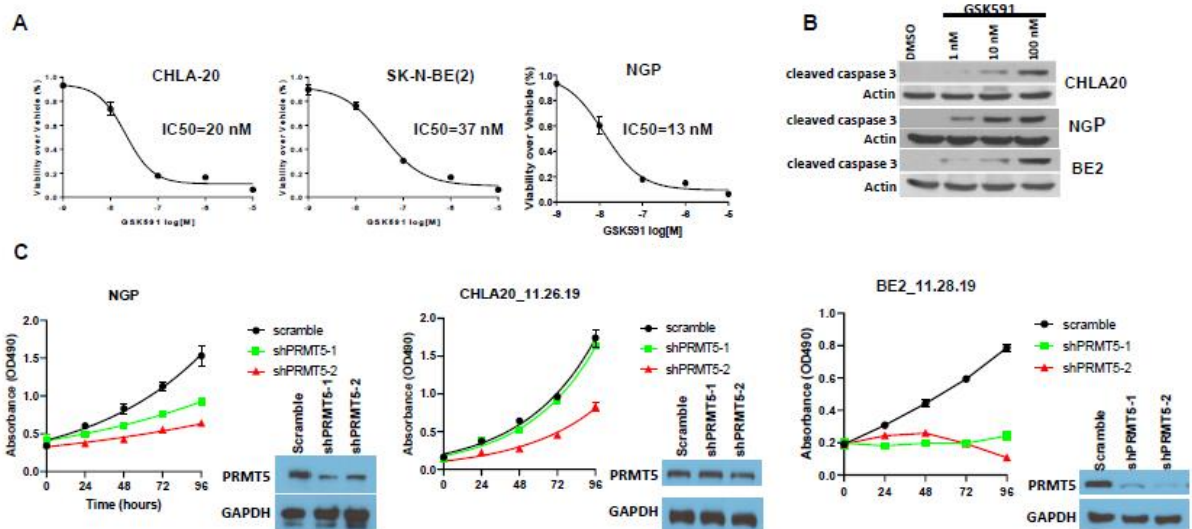
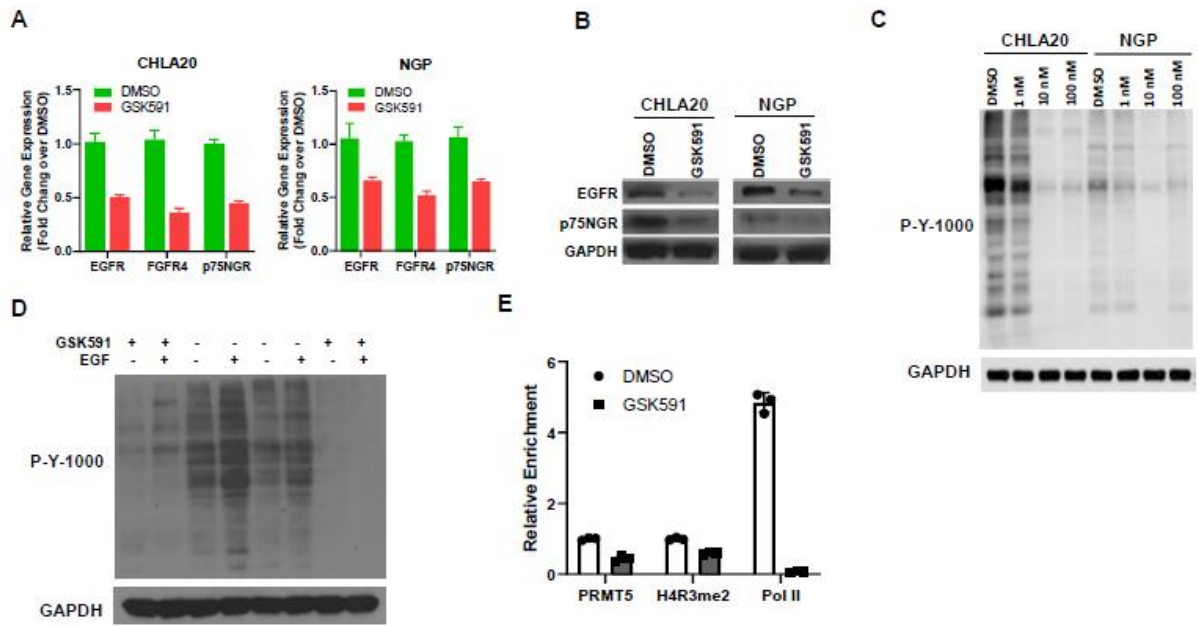


Figure 3. PRMT5 Transcriptionally Regulates EGFR Signaling



## **P161 DISRUPTED ENDOPLASMIC RETICULUM-MITOCHONDRIA INTERACTIONS PROMOTES NEUROBLASTOMA MULTIDRUG RESISTANCE**

*Coku, J.*<sup>2</sup>, *Booth, D.M.*<sup>3</sup>, *Pedrotty, M.*<sup>4</sup>, *Ye, J.C.*<sup>5</sup>, *Scadden, E.*<sup>6</sup>, *Liu, K.*<sup>7</sup>, *Vu, A.*<sup>8</sup>, *Reynolds, C. P.*<sup>9</sup>, *Hajóczky, G.*<sup>10</sup>, *Hogarty, M.D.*<sup>11</sup>

University of Pennsylvania, Cell and Molecular Biology, Philadelphia, USA <sup>2</sup>, Thomas Jefferson University, Department of Pathology, Anatomy & Cell Biology, Philadelphia, USA<sup>3</sup>, Duke University Medical School, Department of Pharmacology and Cancer Biology, Durham, USA<sup>4</sup>, Harvard Medical School, Harvard Medical School, Boston, Harvard Medical School<sup>5</sup>, Harvard Medical School, Department of Stem Cell and Regenerative Biology, Boston, USA<sup>6</sup>, Children's Hospital of Philadelphia, Division of Oncology, Philadelphia, USA<sup>7</sup>, Children's Hospital of Philadelphia, Division of Oncology, Philadelphia, USA<sup>8</sup>, Texas Tech University Health Sciences Center, SCHOOL OF MEDICINE CANCER CENTER, Lubbock, USA<sup>9</sup>, Thomas Jefferson University, Department of Pathology, Anatomy & Cell Biology, Philadelphia, USA<sup>10</sup>, Children's Hospital of Philadelphia, Division of Oncology, Philadelphia, USA<sup>11</sup>

### Background/Introduction:

Many neuroblastoma patients die from progression of multidrug resistant disease, the etiology of which remains poorly understood. Mitochondria integrate diverse stress and survival signals to determine whether a cell lives or dies. We have used functional mitochondrial profiling to show that multidrug resistant neuroblastoma mitochondria are markedly reduced in apoptotic responses to stress. Importantly, apoptotic sensitivity is modulated at mitochondria by interactions with endoplasmic reticulum (ER) at specialized contact sites essential for calcium and lipid transfer between the organelles. ER-mito contacts are enriched for protein complexes, including MFN2 and PACS2, that bridge the organelles. Here we define a novel mechanism for chemotherapy resistance caused by reductions in ER-mito tethers, and provide functional validation for this relationship.

### Aims:

To determine the extent to which reduced ER-mitochondria tethering contributes to therapy resistance and determine whether altered calcium or lipid transfer is causal.

### Methods/Materials:

We studied neuroblastomas from diagnosis (DX, largely chemosensitive) and relapse (REL, chemoresistant) obtained from the same patients. Mitochondrial biomass (citrate synthase activity) and mtDNA content (qPCR and MitoChip) were evaluated. Isolated mitochondria were probed with tBid or Bim peptide to define their responsiveness to apoptotic signals, as measured by cytochrome c release. ER-mito contacts were assessed by physicochemical separation, and by Electron Microscopy (EM). Functional tests of ER-mito tethering involved reducing tethers physically by limited proteolysis, genetically by shRNA silencing (MFN2, PACS2), and chemically by Cyclosporine-A. Mitochondrial calcium levels and transfer were measured using a mitochondrially-targeted calcium reporter and calcium indicator dyes.

### Results:

REL neuroblastoma mitochondria are markedly attenuated in apoptotic responses to tBid and Bim, and this correlates with chemoresistance across drug classes. These differences are highly reproducible and were not caused by changes in mito biomass or mtDNA content. Instead, we find REL cells have reduced ER-mito contact number and/or increased gap-distance compared with patient-matched DX cells using EM, and confirmed by IB for organelle-specific proteins. The impact of reduced ER-mito connectivity was confirmed using multiple orthogonal methods. MFN2 or PACS2 shRNA in DX cells attenuated mitochondrial responses, phenocopied resistance and reduced ER-mitochondria connectivity. ER-mito calcium transfer was decreased in REL cells, as a consequence, while enhancing ER-mito connectivity using synthetic linkers restored calcium transfer.

#### Summary/Conclusions:

ER-mito contacts serve as physiologic regulators of apoptosis, and we show in patient-matched tumor models that these contacts are markedly reduced in therapy resistant cells. Some resistant cells had reduced numbers of ER-mito tethers (and measurably reduced calcium transfer), while other resistant cells had preserved numbers of tethers (and calcium transfer was preserved), but the gap distance was abnormally increased. Ongoing studies are investigating the role of altered lipid transfer as a unifying mechanism for this resistance phenotype. Our work will facilitate the development of clinically useful tools to measure ER-mito interactions and provide a novel framework for testing interventions to prevent emergent therapy resistance or restore mitochondrial competence to resistant cancers.



**P162 Tumor-associated macrophages (TAM) and mesenchymal stromal cells (MSC) augment MYC expression in neuroblastoma (NB) cells by affecting its phosphorylation and stability in a STAT3 dependent manner.**

*Yang, R.<sup>2</sup>, Louault, K.<sup>3</sup>, Asgharzadeh, S.<sup>4</sup>, Shimada, H.<sup>5</sup>, Declerck, Y.<sup>6</sup>*

USC, Children Hospital Los Angeles, Los Angeles, USA <sup>2</sup>, Children hospital Los Angeles, The Saban research insititute, Los Angeles, USA<sup>3</sup>, Children hospital Los Angeles, Hematology, Oncology and Blood and Marrow Transplantation, Los Angeles, USA<sup>4</sup>, Pathology and of Pediatrics at the Stanford University Medical Center., Pathology Clinical, Stanford, Pathology and of Pediatrics at the Stanford University Medical Center.<sup>5</sup>, Children hospital Los Angeles, Cancer and Blood Disease Institute, Los Angeles, USA<sup>6</sup>

**Background/Introduction:**

The presence of TAM in NB tumors is known to be an indicator of aggressivity and poor outcome in particular in NB without amplification of the *MYCN* oncogene (MYC-NA). MYC overexpression in *MYCN*-NA tumors is also a marker of very poor outcome.

**Aims:**

Here we have examined whether TAM affect the expression of MYC in MYC-NA NB tumors.

**Methods/Materials:**

Human monocytes from healthy donors were driven toward TAM by being incubated in the presence of NB cells for 4 days. TAM were then harvested and co-cultured (in Transwells) with *MYCN*-NA NB cells (CHLA255) in the presence or absence of human MSC. After 3 days NB cells were harvested and examined for the expression of MYC and its phosphorylated forms (Ser62 and Thr58) by Western blot analysis. Ruxolitinib (2.5  $\mu$ M), a JAK2/STAT3 inhibitor was added to the co-cultures as indicated.

**Results:**

Exposure of NB cells to TAM but not to untreated human monocytes or to the conditioned medium of co-cultures of TAM and NB cells increased the expression of MYC in *MYCN*-NA cells but not in *MYCN*-A (SK-N-BE(2)) cells. MSC had a similar effect and enhanced the effect of TAM. The increase in protein expression was observed within 30 min. of exposure and was sustained for more than 72 hours. It was not associated with changes in mRNA expression. Increase in MYC expression corresponded to an increase in phosphorylation at Ser62 and a decrease at Thr58. A similar effect was observed in the presence of IL-6 and its agonistic receptor sIL-6R and was blocked in the presence of ruxolitinib.

**Summary/Conclusions:**

The data provide a mechanism explaining the well know contribution of TAM and MSC in the TME of MYC-NA NB tumors by demonstrating that these cells enhance MYC expression by preventing its degradation and suggest that this effect is mediated by IL-6 released by TAM and MSC in the presence of NB cells. In view of the recently identified contribution of MYC to immune escape, the data also suggest that the upregulation of MYC in NB tumors by TAM and MSC could be a mechanism promoting immune escape

## **P163 WIP1 inhibitor SL-176 suppresses neuroblastoma growth and acts synergistically in combination with an H3K27 demethylase Inhibitor**

*Treis, D.*<sup>2</sup>, *Åkerlund, E.*<sup>3</sup>, *Milosevic, J.*<sup>4</sup>, *Pepich, A.*<sup>5</sup>, *Sakaguchi, K.*<sup>6</sup>, *Johnsen, J.*<sup>7</sup>, *Seashore-Ludlow, B.*<sup>8</sup>, *Kogner, P.*<sup>9</sup>, *Wickström, M.*<sup>10</sup>

Karolinska institute, Institution for Women's and Children's Health, Stockholm, Sweden <sup>2</sup>, Karolinska institute, Dept. of Oncology-Pathology, Stockholm, Sweden<sup>3</sup>, Karolinska institute, Dept. of Women's and Children's Health, Stockholm, Sweden<sup>4</sup>, Karolinska institute, Dept. of Women's and Children's Health, Stockholm, Karolinska institute<sup>5</sup>, Hokkaido University, Dept. of Chemistry, Sapporo, Japan<sup>6</sup>, Karolinska institute, Dept. of Women's and Children's Health, Stockholm, Sweden<sup>7</sup>, Karolinska institute, Dept. of Oncology-Pathology, Stockholm, Sweden<sup>8</sup>, Karolinska institute, Dept. of Women's and Children's Health, Stockholm, Sweden<sup>9</sup>, Karolinska institute, Dept. of Women's and Children's Health, Stockholm, Sweden<sup>10</sup>

### Background/Introduction:

The phosphatase WIP1, encoded by *PPM1D* on chromosome 17q, is a putative oncoprotein overexpressed in many malignancies including neuroblastoma. It halts DNA damage response by directly dephosphorylating p53, inhibiting p53 kinases, stabilizing the p53 inhibitor MDM2 and by promoting apoptosis through dephosphorylation of BAX. Knockdown of WIP1 reduces neuroblastoma cell viability. Several WIP1 inhibitors have been preclinically evaluated, among others SL-176 and GSK2830371, but to date, none has reached clinical testing.

### Aims:

We aim to study the effect of WIP1 inhibitors as single treatment and in drug combinations on neuroblastoma growth *in vitro* and *in vivo*.

### Methods/Materials:

Eleven neuroblastoma cell lines were exposed to different concentrations of SL-176; three neuroblastoma cell lines were treated with GSK2830371. After 72 h, cell viability was assessed with a formazan-based colorimetric assay. For the xenograft experiment, mice were injected subcutaneously with SK-N-BE(2) neuroblastoma cells and treated once daily with intraperitoneal SL-176 injections after establishment of macroscopic tumors.

In a semi-automated drug combination screening experiment, the WIP1 inhibitors SL-176 and GSK2830371 were each tested in combination with 528 different clinical and experimental compounds. For this purpose, a fixed concentration of SL-176 or GSK2830371, chosen as the IC<sub>25</sub> of the respective drug in IMR32 cells, or DMSO, was combined with five different concentrations of each of the 528 screening compounds, or DMSO. Both drugs were added to IMR32 or SK-N-AS cells and incubated for 72 h, after which cell viability was assessed using an ATP-based luminescent viability assay. A drug sensitivity score (DSS) was calculated for each dose-response curve, and the  $\Delta$ DSS was calculated as the difference between DSS of the single screening drug and DSS of the combination.

### Results:

SL-176 had cytotoxic effect with similar IC<sub>50</sub> values on all tested neuroblastoma cell lines, while GSK2830371 only seemed effective in the WIP1-overexpressing cells. SL-176 treatment *in vivo* decreased xenograft growth by about 50% without significant side effects.

In the drug combination experiment, the two WIP1 inhibitors SL-176 and GSK2830371 showed different cytotoxic profiles measured by  $\Delta$ DSS, suggesting different mechanisms of WIP1 inhibition. IMR32 cells were sensitive to a greater number of single drugs and drug combinations, as compared to SK-N-AS cells. For SL-176, the best  $\Delta$  DSS in both SK-N-AS and IMR32 cells was found for the combination with GSK-J4, an inhibitor of H3K27 demethylation which has previously been identified as potential target in neuroblastoma. Regarding GSK2830371, combination with several MDM2 inhibitors

yielded high  $\Delta$ DSS in IMR32 cells. The combination of both WIP1 inhibitors with each other also seems beneficial in this cell line.

#### Summary/Conclusions:

The WIP1 inhibitor SL-176 is cytotoxic/cytostatic to neuroblastoma cell lines, but has insufficient effect *in vivo*. Combination with GSK-J4 has shown promising results, and experiments to further investigate synergy *in vitro* and subsequently *in vivo*, are ongoing. The mechanism of WIP1 inhibition by SL-176 also needs to be studied further.

## **P164 Genomic characterization of high-risk neuroblastoma by cancer gene panel tests**

*Ohira, M.*<sup>2</sup>, *Shichino, H.*<sup>3</sup>, *Kubo, T.*<sup>4</sup>, *Haruta, M.*<sup>5</sup>, *Takimoto, T.*<sup>6</sup>, *Nakazawa, A.*<sup>7</sup>, *Yamazaki, F.*<sup>8</sup>, *Matsumoto, K.*<sup>9</sup>, *Nagase, H.*<sup>10</sup>, *Tajiri, T.*<sup>11</sup>, *Nakagawara, A.*<sup>12</sup>, *Ichikawa, H.*<sup>13</sup>, *Hishiki, T.*<sup>14</sup>, *Kamijo, T.*<sup>15</sup>

Saitama Cancer Center, Research Institute for Clinical Oncology, Saitama, Japan<sup>2</sup>, National Center for Global Health and Medicine, Department of Pediatrics, Tokyo, Japan<sup>3</sup>, National Cancer Center Research Institute, Division of Clinical Genomics, Tokyo, Japan<sup>4</sup>, Saitama Cancer Center, Research Institute for Clinical Oncology, Saitama, Saitama Cancer Center<sup>5</sup>, National Center for Child Health and Development, Department of Registration and Research for Childhood Cancer, Tokyo, Japan<sup>6</sup>, Saitama Children's Medical Center, Department of Clinical Research, Saitama, Japan<sup>7</sup>, National Cancer Center Research Institute, Department of Clinical Genomics, Tokyo, Japan<sup>8</sup>, National Center for Child Health and Development, Children's Cancer Center, Tokyo, Japan<sup>9</sup>, Chiba Cancer Center Research Institute, Department of Cancer Genetics, Chiba, Japan<sup>10</sup>, Kyoto Prefectural University of Medicine, Department of Pediatric Surgery, Kyoto, Japan<sup>11</sup>, Saga HIMAT, Saga HIMAT, Saga, Japan,<sup>12</sup> National Cancer Center Research Institute, Department of Clinical Genomics, Tokyo, Japan<sup>13</sup>, Chiba University School of Medicine, Department of Pediatric Surgery, Chiba, Japan<sup>14</sup>, Saitama Cancer Center, Research Institute for Clinical Oncology, Saitama, Japan<sup>15</sup>

### **Background/Introduction:**

Neuroblastoma (NB) is known to exhibit a variety of clinical features and its tumor genome signature has been considered as one of the useful risk classification markers. Cancer gene panel testing can examine mutations of several hundreds of cancer-related genes in tumor specimens, and is one of the promising ways for current tumor diagnosis and therapeutic drugs selection. In Japan, two types of cancer gene panels have been approved for national health insurance coverage from June 2019 and their clinical application have been rapidly spreading mainly for adult cancer, however, such system has not yet been well established for pediatric cancers including NB.

### **Aims:**

To construct a system for better cancer genome medicine for NB by utilizing cancer gene panel, we made a gene panel with pediatric cancer-related genes and evaluated it retrospectively with high-risk NBs.

### **Methods/Materials:**

Specimens were obtained from the patients registered in the clinical studies for high-risk NBs (JN-H-07 and JN-H-11) conducted by the Japan Children's Cancer Group (JCCG) Neuroblastoma Committee (JNBSG). Tumor and paired lymphocyte (if available) DNAs were analyzed by using Ion Torrent Comprehensive Cancer Panel (409 genes) or a custom-made cancer gene panel (211 genes) with Ion Proton (n=45) or Illumina NextSeq sequencers (n=28), respectively (read depth>600).

### **Results:**

Among the 45 NB tumors (JN-H-07 study) analyzed by the Ion Torrent Comprehensive Cancer Panel, 41 tumors had at least one mutation (*ATRX*:3, *ALK*:5, *ARID1A*:1). Ultra-high-risk NBs (died within 2y) had *MYCN* mutation (n=1), *CDK4* amplification (n=1) or tyrosine kinase mutations (*ALK*:1, *EGFR*:1, *FGFR1*:1). As a next step, we modified the "NCC Oncopanel" which is clinically available and approved for national health insurance coverage in Japan to make the customized "NCC Oncopanel Ped". It can survey mutations in 211 genes as well as 9 known gene fusions frequently observed in pediatric cancer. We applied it for sequencing of 28 tumors from the JN-H-11 study. Copy number alterations were clearly detected by both gene panels, and calculated *MYCN* copy numbers showed good correlation with those by quantitative real-time PCR. At least one mutation was detected in 27 tumors, and we found three non-*MYCN*-amplified tumors had actionable gene alterations involved in cell cycle regulations: *CDK4* and *MDM2* amplifications, *CCND1* amplification, and *CDKN2A/2B* homozygous deletion. For these tumors, CDK4/6 inhibitors might be possible candidate drugs. *HRAS* and *NRAS* representative mutations were also observed in other tumors.

#### Summary/Conclusions:

Cancer gene panel analysis will provide beneficial information for considering subsequent therapeutic strategies for high risk NBs by showing targetable gene mutations, mutation frequencies and copy number alterations. We have added analysis of other 24 tumors from JN-H-11 patients (including 3 recurrent tumor samples), so results of 49 cases will be discussed.

## **P165 Generation of patient-specific iPSC from patients with familial neuroblastoma.**

Van Haver, S.<sup>2</sup>, Zhou, T.<sup>3</sup>, Studer, L.<sup>4</sup>, Speleman, F.<sup>5</sup>, Roberts, S.<sup>6</sup>

Ghent University, Biomolecular Medicine, Ghent, Belgium <sup>2</sup>, Memorial Sloan Kettering Cancer Center, Center for Stem Cell Biology, New York, USA<sup>3</sup>, Memorial Sloan Kettering Cancer Center, Center for Stem Cell Biology, New York, USA<sup>4</sup>, Ghent University, Biomolecular Medicine, Ghent, Ghent University<sup>5</sup>, Memorial Sloan Kettering Cancer Center, Pediatrics, New York, USA<sup>6</sup>

### Background/Introduction:

Neuroblastoma (NB) is a developmental disorder whose biology remains poorly understood. Clinical models are essential in gaining a full understanding of NB biology and creating better therapies. One potential approach is using patient-specific induced pluripotent stem cells (iPSCs) derived from patients with familial NB for *in vitro* modelling of the disease by directed differentiation. Leveraging a precision medicine pipeline and the resources of the Paediatric Translational Medicine Program at Memorial Sloan Kettering Cancer Center (MSKCC), and in collaboration with the Developmental Biology Program and Stem Cell Research Core Facility at MSKCC, we prospectively identified four families with germline mutations responsible for familial NB and generated four patient-specific iPSC lines.

### Aims:

Using the existing infrastructure at MSKCC, we aim to develop a pipeline to generate iPSCs from paediatric cancer patients, including NB patients, harbouring mutations of interest. This will lay the foundations for a biobank consisting of rigorously validated iPSC lines with prospectively shown, relevant mutations for further paediatric cancer research.

### Methods/Materials:

After obtaining Institutional Review Board approval, we reprogrammed peripheral white blood cells from four different familial NB patients with the following mutations: TP53 R248Q, PHOX2B p.G197D-BDNFp.R209L, PHOX2B c.234\_240delCGCCGCA and ALK R1275Q. The generated iPSC lines were validated using short tandem repeat testing, shallow Whole Genome Sequencing and targeted sequencing of a panel of 468 cancer-related genes (MSK IMPACT) for lineage tracing and analysis of chromosomal aberrations and mutations, respectively. The pluripotency of the iPSC lines was confirmed using FACS analysis for stem cell markers (i.e., OCT3/4 and SOX2) and tri-lineage differentiation. After validation, the developed cell lines will be submitted to the cell repository in the Antibody and Bioresource core facility at MSKCC.

### Results:

To date, we have successfully generated and validated four different iPSC lines. STR analysis showed correct lineage tracing. sWGS and IMPACT testing verified the presence of the mutation of interest as well as lack of co-mutations or copy number variations. Pluripotency testing using FACS analysis for OCT3/4 and SOX2 confirmed reprogramming to the stem cell state. Further proof of pluripotency is ongoing by performing tri-lineage differentiations. The developed iPSC lines will be used for *in vitro* differentiation experiments to elucidate the biology of NB development. The two PHOX2B mutations reported here represent novel mutations not previously described in familial NB and illustrate the potential of this resource to explore normal differentiation and cancer development.

### Summary/Conclusions:

We have developed a pipeline to create a biobank of iPSC lines carrying prospectively validated mutations of interest. These resources will be made available to interested researchers and will allow researchers to use these generated iPSC lines to further investigate the impact of a mutation-of-interest on development and thus identify essential signalling pathways and/or test promising new therapies. Prospective identification of patients with pathogenic germline mutations is on-going, and

additional iPSC lines will be generated and made available to the research community as a resource for the study of NB and other paediatric cancers.

**P166 Early detection and molecular characterization of therapy-related leukemia in children with neuroblastoma reveals patterns of leukemia transformation with implications for future surveillance protocols**

*Spitzer, B.*<sup>2</sup>, *Gundem, G.*<sup>3</sup>, *Rutherford, K.*<sup>4</sup>, *Farnoud, N.*<sup>5</sup>, *Millard, N.*<sup>6</sup>, *McGovern, E.*<sup>7</sup>, *Patel, M.*<sup>8</sup>, *Levine, M.*<sup>9</sup>, *Medina, J.*<sup>10</sup>, *Zhou, Y.*<sup>11</sup>, *Gao, T.*<sup>12</sup>, *Arango Ossa, J.E.*<sup>13</sup>, *Levine, R.L.*<sup>14</sup>, *Cheung, I.Y.*<sup>15</sup>, *Armstrong, S.A.*<sup>16</sup>, *Cheung, N.V.*<sup>17</sup>, *Papaemmanuil, E.*<sup>18</sup>

Memorial Sloan Kettering Cancer Center, Department of Pediatrics, New York, USA <sup>2</sup>, Memorial Sloan Kettering Cancer Center, Department of Epidemiology & Biostatistics, New York, USA<sup>3</sup>, Memorial Sloan Kettering Cancer Center, Center for Hematologic Malignancies, New York, USA<sup>4</sup>, Memorial Sloan Kettering Cancer Center, Center for Hematologic Malignancies, New York, Memorial Sloan Kettering Cancer Center<sup>5</sup>, Seattle Children's, Hematology-Oncology, Seattle, USA<sup>6</sup>, Memorial Sloan Kettering Cancer Center, Center for Hematologic Malignancies, New York, USA<sup>7</sup>, Memorial Sloan Kettering Cancer Center, Center for Hematologic Malignancies, New York, USA<sup>8</sup>, Memorial Sloan Kettering Cancer Center, Center for Hematologic Malignancies, New York, USA<sup>9</sup>, Memorial Sloan Kettering Cancer Center, Center for Hematologic Malignancies, New York, USA<sup>10</sup>, Memorial Sloan Kettering Cancer Center, Center for Hematologic Malignancies, New York, USA<sup>11</sup>, Memorial Sloan Kettering Cancer Center, Center for Hematologic Malignancies, New York, USA, <sup>12</sup>, Memorial Sloan Kettering Cancer Center, Center for Hematologic Malignancies, New York, USA <sup>13</sup>, Memorial Sloan Kettering Cancer Center, Department of Medicine, Center for Hematologic Malignancies, New York, USA <sup>14</sup>, Memorial Sloan Kettering Cancer Center, Department of Pediatrics, New York, USA <sup>15</sup>, Dana-Farber Cancer Institute, Department of Pediatrics, Boston, USA <sup>16</sup>, Memorial Sloan Kettering Cancer Center, Department of Pediatrics, New York, USA <sup>17</sup>, Memorial Sloan Kettering Cancer Center, Department of Epidemiology & Biostatistics, Center for Hematologic Malignancies, New York, USA <sup>18</sup>

Background/Introduction:

Therapy-related myelodysplastic syndrome (t-MDS) and acute leukemias (AL) represent a major cause of non-relapse morbidity and mortality in childhood cancer survivors. Neuroblastoma (NB) patients receive multimodality therapy and have had high rates of t-MDS/AL. While specific therapeutic modalities have been associated with distinct abnormalities, our understanding of the relationships between the timing of mutation acquisition, dynamics of clonal selection in relation to specific therapeutic modalities, and how these result in overt leukemia, remains limited.

Aims:

Methods/Materials:

We studied 199 samples from 53 NB patients treated at MSKCC (enrolled on NCT00588068), including 17 patients with t-MDS/AL, 14 with transient cytogenetic abnormalities, and 21 matched controls. Comprehensive genomic profiling of bone marrow (BM) was performed with targeted gene sequencing (MSK Hemepact) and fusion detection (Archer FusionPlex Pan-Heme panel) to identify somatic mutations, chromosome level copy number alterations (CNA), and gene fusions at the time of t-MDS/AL diagnosis. Backtracking studies were performed in longitudinal samples with complete molecular characterization. For patients in the transient cohort, samples were sequenced throughout therapy, with a focus on times surrounding cytogenetic abnormalities and treatment windows. In the control cohort, samples were chosen at 1-2 time points at the end of treatment windows.

Results:

We detected at least one disease-defining alteration in all cases with t-MDS/AL at time of diagnosis, most commonly MLL fusions (n=6 patients) or TP53 mutations (n=9 variants, n=5 patients). These events were generally mutually exclusive, with the exception of one patient with an apparent subclonal mutation in TP53 and clonal MLL fusion. The remaining cases acquired gene mutations in NPM1, IDH1, PTPN11, NRAS, CUX1, STAG2, WT1, PPM1D amongst other genes, at a median variant allele frequency (VAF) 21% (range 2.6-74), with or without chromosomal aneuploidies. High-confidence fusions detected by Archer FusionPlex were limited to MLL-rearrangements, though clinical



cytogenetic analysis demonstrated other translocations. All MLL-rearrangements involved the canonical MLL breakpoint region. Backtracking studies identified at least one of these mutations or fusions in the majority (12/17) of patients at a median of 18 months prior to diagnosis (range 1.3-36) using a 0.02 VAF detection threshold.

In contrast, only three patients from the transient cytogenetic cohort, had putative oncogenic somatic mutations identified at any time point sequenced, with a median maximum VAF of 3% (range: 2.5-8.5), with resolution of the molecular alterations in subsequent samples. One control case had an identified presumed oncogenic mutation in BCOR, though this patient died of NB with limited hematologic follow-up after the last sample, >7 years after initial NB diagnosis.

#### Summary/Conclusions:

These findings demonstrate that molecular abnormalities are a hallmark of t-MDS/AL in NB patients and that these are often identifiable months to years prior to leukemia transformation. This presents an opportunity for the development of early detection studies for patients with neuroblastoma undergoing intensive therapy and should inform studies into mechanisms of leukemic transformation.

## **P167 The cfDNA methylome as a complementary assay to classic histopathology for neuroblastoma diagnosis**

*Van Paemel, R.<sup>2</sup>, Vícha, A.<sup>3</sup>, Vandeputte, C.<sup>4</sup>, De Preter, K.<sup>5</sup>, De Wilde, B.<sup>6</sup>*

Ghent University, Biomolecular Medicine, Ghent, Belgium <sup>2</sup>, Charles University in Prague, Second Faculty of Medicine and University Hospital, Pediatric Hematology and Oncology, Prague, Czech Republic<sup>3</sup>, Ghent University, Biomolecular Medicine, Ghent, Belgium<sup>4</sup>, Ghent University, Biomolecular Medicine, Ghent, Ghent University<sup>5</sup>, Ghent University Hospital, Department of Pediatric Hematology, Oncology & Stem Cell Transplantation, Ghent, Belgium<sup>6</sup>

### Background/Introduction:

In the clinical management of pediatric solid tumors, a biopsy remains the gold-standard to establish a diagnosis. The DNA methylation pattern of a tumor is known to correlate to the histopathological diagnosis across cancer types and thus might be of value in the diagnostic workup of tumor samples. It has previously been shown that this methylation pattern can be detected in cell-free DNA (cfDNA).

In a retrospective proof-of-concept study in children with a solid tumor we were able to correctly classify up 80% to 94% of primary diagnostic cfDNA samples according to their histopathological class, solely based on the methylation pattern of cell-free DNA. Our cohort of 60 patients included 10 neuroblastoma cases. Copy number variations could also be assessed directly from the methylation data, enabling the detection of *MYCN* status and segmental chromosomal alterations.

### Aims:

To explore the clinical applicability of this novel methylation profiling technique, we used it to classify a diagnostically challenging case of a three-year old girl with a small round blue cell tumor. Due to the clinical presentation of a primary tumor originating from the distal femur diaphysis with metastasis to the inguinal and pelvic lymph nodes, neuroblastoma was not considered as a diagnosis. Classical pathology and immunohistochemistry did not yield a conclusive diagnosis. The patient started treatment according to EUROEWING 08 protocol because of a suspected hard-to-classify bone tumor. SNP array analysis from the primary tumor revealed 1p deletion, *MYCN* amplification and gain of 17q consistent with neuroblastoma, however MIBG and urinary catecholamines (collected after 2 cycles of VIDE chemotherapy) yielded negative results. We aimed to further explore the possible added value of a liquid biopsy in this challenging diagnostic situation.

### Methods/Materials:

Cell-free DNA from a pretreatment blood sample was isolated. After quality control with capillary electrophoresis, we performed cell-free reduced representation bisulfite sequencing (cf-RRBS) and shallow WGS (sWGS), each on 10 ng of cfDNA. The cf-RRBS methylomes were decomposed according to cellular make-up using a non-negative least squares method. As reference set, publicly available methylome data from primary tumors was used. Copy number aberrations were called on the sWGS data with WisecondorX. Full methods are available on biorxiv (<https://doi.org/10.1101/795047>).

### Results:

The copy number aberrations on the liquid biopsy sample both from the sWGS and methylation profiling data are consistent with the SNP array on the primary tumor (1p deletion, *MYCN* amplification and 17q gain). Decomposition and classification of the cfDNA methylome further supports the diagnosis of neuroblastoma, with approximately 60% of the total cfDNA matching the neuroblastoma reference set. Based on these results, bone marrow of the patient was further investigated with RT-qPCR for expression of TH, PHOX2B, CHRNA3 and GAP43 further supporting neuroblastoma diagnosis.

### Summary/Conclusions:

In this diagnostically challenging case of a patient with a small blue round cell tumor showing little to no lineage specific markers we conclude to a neuroblastoma diagnosis based on the cfDNA copy number and methylation data. This case is an example of how liquid biopsy-based diagnosis can contribute to imaging, classical histopathology and molecular genetics.

## **P168 19p loss is significantly enriched in older age neuroblastoma patients and correlates with poor prognosis**

*Lasorsa, V.A.*<sup>2</sup>, *Cimmino, F.*<sup>3</sup>, *Ognibene, M.*<sup>4</sup>, *Mazzocco, K.*<sup>5</sup>, *Erminio, G.*<sup>6</sup>, *Morini, M.*<sup>7</sup>, *Conte, M.*<sup>8</sup>, *Iolascon, A.*<sup>9</sup>, *Pezzolo, A.*<sup>10</sup>, *Capasso, M.*<sup>11</sup>

CEINGE Biotecnologie Avanzate, Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Università degli St, Napoli, Italy <sup>2</sup>, CEINGE Biotecnologie Avanzate, Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Università degli St, Napoli, Italy<sup>3</sup>, IRCCS Istituto Giannina Gaslini, Laboratorio Cellule Staminali Post Natali e Terapie Cellulari, Genova, Italy<sup>4</sup>, IRCCS Istituto Giannina Gaslini, UOC Anatomia Patologica, Genova, IRCCS Istituto Giannina Gaslini<sup>5</sup>, IRCCS Istituto Giannina Gaslini, Epidemiologia e Biostatistica, Genova, Italy<sup>6</sup>, IRCCS Istituto Giannina Gaslini, Laboratorio di Biologia Molecolare, Genova, Italy<sup>7</sup>, IRCCS Istituto Giannina Gaslini, UOC Oncologia, Genova, Italy<sup>8</sup>, CEINGE Biotecnologie Avanzate, Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Università degli St, Napoli, Italy<sup>9</sup>, IRCCS Istituto Giannina Gaslini, Laboratorio Cellule Staminali Post Natali e Terapie Cellulari, Genova, Italy<sup>10</sup>, CEINGE Biotecnologie Avanzate, Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Università degli St, Napoli, Italy<sup>11</sup>

### Background/Introduction:

Neuroblastoma is an embryonal tumor in which the normal developing process of adrenal medulla and paravertebral sympathetic ganglia is affected. The cell origin and initiation mechanism of neuroblastoma has been long-standing mysteries. Therefore, investigation of differentiation and migration of neural crest cells no doubt will promote further understanding of initiation of neuroblastoma. Single cell transcriptome analysis is a powerful tool in uncovering the differentiation process of neural crest cells.

### Aims:

To delineate development trajectory of human neural crest cells at single cell level and to infer putative cell origin of NB

### Methods/Materials:

We performed single cell sequencing on two human embryos at gestational age (GA) of about 4-5 weeks and two fetal adrenal glands at GA of 10 weeks and 16 weeks respectively. Development trajectory was delineated using Monocle. Cell types was identified by well-defined markers. CIBERSORT was used to infer NB expression pattern of RNA-seq data by utilizing differentially expressed genes of neural crest cell derivatives as reference.

### Results:

After qualification control and unsupervised clustering, there are all together 36,839 cells from embryos divided into 10 cell subgroups and 25,615 cells from fetal adrenal glands with 13 cell types. Cell trajectory reveals the cell fate transition from Schwann cell progenitors to chromaffin cells, through the stage of chromaffin progenitors. Along with this process, there is a synergetic expression of *PHOX2B* and proliferation genes such as *MK167*. By analyzing pseudotime-dependent transcription factor (TF), several previously unknown TFs associated with chromaffin progenitors, such as *SOX11* and *TCF4*, were identified. Putative cell origin of neuroblastoma was inferred using RNA-seq data of NB, which shows that inspite of primary site, either sympathetic ganglia or adrenal medulla, the expression pattern of tumor cells is more similar to more mature chromaffin cell type.

### Summary/Conclusions:

Schwann cell progenitors, belonging to the late migrating neural crest cells, differentiate into chromaffin cells, through the stage of chromaffin progenitors. Several TFs are dynamically expressed during the process. In spite of primary site of the tumor, the expression pattern of NB cells resembles more mature chromaffin cell type, implying a putative cell origin.



## **P169 Neuropeptide Y/Y5 receptor axis as a hypoxia-inducible pro-metastatic pathway in neuroblastoma.**

*Kitlinska, J.*<sup>2</sup>, *Abu Alsaud, N.*<sup>3</sup>, *Caprio, L.*<sup>4</sup>, *Hong, S.H.*<sup>5</sup>, *Galli, S.*<sup>6</sup>

Georgetown University, Biochemistry and Molecular & Cellular Biology, Washington, USA <sup>2</sup>,  
Georgetown University, Biochemistry and Molecular & Cellular Biology, Washington, USA<sup>3</sup>,  
Georgetown University, Biochemistry and Molecular & Cellular Biology, Washington, USA<sup>4</sup>,  
Georgetown University, Biochemistry and Molecular & Cellular Biology, Washington, Georgetown  
University<sup>5</sup>, Georgetown University, Biochemistry and Molecular & Cellular Biology, Washington, USA<sup>6</sup>

### **Background/Introduction:**

As tumors originating from precursors of sympathetic neurons, neuroblastomas (NBs) expresses neuronal proteins, such as neuropeptide Y (NPY) and its receptors. NB cells constitutively express Y2 receptor (Y2R), which maintains their proliferation and tumor vascularization. However, pro-apoptotic conditions additionally induce expression of Y5 receptor (Y5R), which acts as a survival factor. High Y5R expression was also observed in angioinvasive NB cells surrounding blood vessels, suggesting a role for this receptor in NB dissemination. Indeed, in NB patients, high serum levels of NPY associated with the presence of metastases. Furthermore, our studies in other tumor types implicated that Y5R can be up-regulated by hypoxia - a known stimulator of the metastasis.

### **Aims:**

The goal of this study was to identify mechanisms that may underlie potential pro-metastatic effects of NPY in NB.

### **Methods/Materials:**

Expression of NPY and its receptors in NB cells, SK-N-BE(2) and SK-N-AS, with or without exposure to hypoxia (0.1% oxygen for 72h) was assessed by RT-PCR, Western blot and immunocytochemistry. The effect of NPY on migration of NB cells was tested using transwell assay, while interactions with RhoA pathway were measured by RhoA pull-down and proximity ligation assay. To determine the effect of hypoxia on NB metastasis, SK-N-BE(2) and SK-N-AS cells were pre-exposed to hypoxia for 72h and injected into adrenal fat pads of SCID/bg mice.

### **Results:**

We have found that NPY, acting via Y5R, stimulates NB cell motility and acts as a chemotactic factor for these cells in a concentration-dependent manner. Hypoxia increased expression of Y5R in NB cells and their migratory response to NPY. This increase in NPY-driven migration observed in hypoxic NB cells was fully blocked by Y5R antagonist. Notably, SK-N-BE(2) cells pre-exposed to hypoxia had also increased metastatic potential, as evidenced by higher infiltration of lung tissues by NB cells upon their orthotopic injections. The effect of NPY/Y5R pathway on NB cell migration was associated with marked cytoskeleton remodeling, manifested by an increase in the number of filopodia. In migratory cells, the active fraction of Y5R present on the cell surface was located in the leading and trailing edges of NB cells, and co-localized with RhoA-GTP, a known cytoskeleton regulator. RhoA-GTP pull-down assay confirmed NPY-induced RhoA activation in NB cells, while proximity ligation assay revealed direct interactions between Y5R and RhoA-GTP triggered by NPY stimulation.

### **Summary/Conclusions:**

Altogether, our data indicate that NPY/Y5R/RhoA pathway stimulates overall motility of NB cells and their directional migration toward NPY. This effect is exacerbated in hypoxia. Further studies are required to determine the contribution of these processes to NB metastasis.

## **P170 The molecular aetiology of ATRX aberrations in neuroblastoma**

*Van Gerven, M.R.<sup>2</sup>, Van den Boogaard, M. L.<sup>3</sup>, Schild, L.<sup>4</sup>, Debets, D. O.<sup>5</sup>, Van Arkel, J.<sup>6</sup>, Broeils, L. A.<sup>7</sup>, Damen, M. J.<sup>8</sup>, De Vries, I.<sup>9</sup>, Altelaar, M.<sup>10</sup>, Molenaar, J. J.<sup>11</sup>*

Princess Máxima Center, Research, Utrecht, Netherlands<sup>2</sup>, Princess Máxima Center, Research, Utrecht, Netherlands<sup>3</sup>, Princess Máxima Center, Research, Utrecht, Netherlands<sup>4</sup>, Utrecht University, Pharmaceutical Sciences, Utrecht, Utrecht University<sup>5</sup>, Princess Máxima Center, Research, Utrecht, Netherlands<sup>6</sup>, Princess Máxima Center, Research, Utrecht, Netherlands<sup>7</sup>, Utrecht University, Pharmaceutical Sciences, Utrecht, Netherlands<sup>8</sup>, Princess Máxima Center, Research, Utrecht, Netherlands<sup>9</sup>, University Utrecht, Pharmaceutical Sciences, Utrecht, Netherlands<sup>10</sup>, Princess Máxima Center, Research, Utrecht, Netherlands<sup>11</sup>

### **Background/Introduction:**

A subgroup of high-risk neuroblastoma patients with very poor clinical outcome is characterised by genetic aberrations in the chromatin-remodeler ATRX. The majority of these tumours have in-frame ATRX deletions while some rare cases show ATRX stop-codons. ATRX is known to be involved in the removal of G-quadruplexes, DNA repair, H3.3 incorporation, inhibition of macroH2A1 disposition and indirectly in H3K27 tri-methylation. ATRX aberrations are known to cause alternative lengthening of telomeres (ALT), in which the telomeres are elongated by a homologous recombination-like mechanism resulting in C-circles, elongated telomeres and ALT-associated PML bodies (APBs). There is limited knowledge of the role of (in-frame deleted) ATRX in neuroblastoma.

### **Aims:**

The aim for this project is to create models of ATRX alterations to study the molecular aetiology of ATRX aberrations in neuroblastoma and to identify effective therapeutic interventions for this subgroup of patients.

### **Methods/Materials:**

Currently, we have one cell lines (CHLA-90) and one organoid line (AMC772T2) with different ATRX deletions. For both mutant lines and an ATRX wildtype cell line (CHP134) we performed ATRX immunoprecipitation (IP) followed by mass spectrometry to identify the common lost or gained ATRX protein-binding partners.

To expand the number of neuroblastoma models with ATRX alterations we used CRISPR-Cas9 to create isogenic cell line and organoid models with ATRX knock-out (KO) mutations and different patient-specific in-frame ATRX deletions. We also created a PiggyBac doxycycline-inducible wildtype ATRX plasmid and a PiggyBac doxycycline-inducible in-frame deleted ATRX plasmid.

We used the C-circle assay, telomere southern blotting and an APB FISH/IF to investigate ALT characteristics in these models. We also performed high-throughput compound screens of AMC772T2 and CHLA-90 and will extend this to the newly created models.

### **Results:**

We identified that the ATRX deletions in CHLA-90 and AMC772T2 result in in-frame deleted ATRX protein products. The in-frame deleted ATRX protein from CHLA-90 is able to interact with DAXX, a known ATRX interaction partner, while this interaction seems lost in AMC772T2. We obtained mass spectrometry results from the ATRX IPs of CHLA-90, AMC772T2 and CHP134 (ATRX wild-type). These data are further analysed and (the absence of) interaction partners will be validated.

In addition, we have generated isogenic ATRX KO GIMEN clones and isogenic ATRX KO SKNAS clones. These clones were negative for ALT characteristics in the C-circle assay, telomere southern and ABP FISH/IF. Since these clones are still telomerase dependent, we will additionally inactivate/reduce telomerase activity to enable them to switch to ALT. Furthermore, we created

models with inducible ATRX overexpression in CHLA-90 and AMC772T2 and models with ATRX deletions. We will investigate ALT characteristics in these lines.

The isogenic ATRX KO and overexpression models will be used in high-throughput compound screens and will be compared with the compound sensitivity data of CHLA-90 and AMC772T2 to identify possible therapeutic targets.

Summary/Conclusions:

We have created models of ATRX KO, in-frame deletions and overexpression in neuroblastoma cell lines and organoids. These models will be used to increase the understanding of the molecular aetiology of neuroblastoma tumours with ATRX aberrations and to identify effective therapeutic interventions.



## **P171 A Precision Medicine Biology Trial for patients with refractory or relapsed neuroblastoma: A New Approaches to Neuroblastoma Therapy Consortium (NANT) Study**

*Asgharzadeh, S.,<sup>1</sup> Goldsmith, K.<sup>2</sup>, Goldsmith, K.<sup>3</sup>, Kennedy, R.<sup>4</sup>, Shamirian, A.<sup>5</sup>, Chunqiao, C.<sup>6</sup>, Hung, L.<sup>7</sup>, Granger, M.<sup>8</sup>, Weiss, B.<sup>9</sup>, Macy, M.<sup>10</sup>, Cohn, S.<sup>11</sup>, Matthay, K.<sup>12</sup>, Park, J.<sup>13</sup>, Pawel, B.<sup>14</sup>, Hiroyuki, H.<sup>15</sup>, Groshen, S.<sup>16</sup>, Marachelian, A.*

CHILDREN'S HOSPITAL LOS ANGELES, Pediatrics, Los Angeles, USA<sup>1</sup>, Emory University School of Medicine, Pediatrics, Atlanta, USA<sup>2</sup>, Emory University School of Medicine, Pediatrics, Atlanta, USA<sup>3</sup>, CHILDREN'S HOSPITAL OF LOS ANGELES, Pediatrics, Los Angeles, USA<sup>4</sup>, CHILDREN'S HOSPITAL OF LOS ANGELES, Pediatrics, Los Angeles, CHILDREN'S HOSPITAL OF LOS ANGELES<sup>5</sup>, University of Southern California, Pediatrics, Los Angeles, USA<sup>6</sup>, CHILDREN'S HOSPITAL OF LOS ANGELES, Childrens Hospital Los Angeles, Los Angeles, USA<sup>7</sup>, Cook Children's Hospital, Pediatrics, Fort Worth, USA<sup>8</sup>, Cincinnati Children's Hospital, Pediatrics, Cincinnati, USA<sup>9</sup>, Children's Hospital of Colorado, Pediatrics, Denver, USA<sup>10</sup>, University of Chicago, Pediatrics, Chicago, USA<sup>11</sup>, University of California San Francisco, Pediatrics, San Francisco, USA<sup>12</sup>, Seattle Children's Hospital, Pediatrics, Seattle, USA<sup>13</sup>, CHILDREN'S HOSPITAL OF LOS ANGELES, Pathology, Los Angeles, USA<sup>14</sup>, Stanford, Pediatrics, San Francisco, USA<sup>15</sup>, University of Southern California, Preventative Medicine, Los Angeles, USA<sup>16</sup>, CHILDREN'S HOSPITAL OF LOS ANGELES, Pediatrics, Los Angeles, USA<sup>17</sup>

### Background/Introduction:

Limited data exists regarding the genetic and immunologic predictive biomarkers in recurrent or refractory neuroblastoma (rNB). Understanding rNB mechanisms of resistance through in depth genomic or tumor microenvironment (TME) alterations is critical to devising novel targeted therapies.

### Aims:

This study describes the feasibility of performing next generation sequencing, assessing the landscape of genomic and immunologic alterations of rNB tissues, and feasibility of obtaining genomic data from tumor enriched bone marrow aspirates.

### Methods/Materials:

Patients with rNB were enrolled on the IRB-approved NANT 15-01 protocol. Tumor from bone, bone marrow or soft tissue disease, as well as germline blood were analyzed using Ashion's GEM 600-gene panel or GEM Extra Platform with the latter platform including full transcriptome (RNASeq) and whole-exome (WES) sequencing coupled with germline subtraction. In addition, soft tissue specimens were evaluated by immunohistochemistry (IHC) for C-MYC and *MYCN* expression, CD163 (tumor associated macrophages (TAM)) and PDL-1. The generated report on samples with adequate genomic material for testing was returned back to the treating physician to aid in clinical decision making. Bone marrow aspirate specimens were enriched for tumor cells using positive selection for GD2 and analyzed pre- and post-enrichment by flow for GD2, CD45, NCAM and Phox2B.

### Results:

To date, 83 biopsy specimens from 85 enrollments (77 patients) have been submitted, 52 (68%) of which completed all study analyses and generated a genomic report. Of the 83 specimens, 32/41 (78%) were from soft tissue, 14/29 (48%) bone, and 6/13 (46%) bone marrow. 46/77 (61%) had a report generated from the first specimen submitted. Thirty tumor specimens had insufficient quality for sequencing. Of those, 8 (27%) patients submitted a second specimen (re-enrolled) with 6/8 (75%) resulting in a report. Physicians who received a complete genomic report, were asked to fill out a one year follow up survey, which showed 12/45 (27%) utilized the genetic results to enroll patients on targeted therapies. From the 52 reports generated, actionable genetic variants were identified in 43/52 (83%), 7/52 (13%) identified non-actionable genetic variant, and 4/52 (8%) identified no genetic variant. The most common mutations identified included ALK mutations (33%), which is higher than previously reported for relapsed patients. Additional alterations in *MYCN* (25%), *ATRX* (15%),

RAS/MAPK pathway-related genes (11%), and CDKN2A (8%) were identified. Soft tissue samples were also analyzed for TME markers, showing heavy infiltration of TAMs in 17/32 (53%) including 7/8 (88%) refractory specimens. PDL1 (> 5%) was detected in 5/32 (16%) of soft tissue samples. 12/46 (26%) bone marrow aspirate samples submitted for enrichment were found positive for neuroblastoma (>1% GD2+ by flow). 9/12 (75%) were enriched to >30% GD2+ cells for genetic sequencing.

#### Summary/Conclusions:

A multi-institution clinical genomics study in neuroblastoma is feasible with high chance of success in obtaining useful samples with guided biopsies, including bone metastases. Enrichment of bone marrow aspirates was feasible in a subset of these patients. A substantial proportion of children with rNB have actionable alterations with significant ALK enrichment in this multiply recurrent patient cohort. Approximately half of rNBs show evidence of an active tumor microenvironment.

## **P172 Translation addiction as a novel therapeutic target in neuroblastoma**

*Durinck, K.*<sup>2</sup>, *Nunes, C.*<sup>3</sup>, *Olexiouk, V.*<sup>4</sup>, *Sanders, E.*<sup>5</sup>, *De Vloed, F.*<sup>6</sup>, *Parsa, S.*<sup>7</sup>, *Dolman, E.*<sup>8</sup>, *De Wilde, B.*<sup>9</sup>, *Molenaar, J.J.*<sup>10</sup>, *Wendel, H.G.*<sup>11</sup>, *Speleman, F.*<sup>12</sup>

Ghent University, Department of Biomolecular Medicine, Ghent, Belgium<sup>2</sup>, Ghent University, Department of Biomolecular Medicine, Ghent, Belgium<sup>3</sup>, Ghent University, Department of Biomolecular Medicine, Ghent, Belgium<sup>4</sup>, Ghent University, Department of Biomolecular Medicine, Ghent, Ghent University<sup>5</sup>, Ghent University, Department of Biomolecular Medicine, Ghent, Belgium<sup>6</sup>, Memorial Sloan Kettering Center, Cancer Biology and Genetics, New York, USA<sup>7</sup>, Princess Máxima Center, Child oncology, Utrecht, Netherlands<sup>8</sup>, Ghent University Hospital, Department of Pediatric Hematology-Oncology and Stem Cell Transplantation, Ghent, Belgium<sup>9</sup>, Princess Máxima Center, Child oncology, Utrecht, Netherlands<sup>10</sup>, Memorial Sloan Kettering Center, Cancer Biology and Genetics, New York, USA<sup>11</sup>, Ghent University, Department of Biomolecular Medicine, Ghent, Belgium,<sup>12</sup>

### **Background/Introduction:**

Translational control relies on the orchestrated activity of the ribosomal complexes as well as initiation and elongation machineries. In addition, also inherent properties of the RNA-sequence and structures commonly located in the untranslated regions (UTR) of the transcript, such as G-quadruplexes (G4s), also play a key role. Recent work increasingly supports a role for translational control as a hallmark for cancer. The role of adaptive changes in protein synthesis during tumorigenesis is supported by the identification of recurrent mutations affecting both components of the translation machinery itself, mRNA *cis*-regulatory elements as well as multiple oncogenic pathways activating translation. While these specific cancer dependencies related to translation have emerged as innovative entry points for precision oncology, they have not been explored in depth in neuroblastoma.

### **Aims:**

To perform phenotypic and molecular evaluation of pharmacological eIF4A inhibition in neuroblastoma and scrutinize the mode-of-action through a comprehensive and integrated proteome profiling approach.

### **Methods/Materials:**

We tested the response of a panel of 7 neuroblastoma cell lines to the eIF4A inhibitor CR31B, a synthetic silvestrol analogue. Next, shotgun proteome profiling of IMR32 cells treated with CR31B (or DMSO as a reference) in a time series (3h, 6h, 12h, 24h, 48h and 72h) was performed followed by the identification of patterns of differential protein expression.

### **Results:**

The eIF4A inhibitor CR31B treatment had strong effects on cell viability within low nanomolar concentration range in all cell tested cell lines. Comparative proteomics analysis before and after CR31B exposure in neuroblastoma cells revealed 600 significantly differentially expressed proteins from a total of 4452 quantified proteins. Further filtering towards hits displaying at least a 2-fold absolute fold change in expression level, lead to the discovery of TPX2 as one of the top targets, with TPX2 downregulation upon CR31B treatment confirmed by western blot analysis. While TPX2 is a crucial regulator of Aurora kinase A (AURKA, a critical factor of *MYCN* stability in neuroblastoma and mitotic regulator) and acts as spindle assembly factor, recent evidence also points towards a moonlighting function for TPX2 in DNA repair and replication fork stability, in concert with AURKA. Shotgun proteome profiling will be further complemented with ribosome profiling (ribo-seq) and total RNA-seq. Initial knock down of TPX2 in neuroblastoma cells support its role as dependency factor. We will also perform *in vivo* experiments to scrutinize the functional role of TPX2 in replicative stress resistance and evaluate the pharmacological potential of CR31B using the d $\beta$ h-eGFP-*MYCN* driven zebrafish neuroblastoma model.

Summary/Conclusions:

Our data point at TPX2 as a putative key downstream effector of eIF4A driven translational addiction in neuroblastoma and should stimulate further investigation of this druggable vulnerability.

## **P173 Validation of a standardized diagnostic workflow for analyzing circulating tumor DNA in Neuroblastoma**

Walter, C.<sup>2</sup>, Gerber, T.<sup>3</sup>, Six, V.<sup>4</sup>, Ambros, I.M.<sup>5</sup>, Ambros, P.F.<sup>6</sup>, Ladenstein, R.<sup>7</sup>, Taschner-Mandl, S.<sup>8</sup>, Bernkopf, M.<sup>9</sup>

LabDia Labordiagnostik GmbH, Children's Cancer Research Institute, Tumorbiology, Vienna, Austria<sup>2</sup>, Children's Cancer Research Institute, Tumorbiology, Vienna, Austria<sup>3</sup>, LabDia Labordiagnostik GmbH, Children's Cancer Research Institute, Tumorbiology, Vienna, Austria<sup>4</sup>, LabDia Labordiagnostik GmbH, Children's Cancer Research Institute, Tumorbiology, Vienna, LabDia Labordiagnostik GmbH, Children's Cancer Research Institute<sup>5</sup>, LabDia Labordiagnostik GmbH, Children's Cancer Research Institute, Tumorbiology, Vienna, Austria<sup>6</sup>, Children's Cancer Research Institute, Clinical Research, Vienna, Austria<sup>7</sup>, LabDia Labordiagnostik GmbH, Children's Cancer Research Institute, Tumorbiology, Vienna, Austria<sup>8</sup>, LabDia Labordiagnostik GmbH, Children's Cancer Research Institute, Tumorbiology, Vienna, Austria<sup>9</sup>

### Background/Introduction:

The minimal invasive nature of liquid biopsies and their application as a potential biomarker for cancer diagnosis and monitoring has already been widely demonstrated in various research studies. Especially in neuroblastoma extensive tumor heterogeneity has been observed and could lead to biases in diagnostic tumor profiling. Therefore the additional analysis of circulating tumor DNA (ctDNA) could be beneficial for the use in clinical diagnostics; however, a fully validated standard protocol is not yet available.

### Aims:

Our aim was to establish a diagnostic workflow by (1) investigating pre-analytical conditions including blood collection tubes, transport and storage, (2) comparing manual and automated cell-free DNA extraction methods and (3) validating the molecular analysis of neuroblastoma-specific tumor markers by digital droplet PCR (ddPCR).

### Methods/Materials:

Healthy donor blood was collected in standard blood collection tubes (EDTA, Heparin) and leucocyte stabilizing tubes (PAXgene ccfDNA, Roche cfDNA and Streck BCT) and cfDNA was extracted from plasma after storage of 0-48 hours at RT or +4°C, respectively. Extracted cfDNA was analyzed for yield and fragment size distribution (genomic DNA contamination) by TapeStation (Agilent, USA), Qubit fluorometry (ThermoFisher, USA) and applied on down-stream applications (NGS, ddPCR). The most commonly used QIAamp circulating nuclear acid kit (Qiagen, Germany) was compared to the fully automated extraction protocol Maxwe I® RSC ccfDNA plasma kit (Promega, USA), and the quality and quantity of the extracted cfDNA analyzed with the methods described above. ddPCR with Bio-Rad's (USA) wet-lab validated ddPCR Assays was applied for the detection of *MYCN* amplification, *ALK* amplification and *ALK* hotspot mutations (*ALK* p.F1174L and p.R1275Q) in cfDNA reference standards (Horizon, UK) and spike-in samples.

### Results:

The cfDNA extracted from widely used EDTA-samples show good results for liquid biopsy analyses if stored for a maximum of 4 hours at room temperature or for up to 24 hours at +4°C. For longer storage or transportation of blood samples leucocyte stabilizing tubes are recommended. Furthermore, using Streck BCT or EDTA blood samples, stored for maximum 24 hours at +4°C, leucocytes and low amounts of tumor cells can be isolated and analyzed in addition to the ctDNA.

Concerning the extraction methods and ddPCR we were able to meet the required quality criteria in order to implement the workflow for analyzing circulating tumor DNA from neuroblastoma blood samples in a diagnostic laboratory. This includes partial automation which allows us to analyze more samples in a standardized manner, leading to fast turnaround times.

#### Summary/Conclusions:

Taken together, we established a validated partially automated diagnostic workflow including guidelines for pre-analytical conditions adapted to small blood volumes for processing of peripheral blood and cfDNA extraction for reliable downstream ctDNA analysis compliant with real-life conditions in clinics and diagnostic labs. By using the ddPCR we implemented a highly sensitive and specific detection method for *MYCN* and *ALK* alterations in ctDNA of neuroblastoma patients. These additional genetic results supplied by molecular diagnostics could improve disease stratification, therapeutic target detection and monitoring and allow researchers to study tumor heterogeneity and evolution in the future.

**P174 Human stem cell based models of neuroblastoma provide a complementary system to address critical biological questions in neuroblastoma.**

*Huang, M.<sup>2</sup>, Zheng, T.<sup>3</sup>, Nasholm, N.<sup>4</sup>, Menard, M.<sup>5</sup>, Farrel, A.<sup>6</sup>, Ghiglieri, L.<sup>7</sup>, Gustafson, W.<sup>8</sup>, Maris, J.<sup>9</sup>, Matthay, K.<sup>10</sup>, Weiss, W.<sup>11</sup>*

Children's Hospital Los Angeles (CHLA), Pediatrics, Los Angeles, USA <sup>2</sup>, UCSF, Neurology, San Francisco, USA<sup>3</sup>, UCSF, Pediatrics, San Francisco, USA<sup>4</sup>, UCSF, Neurology, San Francisco, UCSF<sup>5</sup>, Children's Hospital of Philadelphia, Pediatrics, Philadelphia, USA<sup>6</sup>, UCSF, Neurology, San Francisco, USA<sup>7</sup>, UCSF, Pediatrics, San Francisco, USA<sup>8</sup>, Children's Hospital of Philadelphia, Pediatrics, Philadelphia, USA<sup>9</sup>, UCSF, Pediatrics, San Francisco, USA<sup>10</sup>, UCSF, Neurology, San Francisco, USA<sup>11</sup>

**Background/Introduction:**

Current human models of neuroblastoma rely on PDX and human cell lines. While these models can provide important insight into how specific mutations impact the biology of neuroblastoma, the mutation being investigated is influenced by the array of other mutations present in the genome. In addition, these tumors have already been transformed and it is difficult to determine which mutation initiated tumorigenesis. We propose a human stem cell based model of neuroblastoma would provide a complementary system to investigate these important questions in neuroblastoma biology.

**Aims:**

The aim of this study is to utilize human stem cell based models of neuroblastoma to identify how various MYC-driven tumors differ molecularly in driving tumorigenesis.

**Methods/Materials:**

Human induced pluripotent stem cells (iPSC) derived integration free from healthy human adults were transduced with inducible *MYCN*<sup>WT</sup> *MYCN*<sup>P44L</sup> and *CMYC*. *MYCN*<sup>WT</sup> were also transduced with either empty vector or constitutive expression of *ALK*<sup>F1174L</sup>. iPSC were differentiated toward trunk neural crest cells implanted into the renal capsules of immunocompromised mice. Mice were euthanized when tumors grew to 2cm in diameter. Nucleic acids were extracted from flash frozen tumor tissue and submitted for RNA-seq and ChIP-seq analysis.

**Results:**

*ALK/MYCN* tumors had lower latency than *MYCN* tumors. Differential gene expression analysis revealed *ALK/MYCN* tumors, compared to *MYCN* tumors, upregulated genes involved in integrin signaling including *ITGB1* and *ITGB5*.

*MYCN*<sup>P44L</sup> and *CMYC* tumors were more penetrant than *MYCN*<sup>WT</sup> tumors. RNA and DNA have been extracted and submitted for RNA-seq and ChIP-seq analysis, respectively, to identify potential differences in genes driven by each transcription factor.

**Summary/Conclusions:**

Here, we show that human stem cell based models of neuroblastoma can identify important biological differences in how specific genetic mutations influence neuroblastoma tumorigenesis. In the case of *ALK/MYCN* tumors, our study suggests blocking integrin signaling may represent a new therapeutic strategy to treat tumors, particularly in tumors resistant to *ALK* inhibitors.

## **P175 Dynamic Contrast-Enhanced Perfusion MRI and Diffusion-Weighted Imaging as an imaging biomarker for neuroblastic tumors.**

*Cerdá Alberich, L.<sup>2</sup>, Martí-Bonmatí, L.M.B.<sup>3</sup>*

Health Research Institute La Fe, Biomedical Imaging Research Group, Valencia, Spain <sup>2</sup>, University and Polytechnic La Fe Hospital, Clinical Area of Medical Imaging, Valencia, Spain<sup>3</sup>

### Background/Introduction:

Neuroblastoma (NB) is the most frequent solid extracranial cancer in childhood. Its diagnosis, prognosis and monitoring are based on the information provided by multiparametric magnetic resonance (MR) and MIBG images.

### Aims:

Our focus in this work is to explore the utility of diffusion and perfusion changes in NB as an early biomarker of diagnosis, using diffusion weighted (DWI) and dynamic contrast enhanced (DCE) MRI.

### Methods/Materials:

Multiple MR imaging real-word sequences, available within the H2020 PRIMAGE project, were used from 50 patients. Volumes-of-interest were calculated and transferred to DCE perfusion and apparent diffusion coefficient (ADC) maps. Histogram analysis and clustering based unsupervised machine learning algorithms were used to determine the values of mean and standard deviation (SD) of initial area under the curve at 60 seconds (IAUC60) and ADC for automatic differentiation of neuroblastic tumors. The resolution of voxel intensity was estimated and the IAUC60 and ADC data was smeared accordingly to allow us to identify and remove the noise and low-quality voxels (defined as those voxels with an uncertainty larger than 10%).

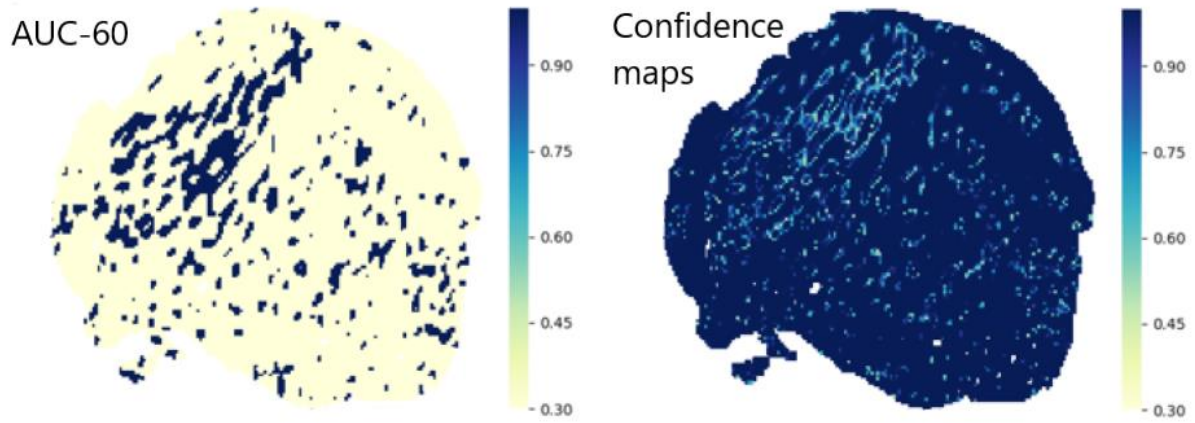
### Results:

Significant differences in mean ADC (expressed in  $10^{-3}$  mm<sup>2</sup>/s) were found for neuroblastic tumors: 1.2 for ganglioneuroma, 0.74 for ganglioneuroblastoma, and 0.54 for neuroblastoma, with an uncertainty of 37%, 43% and 20%, respectively. This result improves tumor differentiation with respect to state-of-the-art voxel-by-voxel methodologies, which were found to be: 1.6 for ganglioneuroma, 1.7 for ganglioneuroblastoma, and 1.3 for neuroblastoma, with an uncertainty of 45%, 63% and 23%, respectively. Mean IAUC60 was found to have a value of 43 (and 14% uncertainty) for neuroblastoma, as opposed to a value of 17 (and 17% uncertainty) with state-of-the-art voxel-by-voxel methodologies.

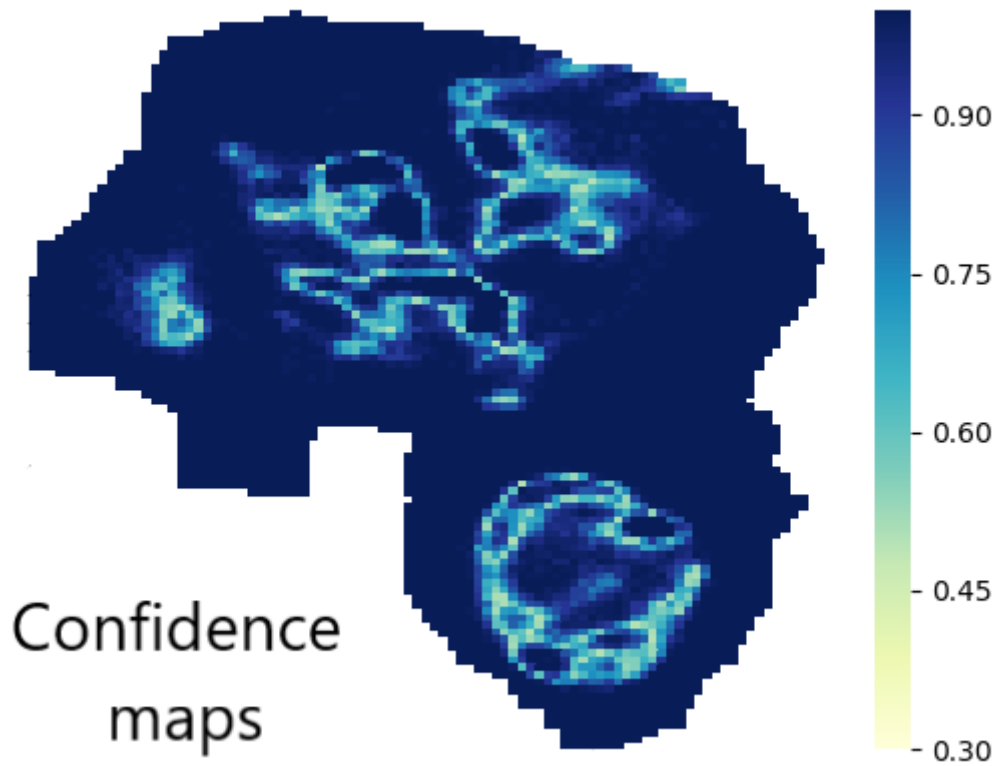
### Summary/Conclusions:

The proposed novel technique to determine IAUC60 and ADC parameters holds promise for differentiating benign and malignant neuroblastic tumors. This work lays the foundation for future advances in reproducible imaging biomarkers for paediatric solid tumor cancers.





Caption 1: Confidence maps based on the Apparent Diffusion Coefficient (ADC) to account for neuroblastic intra-tumor heterogeneity.



Caption 2: Representation of neuroblastic tumor habitats (left) and confidence maps based on the initial area under the curve after 60 seconds (iAUC-60) (right).

## **P176 WHOLE TRANSCRIPTOME PROFILING OF LIQUID BIOPSIES FROM TUMOR XENOGRAFTED MOUSE MODELS ENABLES SPECIFIC MONITORING OF TUMOR-DERIVED RNA**

*Vermeirssen, V.,<sup>1</sup> Deleu, J.<sup>2</sup>, Deleu, J.<sup>3</sup>, Everaert, C.<sup>4</sup>, Morlion, A.<sup>5</sup>, Verniers, K.<sup>6</sup>, Anckaert, J.<sup>7</sup>, Vandesompele, J.<sup>8</sup>, Van Maerken, T.<sup>9</sup>, Decock, A.<sup>10</sup>, De Wilde, B.<sup>11</sup>*

Ghent University, Biomedical Molecular Biology & Biomolecular Medicine, Ghent, Belgium<sup>1</sup>, Ghent University, Biomolecular Medicine, Ghent, Belgium<sup>2</sup>, Ghent University, Biomolecular Medicine, Ghent, Belgium<sup>3</sup>, Ghent University, Biomolecular Medicine, Ghent, Belgium<sup>4</sup>, Ghent University, Biomolecular Medicine, Ghent, Belgium<sup>5</sup>, Ghent University, Biomolecular Medicine, Ghent, Belgium<sup>6</sup>, Ghent University, Biomolecular Medicine, Ghent, Belgium<sup>7</sup>, Ghent University, Biomolecular Medicine, Ghent, Belgium<sup>8</sup>, Ghent University, Biomolecular Medicine & Clinical Biology, Ghent, Belgium<sup>9</sup>, Ghent University, Biomolecular Medicine, Ghent, Belgium<sup>10</sup>, Ghent University, Biomolecular Medicine & Pediatric Haematology, Oncology and Stem Cell Transplant, Ghent, Belgium<sup>11</sup>

### **Background/Introduction:**

Liquid biopsies enable disease diagnosis and treatment response monitoring. While the use of cell-free DNA is well established, extracellular RNA has particular advantages, mostly because of its dynamic nature. Besides free-floating (circular) RNA, RNA is also present in extracellular vesicles, lipo-protein complexes and platelets. More recently, the concept of tumor educated platelets was established whereby tumor-derived RNA ends up in platelets and the overall splicing pattern of platelet RNA is altered. In this study, we used tumor xenografts as an elegant model system, to assess to which extent tumor (human) RNA ends up in different blood (plasma) fractions. This will support the decision to select the fraction which is best suited to probe tumor-derived extracellular transcriptomes. Based on proof-of-concept data, we anticipate that monitoring through liquid biopsies will enable the assessment of therapeutic effects on tumor cells.

### **Aims:**

We aim to determine in which blood-derived plasma fraction (from tumor educated platelets to platelet-free plasma) resides the highest tumor-derived RNA concentration using xenograft model systems and to establish standardized experimental and computational procedures for plasma preparation and sequencing-based profiling of extracellular messenger RNA (mRNA).

### **Methods/Materials:**

We examined the circulating transcriptome of extracellular mRNA in two different subcutaneous xenograft models, i.e. SK-N-BE(2C) cells and the BRC016 breast cancer PDX model, as well as in non-tumor bearing control mice. From 5 mice per group, blood was collected via cardiac puncture using an EDTA tube, followed by platelet, platelet-rich, platelet-poor and platelet-free plasma preparation. Using 60 µl input volumes, an established total RNA sequencing workflow to profile extracellular mRNA (Everaert, Helsmoortel et al., 2019) with synthetic spike-in RNA for calibration purposes, and a dedicated data processing pipeline to unambiguously distinguish human (tumor) from murine (host) mRNA, we charted the extracellular transcriptomes.

### **Results:**

Total RNA sequencing of small volumes of biofluids with (partially) degraded RNA is technically challenging. The use of synthetic spike-in RNA and downsampling of sequencing libraries to equal depth (1 or 5 million reads, depending on the model) enable robust data comparison.

Despite the high RNA concentration variability among individual mice, we were able to detect human RNA in all plasma fractions in a ratio that was on average ten to hundred times lower than murine RNA. In line with the large RNA cargo in platelets, the murine RNA concentration in plasma increased with increasing platelet concentration. In contrast, human RNA concentrations were relatively

constant. As such, we did not find supporting evidence for preferential loading of tumor RNA in platelets.

Using small biofluid input volumes and low (down-sampled) sequencing depth, we detected up to 1500 human tumor-derived protein-coding genes and over 7000 murine genes. Functional exploration of the resulting transcriptomes revealed various enriched gene sets and pathways, indicative of biological signal. We are currently conducting neuroblastoma xenograft studies with targeted therapeutic agents, in order to assess whether on-target activity of the drug on the tumor cells can be monitored in plasma.

Summary/Conclusions:

We provide a firm proof-of-concept for the RNA-based detection of specific tumor signal in plasma from xenografted mice. We conclude that platelet-free plasma is the preferred liquid biopsy type for therapeutic response monitoring.

\*Vanessa Vermeirssen and Jill Deleu contributed equally to this work.

\*All authors are also affiliated to the *Cancer Research Institute Ghent (CRIG)*.

## **P177 Recurrent rare high level amplification of the BRISC and BRCA1-A complex member 2 (BABAM 2) gene in high-risk neuroblastoma**

*Durinck, K.*<sup>2</sup>, *Nunes, C.*<sup>3</sup>, *Everaert, C.*<sup>4</sup>, *Depuydt, P.*<sup>5</sup>, *De Preter, K.*<sup>6</sup>, *Bartenhagen, C.*<sup>7</sup>, *De Wilde, B.*<sup>8</sup>, *Fischer, M.*<sup>9</sup>, *Devalck, C.*<sup>10</sup>, *Speleman, F.*<sup>11</sup>, *Van Roy, N.*<sup>12</sup>

Ghent University, Department of Biomolecular Medicine, Ghent, Belgium<sup>2</sup>, Ghent University, Department of Biomolecular Medicine, Ghent, Belgium<sup>3</sup>, Ghent University, Department of Biomolecular Medicine, Ghent, Belgium<sup>4</sup>, Ghent University, Department of Biomolecular Medicine, Ghent, Ghent University<sup>5</sup>, Ghent University, Department of Biomolecular Medicine, Ghent, Belgium<sup>6</sup>, Center for Molecular Medicine Cologne, Experimental pediatric oncology, Cologne, Germany<sup>7</sup>, Ghent University Hospital, Department of Pediatric Hematology-Oncology and Stem Cell Transplantation, Ghent, Belgium<sup>8</sup>, Center for Molecular Medicine Cologne, Experimental Pediatric Oncology, Cologne, Germany<sup>9</sup>, Queen Fabiola University Hospital for children, Department of Pediatric Hematology-Oncology, Brussels, Belgium<sup>10</sup>, Ghent University, Department of Biomolecular Medicine, Ghent, Belgium<sup>11</sup>, Ghent University, Biomolecular Medicine, Ghent, Belgium,<sup>12</sup>

### Background/Introduction:

Neuroblastoma (NB) is a childhood cancer characterized by recurrent copy number aberrations and a low mutational burden. Around 25% of the tumors, usually high stage, present with high level amplification of the *MYCN* gene, one of the most important genetic markers in terms of prognosis and treatment impact, together with other rare but recurrent amplifications. The prognosis of children with NB tumors carrying amplified genes is unfavorable. Previous studies from our lab and others have shown that rare recurrent amplicons harbour genes critically involved in NB, including *MYC*, *LIN28B*, *ALK*, *MDM2* and *CDK4*.

### Aims:

Unraveling the nature of a novel rare recurrent amplicon encompassing the *BABAM2* locus in neuroblastoma

### Methods/Materials:

Shallow whole genome sequencing was performed to map copy number imbalances. Whole genome DNA and RNA sequencing were done respectively to unravel the nature of the amplicon and to determine RNA expression level and identify alternative or fusion transcripts. In search for additional cases with focal *BABAM2* amplification, TARGET and TCGA databases were mined.

### Results:

A first case of NB with high level amplification of the exons 4-5 of the *BABAM2* coding region was identified in a high stage neuroblastoma of a 7-month old infant diagnosed presenting with liver metastases, but without bone marrow involvement. Shallow whole genome sequencing of the tumor revealed the presence of typical segmental aberrations including *MYCN* amplification, 1p deletion, 2p gain (including *ALK*), 17q gain in addition to whole chromosome imbalances (losses of whole chromosome 9, 16, 19 and X and gain of chromosome 22). Whole genome sequencing was performed to study the *BABAM2* amplicon in further detail and revealed an integration of *BABAM2* exon 4 into the *MYCN* amplicon. RNA sequencing showed an overexpression of *BABAM2*. Comparison of the RNA sequencing profile of this case with >500 other NB cases led to the identification of an extra NB case with an amplicon encompassing *BABAM2* and *ALK*. In addition, database mining revealed another NB case with *BABAM2* amplification and one case of pheochromocytoma/paraganglioma. The *BABAM2* protein acts as an adapter in several protein complexes, including the BRCA1-A complex and the BRISC complex, involved in DNA double strand break repair and mitotic spindle assembly, thereby providing new therapeutic opportunities. The *BABAM2* gene is upregulated in several types of cancer, including ovarian cancer and in AML with 11q23 rearrangements and t(8;16), where *BABAM2* was found to be overexpressed due to intragenic transcription initiation.

Summary/Conclusions:

In this study, we describe the first neuroblastoma cases presenting with amplification of the *BABAM2* gene. A systematic search revealed this alteration to be rare but recurrent strongly suggesting a tumor driving role in NB.

## **P178 Angiogenic potential of small extracellular vesicles in drug resistant neuroblastoma**

Frawley, T.<sup>2</sup>

Royal College of Surgeons, Pharmacy and Biomolecular Sciences, Dublin, Ireland <sup>2</sup>

### Background/Introduction:

Neuroblastoma is a paediatric cancer of the sympathetic nervous system and the most common solid tumour of infancy which accounts for approximately 15% of all paediatric oncology deaths. The main challenge in treating high risk neuroblastoma is metastatic spread and development of chemotherapeutic resistance. Extracellular vesicles are membrane bound vesicles containing bioactive molecules including RNAs and proteins that play an important role in cancer through cell-cell communication. We hypothesise that the development of cisplatin resistance in neuroblastoma cells increases the angiogenic potential and alters the proteome of their small extracellular vesicles (sEV).

### Aims:

### Methods/Materials:

The cell lines Kelly (cisplatin sensitive) and KellyCis83 (cisplatin resistant) were cultured in high density bioreactors with sEV depleted media, which was harvested weekly for sEV isolation by differential centrifugation. sEV were characterised by Western blot analysis for positive protein markers HSP70, TSG101 and CD9, and the negative marker VDAC-1. Nano-particle tracking analysis (NTA) and transmission electron microscopy (TEM) was performed to characterise sEV size range and morphology, respectively. To assess angiogenic potential, human umbilical vein endothelial cells (HUVEC) were incubated with Kelly and KellyCis83 sEV on Matrigel and tubule formation and length were measured at 8 and 24 hours.

### Results:

Kelly and KellyCis83 sEV were positive for HSP70, TSG101 and CD9 and negative for VDAC-1 by Western blot. TEM revealed spherical membrane bound particles with a NTA consistent size profile between 80-200nm. There was a significant increase in the number and length of tubules formed in HUVECs treated with KellyCis83 sEV compared to Kelly sEV and untreated HUVECs.

### Summary/Conclusions:

Our data indicates that the development of cisplatin resistance results in the secretion of sEV that bear an increased angiogenic potential compared to cisplatin sensitive cells. In future studies we aim to investigate the sEV proteome of Kelly and KellyCis83 cells to identify which proteins are involved in driving this angiogenic potential.

## **P179 MYCN mediated metabolic processes in childhood neuroblastoma**

*Arsenian Henriksson, M.*<sup>2</sup>, *Oliynyk, G.*<sup>3</sup>, *Ruiz-Pérez, M.*<sup>4</sup>, *Liu, T.*<sup>5</sup>, *Sainero Alcolado, L.*<sup>6</sup>, *Rodriguez Garcia, A.*<sup>7</sup>, *Zirath, H.*<sup>8</sup>, *Dzieran, J.*<sup>9</sup>

Karolinska Institutet, Microbiology and Tumor Biology (MTC), Stockholm, Sweden <sup>2</sup>, Karolinska Institutet, Microbiology, Tumor and Cell Biology (MTC), Stockholm, Sweden<sup>3</sup>, Karolinska Institutet, Microbiology, Tumor and Cell Biology (MTC), Stockholm, Sweden<sup>4</sup>, Karolinska Institutet, Microbiology, Tumor and Cell Biology (MTC), Stockholm, Karolinska Institutet<sup>5</sup>, Karolinska Institutet, Microbiology, Tumor and Cell Biology (MTC), Stockholm, Sweden<sup>6</sup>, Karolinska Institutet, Microbiology, Tumor and Cell Biology (MTC), Stockholm, Sweden<sup>7</sup>, Karolinska Institutet, Microbiology, Tumor and Cell Biology (MTC), Stockholm, Sweden<sup>8</sup>, Karolinska Institutet, Microbiology, Tumor and Cell Biology (MTC), Stockholm, Sweden<sup>9</sup>

### Background/Introduction:

Neuroblastoma (NB) is one of the most aggressive solid tumors of early childhood. Amplification of the *MYCN* oncogene can be found in around 30% of patients and is associated with rapid tumor progression and poor prognosis. In spite of treatment development, overall and event-free survival for high-risk neuroblastoma patients remains around 40-50%, and survivors often suffer long-term side effects. Alternative approaches, as differentiation-inducing therapies, are especially interesting for high-risk patients in which resistance to conventional chemotherapy arises.

We have shown that a small chemical molecule, 10058-F4, previously identified as a c-MYC inhibitor also targets the *MYCN*/MAX complex resulting in apoptosis and neuronal differentiation in *MYCN*-amplified NB cells. Importantly, we demonstrated that inhibition of MYC results in metabolic changes including mitochondrial dysfunction leading to accumulation of lipid droplets (Zirath 2013).

We have previously also shown that the *miR-17-92* locus is directly activated by *MYCN*, highlighting the importance of this cluster in tumorigenesis. We have identified several nuclear hormone receptors including estrogen receptor alpha (ER $\alpha$ ) and the glucocorticoid receptor (GR) as targets of *miR-17-92* and have demonstrated their connection to neural differentiation and that overexpression of ER $\alpha$  led to reduced tumor burden (Lovén 2010; Ribeiro 2016; Dzieran 2018).

### Aims:

To study the impact of *MYCN* on neuroblastoma cell metabolism and the possible synergy of combined activation of estrogen receptor alpha and the glucocorticoid receptor for neuronal differentiation.

### Methods/Materials:

Here, we have combined proteomics, gene expression profiling, functional analysis, and metabolic tracing as well as *in vitro* differentiation analysis and xenograft studies.

### Results:

Analysis of protein and gene expression highlighted changes in key enzymes involved in glycolysis and  $\beta$ -oxidation in *MYCN*-amplified NB. Metabolic profiling showed that these cells display both enhanced glycolytic and respiratory capacity compared to non-*MYCN* amplified NB cells. In response to high energy demand conditions *MYCN*-amplified cells significantly increased the glycolytic rate and the mitochondrial capacity. In addition, we discovered that the cells showed *de novo* glutamine synthesis. Importantly, we found that high expression of the key enzyme of  $\beta$ -oxidation, CPT1, correlates with reduced survival of NB patients and that inhibition of CPT1 with etomoxir reduced tumor burden in NB xenografts *in vivo* (Oliynyk 2019). We are now further examining the contribution of glutamine and glucose to fatty acids in high versus low *MYCN* expressing neuroblastoma cells by metabolic tracing followed by mass spectrometry analysis. We are also analysing a second inhibitor of CPT1, teglicar, in neuroblastoma xenograft experiments.

We have injected GR-overexpressing neuroblastoma cells into mice and show that high levels of GR reduces tumor growth. We further found that coactivation of ER $\alpha$  and GR in BE(2)-GR/ER $\alpha$  cells resulted in a similar robust neurite outgrowth as with retinoic acid treatment alone. Importantly, the neuronal differentiation markers TH and p75<sup>NTR</sup> were upregulated by estradiol (E2) but not by dexamethasone (dex). Furthermore, TH and p75<sup>NTR</sup> expression levels were higher by E2+dex treatment than by E2 treatment alone. Collectively, these data show that co-expression of GR and ER $\alpha$  possess synergistic effects on neuronal differentiation in *MYCN*-amplified NB cells.

#### Summary/Conclusions:

Taken together, our findings provide novel information on *MYCN*-mediated high-energetic metabolic phenotype, which can be a possible mechanism of cancer cell survival during NB pathogenesis. We also present novel data on induction of neuronal differentiation in neuroblastoma. These new knowledge may form the basis for novel cancer therapies for children with neuroblastoma.



## **P180 VISIOMICS – a docker-based platform supporting the exploration and integrated analysis of multiOMICs data for tumor diagnostics**

*Kromp, F.*<sup>2</sup>, *Lazic, D.*<sup>3</sup>, *Bozsaky, E.*<sup>4</sup>, *Rifatbegovic, F.*<sup>5</sup>, *Abbasi, R.*<sup>6</sup>, *Popitsch, N.*<sup>7</sup>, *Kauer, M.*<sup>8</sup>, *Poetsch, A.*<sup>9</sup>, *Mivalt, F.*<sup>10</sup>, *Fischer, L.*<sup>11</sup>, *Kopetzky, T.*<sup>12</sup>, *Eghbal-Zadeh, H.*<sup>13</sup>, *Buehler, K.*<sup>14</sup>, *Alemzadeh, S.*<sup>15</sup>, *Matkovic, K.*<sup>16</sup>, *Kriegner, A.*<sup>17</sup>, *Palme, J.*<sup>18</sup>, *Dulovijs, M.*<sup>19</sup>, *Hanbury, A.*<sup>20</sup>, *Lupu, M.*<sup>21</sup>, *Taha, A.A.*<sup>22</sup>, *Bampoulidis, A.*<sup>23</sup>, *Ostalecki, C.*<sup>24</sup>, *Brandstötter, M.*<sup>25</sup>, *Atanasov, M.*<sup>26</sup>, *Gerber, T.*<sup>27</sup>, *Beneder, M.*<sup>28</sup>, *Ziegler, A.*<sup>29</sup>, *Fiedler, S.*<sup>30</sup>, *Ladenstein, R.*<sup>31</sup>

Children's Cancer Research Institute, Tumor biology, Vienna, Austria <sup>2</sup>, Children's Cancer Research Institute, Tumor biology, Vienna, Austria<sup>3</sup>, Children's Cancer Research Institute, Tumor biology, Vienna, Austria<sup>4</sup>, Children's Cancer Research Institute, Tumor biology, Vienna, Children's Cancer Research Institute<sup>5</sup>, Children's Cancer Research Institute, Clinical genetics, Vienna, Austria<sup>6</sup>, Children's Cancer Research Institute, Bioinformatics, Vienna, Austria<sup>7</sup>, Children's Cancer Research Institute, Bioinformatics, Vienna, Austria<sup>8</sup>, Children's Cancer Research Institute, Bioinformatics, Vienna, Austria<sup>9</sup>, Children's Cancer Research Institute, Tumor biology, Vienna, Austria<sup>10</sup>, Software Competence Center Hagenberg, Knowledge-based Vision Systems, Hagenberg, Austria<sup>11</sup>, Software Competence Center Hagenberg, Knowledge-based Vision Systems, Hagenberg, Austria, <sup>12</sup>, Johannes Kepler University of Linz, Institute of Computational Perception, Linz, Austria <sup>13</sup>, VrVis Center for Virtual Reality and Visualization GmbH, Biomedical image informatics, Vienna, Austria <sup>14</sup>, VrVis Center for Virtual Reality and Visualization GmbH, Biomedical image informatics, Vienna, Austria <sup>15</sup>, VrVis Center for Virtual Reality and Visualization GmbH, Interactive visualization, Vienna, Austria <sup>16</sup>, Platomics GmbH, -, Vienna, Austria <sup>17</sup>, Platomics GmbH, -, Vienna, Austria <sup>18</sup>, Platomics GmbH, -, Vienna, Austria <sup>19</sup>, TU Wien, Institute of Information Systems Engineering, Vienna, Austria <sup>20</sup>, Research Studios Austria FG, Data Science, Vienna, Austria <sup>21</sup>, Research Studios Austria FG, Data Science, Vienna, Austria <sup>22</sup>, Research Studios Austria FG, Data Science, Vienna, Austria <sup>23</sup>, Universitätsklinikum Erlangen, Department of Dermatology, Erlangen, Germany <sup>24</sup>, CogVis Software and Consulting GmbH, -, Vienna, Austria <sup>25</sup>, CogVis Software and Consulting GmbH, -, Vienna, Austria <sup>26</sup>, Children's Cancer Research Institute, Tumor biology, Vienna, Austria<sup>27</sup>, Children's Cancer Research Institute, Tumor biology, Vienna, Austria <sup>28</sup>, Children's Cancer Research Institute, Tumor biology, Vienna, Austria<sup>29</sup>, Children's Cancer Research Institute, Tumor biology, Vienna, Austria<sup>30</sup>, Children's Cancer Research Institute, Studies&Statistics for Integrated Research and Projects, vienna <sup>31</sup>

### **Background/Introduction:**

In cancer diagnostics tumor sub-grouping, risk stratification and/or survival prediction typically involve either genomics, gene expression or epigenetic analysis. If combined with bioimaging and clinical data and integrated based on expert knowledge and statistical methods, multi-scalar genomics (multiOMICs) analyses have demonstrated feasibility in terms of time-to-analysis and promise to improve patient outcome. However, there is an urgent need, but lack of platforms supporting a technical and biological data integration.

### **Aims:**

The aim was to build a platform allowing integration and visualization of multiOMICs, cell/tissue imaging and clinical datasets as a basis to explore biological data, to unravel yet unidentified causal relations and to finally translate the gained knowledge into certified clinical analysis workflows.

### **Methods/Materials:**

We use state-of-the-art technologies to develop a datastore to host raw data files and metadata information as output from multiOMICs data analysis and tissue image analysis. Image-based deep learning frameworks are used to extract marker expression/gene features from interphase fluorescence in situ hybridization (iFISH) and multi-epitope ligand cartography (MELC) images whereas single nucleotide polymorphism array (SNPa)-based deep learning frameworks are developed to support efficient genomic data annotation. Based on statistical methods for feature

selection and correlation analysis and combined with innovative concepts for visual analytics, tools allowing an explorative visualisation and analysis of multiOMICs, clinical and imaging datasets are developed.

#### Results:

We established a modular system to address biological research questions for cancer diagnostics consisting of dockerized components, orchestrated by a central workflow manager. Data extraction modules, including bioinformatics pipelines, image processing pipelines and genomics data annotation workflows, extract and store linkable data from all data sources in a central database. The system then allows integration and visual exploration of multiOMICs data using a data integration module and a visual analytics framework embedded in a React-based webclient. All developed modules interact using REpresentational State Transfer (REST) interfaces and are being integrable into certifiable clinical diagnostic workflows.

#### Summary/Conclusions:

In VISIOMICS, experts from biology, bioinformatics, data analysis, bioimage analysis, deep learning and visual analytics use state-of-the-art methods to develop a platform achieving multiOMICs and histopathology data integration and visualization gaining new knowledge and serving as basic components to develop certifiable clinical diagnostic workflows.

## **P181 Defining the effects of polyamine synthesis inhibition on the neuroblastoma immune microenvironment**

*Benavides, A.*<sup>1</sup>, *Bassiri, H.*<sup>2</sup>, *Karageorgos, S.*<sup>3</sup>, *Khurana, P.*<sup>4</sup>, *Vu, A.*<sup>5</sup>, *Ferry, G.*<sup>6</sup>, *Ziegler, A.*<sup>7</sup>, *Norris, M.*<sup>8</sup>, *Haber, M.*<sup>9</sup>, *Silverman, M.A.*<sup>10</sup>, *Planet, P.J.*<sup>11</sup>, *Hogarty, M.*<sup>12</sup>, *Bassiri, H.*<sup>13</sup>

American Association for Cancer Research, Cancer Immunology Research journal, Philadelphia, USA<sup>1</sup>, Children's Hospital of Philadelphia, Pediatrics, Philadelphia, USA<sup>2</sup>, European University Cyprus, Pediatrics, Engomi, Cyprus<sup>3</sup>, University of Pennsylvania, Biomedical Graduate Studies, Philadelphia, USA<sup>4</sup>, Children's Hospital of Philadelphia, Oncology, Philadelphia, Children's Hospital of Philadelphia<sup>5</sup>, University College London, Institute of Child Health, London, England<sup>6</sup>, St. Anna Children's Cancer Research Institute, Tumor Biology, Vienna, Austria<sup>7</sup>, Children's Cancer Institute, Professor and Deputy Director, Sydney, Australia<sup>8</sup>, Children's Cancer Institute, Embryonal Cancer Therapeutics and Prevention, Sydney, Australia<sup>9</sup>, , Nederland<sup>10</sup>, Nederland<sup>11</sup>, Children's Hospital of Philadelphia, Pediatrics, Philadelphia, USA, <sup>12</sup>, Children's Hospital of Philadelphia, Pediatrics, Philadelphia, USA <sup>13</sup>

### Background/Introduction:

*MYCN* remains the primary oncogenic driver, and *MYC* signaling is often deregulated in high-risk neuroblastoma (NB). Although direct *MYC* antagonism has not been achievable, *MYC* regulates several downstream cellular modules (including polyamine (PA) metabolism), that are druggable. Indeed, the FDA-approved drug difluoromethylornithine (DFMO) significantly decreases PA synthesis by irreversibly inactivating the enzyme ornithine decarboxylase. In a preclinical model of *MYCN*-driven high-risk NB (*TH-MYCN*<sup>+/+</sup> transgenic mice), we find that DFMO significantly extends survival. We hypothesized that in addition to its tumor-intrinsic effects, DFMO may be altering the tumor microenvironment (TME) to enhance antitumor immunity.

### Aims:

To gain a better understanding of the ways in which DFMO alters the NB TME, we set out to characterize the frequencies and functional capacities of the hematopoietic cells that infiltrate the tumors of *TH-MYCN*<sup>+/+</sup> transgenic mice.

### Methods/Materials:

Tumors from *TH-MYCN*<sup>+/+</sup> transgenic mice were excised and subjected to mechanical and enzymatic dissociation. Single cell suspensions were subjected to multiparameter flow cytometry using a variety of panels aimed at determining the frequencies of live CD45<sup>+</sup> cells and their expression of markers of functional capacity. Cytokine levels were assayed using quantitative PCR and ELISA. The microbiota of DFMO-treated and untreated mice were assayed using 16S rRNA deep sequencing.

### Results:

We find that DFMO administration results in multiple alterations in the frequency and phenotype of tumor-infiltrating immune cells. Given DFMO's known anti-protozoal activity, we examined the microbiota of the respiratory and intestinal tracts of treated and untreated mice; these analyses revealed no significant changes in the microbiota of DFMO-treated mice when compared to age-matched untreated mice, suggesting that the observed TME effects were not driven by microbiome alterations. Most notable amongst the changes induced by DFMO in the tumor-infiltrating hematopoietic cells, was a dose-dependent increase in the frequency of natural killer (NK) cells. Additionally, while NK cells in untreated mice displayed significantly decreased expression of the stimulatory receptor NKG2D, DFMO partially restored this loss. Concomitantly,

tumor cells from DFMO-treated mice expressed higher levels of Rae1- $\gamma$ , a known ligand for NKG2D. Decreases in NKG2D expression had previously been described in human NK cells exposed to transforming growth factor beta 1 (TGF $\beta$ 1), a multifunctional cytokine that is produced in several solid tumors including NB. TGF $\beta$ 1 is synthesized as a pro-peptide that undergoes further processing for generation of its free (and active) form. Our preliminary analyses indicate

that DFMO-treated and untreated tumors express similar levels of TGF $\beta$ 1 transcripts, yet treated tumors contain lower concentrations of free TGF $\beta$ 1 protein. Studies are now ongoing to determine how DFMO treatment alters the processing of pro-TGF $\beta$ 1 to its active form.

#### Summary/Conclusions:

These findings collectively demonstrate that DFMO increases intra-tumoral NK cell frequencies and protects them from loss of anti-tumor activity. Understanding the mechanisms by which interference with PA synthesis results in reduction in intra-tumoral NK cell immunosuppression bears significant clinical relevance, as dinutuximab is believed to exert its effects via NK cell-mediated antibody-dependent cellular cytotoxicity. Future studies will be aimed at determining if inhibition of TGF $\beta$ 1 activity could synergize with PA interference to enhance NK cell-mediated antitumor immunity in NB.

## **P182 MYC transcription activation mediated by OCT4 as a mechanism of resistance to 13-cisRA-induced differentiation in neuroblastoma**

*Kang, M.*<sup>2</sup>, *Wei, S.*<sup>3</sup>, *Nguyen, T.*<sup>4</sup>, *Mook, D.*<sup>5</sup>, *Makena, M.*<sup>6</sup>, *Verlekar, D.*<sup>7</sup>, *Hindle, A.*<sup>8</sup>, *Martinez, G.*<sup>9</sup>, *Yang, S.*<sup>10</sup>, *Shimada, H.*<sup>11</sup>, *Reynolds, P.*<sup>12</sup>

Texas Tech University Health Sciences Center, Pediatrics, Lubbock, USA<sup>2</sup>, Texas Tech University Health Sciences Center, Pediatrics, Lubbock, USA<sup>3</sup>, Texas Tech University Health Sciences Center, Pediatrics, Lubbock, USA<sup>4</sup>, Texas Tech University Health Sciences Center, Pediatrics, Lubbock, Texas Tech University Health Sciences Center<sup>5</sup>, The Johns Hopkins University, Physiology, Baltimore, USA<sup>6</sup>, Texas Tech University Health Sciences Center, Pediatrics, Lubbock, USA<sup>7</sup>, Texas Tech University Health Sciences Center, Pediatrics, Lubbock, USA<sup>8</sup>, Texas Tech University Health Sciences Center, Pediatrics, Lubbock, USA<sup>9</sup>, Pennington Biomedical Research Center, Pathology, Baton Rouge, USA<sup>10</sup>, Stanford University, Pathology, Stanford, USA<sup>11</sup>, Texas Tech University Health Sciences Center, Pediatrics, Lubbock, USA,<sup>12</sup>

### Background/Introduction:

*MYCN* genomic amplification is one of the risk factors in neuroblastoma. 13-*cis* retinoic acid (13-*cis*RA), a differentiating agent, down-regulates *MYCN* expression and is part of neuroblastoma maintenance therapy. Despite the improvement in clinical outcome from maintenance therapy of neuroblastoma given in first response with 13-*cis*RA and anti-GD2 monoclonal antibody plus cytokine, ~40% of high-risk neuroblastoma patients still die of recurrent disease. Although *MYC* genomic amplification is rare in neuroblastoma (~1%), 11% of neuroblastoma primary tumors collected at diagnosis have high c-MYC protein suggesting that *MYC* transcriptional activation rather than genomic amplification drives such tumors.

### Aims:

We sought to investigate the role of *MYC* oncogene in 13-*cis*RA resistance and to molecularly characterize mechanisms of *MYC* expression in neuroblastoma.

### Methods/Materials:

All neuroblastoma models were obtained from the COG repository for cell lines and xenografts (<http://www.CCcells.org>). Transcription factors associated with increased expression of c-MYC in 13-*cis*RA-resistant neuroblastoma cell lines were identified using protein/DNA hybridization arrays. Binding of OCT4 to the MYC enhancer/promoter region was identified and confirmed by EMSA and ChIP assay. The binding kinases to OCT4, MAPKAPK2 (MK2) and DNA-PK, and the phosphorylation sites of OCT4 were identified by mass spectrometry. RNAi-mediated gene silencing was used to validate the MK2-OCT4-MYC pathway.

### Results:

Neuroblastoma cell lines selected for resistance to 13-*cis*RA showed low *MYCN* and high c-MYC expression. Transcriptional activation of *MYC* mediated by OCT4 (encoded by *POU5F1*) functionally replaced *MYCN* in 13-*cis*RA-resistant cell lines. In neuroblastoma patient-derived cell lines (PCLs), 19 established at diagnosis (Dx) and 16 at progressive disease (PD), and patient-derived xenografts (PDXs) (8 Dx and 9 PD), we confirmed that c-MYC expression levels were higher in PD relative to Dx lines ( $P = 0.0005$ ). We identified OCT4 and TCF3 as transcription factors highly expressed in neuroblastoma cells with high c-MYC expression. We then identified two novel OCT4-binding sites (including OBS1 and OBS2) located in the *MYC* promoter/enhancer region: -1209 to -1140 and found that OCT4 NH<sub>2</sub>-terminal domain (NTD) and POU specific domain (POUs) are critical for *MYC* transcriptional activation. To identify kinases that are associated with OCT4-induced c-MYC activation, we used mass spectrometry and PhosphoMotif Finder<sup>®</sup> and identified MK2 as one of the upstream kinases that can bind to and directly regulate the OCT4 biological function by phosphorylation at its amino acid Ser<sup>111</sup> residue to transcriptionally activate *MYC* expression. RNA expression in 175 *MYCN* non-amplified high-risk primary tumors (TARGET database) showed that

*MAPKAPK2* positively correlated with *MYC* expression ( $P < 0.001$ ) and overall survival was lower ( $P < 0.001$ ) for patients with high *MAPKAPK2*. Also, OCT4, MK2, and c-MYC were higher in PD relative to Dx neuroblastoma PCLs and PDXs. Functional studies by gene knockdown of the *POU5F1* or *MAPKAPK2* using shRNAs showed decreased c-MYC expression, inhibition of cell proliferation, and restoring growth arrest and neurite outgrowth in response to 13-*cisRA*.

Summary/Conclusions:

High c-MYC expression, independent of genomic amplification, is associated with disease progression and resistance to 13-*cisRA* in neuroblastoma. The MK2-mediated OCT4 transcriptional activation is a novel mechanism for *MYC* activation in PD neuroblastoma and provides a potential therapeutic target.

## **P183 Characterization of the anti-tumor effects of Diphenyleneiodonium Chloride in *MYCN*-amplified neuroblastoma**

Halasz, M.<sup>2</sup>, Abdul Aziz, A.A.<sup>3</sup>, Maher, S.<sup>4</sup>

University College Dublin, Systems Biology Ireland, School of Medicine, Dublin, Ireland <sup>2</sup>, University College Dublin, Systems Biology Ireland, School of Medicine, Dublin, Ireland<sup>3</sup>, University College Dublin, Systems Biology Ireland, School of Medicine, Dublin, Ireland<sup>4</sup>

### Background/Introduction:

Neuroblastoma is one of the deadliest childhood cancers. Despite extensive research, the prognosis for children with high-risk neuroblastoma remains as low as 50%. Amplification of *MYCN* drives half of the high-risk cases. Previously, we reported that Diphenyleneiodonium Chloride (DPI) induces apoptosis and differentiation of *MYCN*-amplified neuroblastoma cells and inhibits NB growth both *in vitro* and *in vivo* in zebrafish.

### Aims:

In this project, our aim was to shed light on the mechanisms of action of DPI in *MYCN*-amplified neuroblastoma cells.

### Methods/Materials:

We applied mass spectrometry-based label-free quantitative proteomics and phosphoproteomics to study the DPI induced molecular changes in *MYCN* amplified neuroblastoma cells.

### Results:

We found that DPI interferes with cell cycle progression and reduces *MYCN* levels in addition to the induction of mitochondrial superoxide-mediated apoptosis in *MYCN*-amplified neuroblastoma cells.

### Summary/Conclusions:

Diphenyleneiodonium Chloride is a potential novel treatment strategy for the therapy of *MYCN*-amplified neuroblastoma.

## **P184 ABCC3 drug transporter in Neuroblastoma cell invasion**

Milazzo, G.<sup>2</sup>, Mercatelli, D.<sup>3</sup>, De Rosa, P.<sup>4</sup>, Di Muzio, G.<sup>5</sup>, Di Giacomo, S.<sup>6</sup>, Ciaccio, R.<sup>7</sup>, Perini, G.<sup>8</sup>, Giorgi, F.<sup>9</sup>

University of Bologna, FaBiT, Bologna, Italy <sup>2</sup>, University of Bologna, FaBiT, Bologna, Italy<sup>3</sup>, University of Bologna, FaBiT, Bologna, Italy<sup>4</sup>, University of Bologna, FaBiT, Bologna, University of Bologna<sup>5</sup>, University of Bologna, FaBiT, Bologna, Italy<sup>6</sup>, University of Bologna, FaBiT, Bologna, Italy<sup>7</sup>, University of Bologna, FaBiT, Bologna, Italy<sup>8</sup>, University of Bologna, FaBiT, Bologna, Italy<sup>9</sup>

### Background/Introduction:

Neuroblastoma, a neural crest derived tumor, is one of the most common extra cranial solid cancer in childhood. The amplification of *MYCN* oncogene is the most important marker of aggressive neuroblastomas. This subgroup of tumors often shows a multiple drug resistant phenotype and although many high-risk neuroblastoma tumors initially respond to the first cycles of intensive chemotherapy, they frequently become refractory to treatment as the disease progresses. Chemoresistance can be due to several cellular mechanisms, including those related to altered activity of specific ATP-binding cassette (ABC) drug transporters. ABC transporters shuttle hydrophobic lipophilic compounds across cell membranes in an ATP-dependent manner. ABCC3 is one of the most de-regulated ABC transporter gene whose expression is strongly down-regulated in case of high *MYCN* levels.

### Aims:

To test if the low ABCC3 expressing neuroblastoma cells SK-N-BE 2c could change their invasivity potential, we've measured invasiveness by transwell-matrigel invasion assay of neuroblastoma cells over-expressing both ABCC3 wt and the ATP binding site mutant. Transcriptomic analysis has been performed by a Nimblegen oligonucleotide microarray and QRT-PCR assay has been used to validate microarray data. Bioinformatic analyses were performed to determine which specific pathways are significantly de-regulated in case of ABCC3 WT over-expression when compared with both control and ABCC3 ATP binding site mutant cells.

### Methods/Materials:

We've measured invasiveness of cells over-expressing ABCC3 by transwell-matrigel invasion assay. Transcriptomic analysis has been performed by a Nimblegen oligonucleotide microarray. QRT-PCR and luciferase gene reporter assays have been used to validate microarray data. We've also analysed the potential perturbation of key Transcription Factor activity by using the VIPER algorithm (Virtual Inference of Protein-activity by Enriched Regulon analysis). Dichlorofluorescein-diacetate assay has been employed to measure intracellular ROS level.

### Results:

*MYCN* amplified neuroblastoma cell clones stably over-expressing ABCC3\_WT coding sequence were found to be less invasive in-vitro if compared to control cells. Gene ontology analysis of microarray data have revealed a strong de-regulation of genes involved in cell migration/invasion pathways. Among them, MET is the most down-regulated gene. Met encodes for the Hepatocyte Growth Factor Receptor (HGFR) whose expression have been largely demonstrated to be strictly correlated to cell invasiveness and advance stage of many types of tumors. Transcription factor network analysis (VIPER) has shown multiple de-regulated TF-networks including key cancer related factors such as HAND2, GATA3 and TWIST1.

### Summary/Conclusions:

High level of *MYCN* expression in tumors may trigger an altered ABC transporter expression profile, hence contributing to the control of drug and physiological substrate efflux in cancer cells. Further investigations on *MYCN*-ABCC3 axis could reveal key druggable pathways correlated with metastatic behavior of neuroblastoma cells. Our results suggest that *MYCN* amplification and the resulting



ABCC3 repression lead to an aggressive cell phenotype due in part to the inhibition of the transporter function of ABCC3. High level of *MYCN* expression in neuroblastoma tumours may trigger an altered ABC transporter expression profile, hence contributing to the control of drug and physiological substrate efflux in cancer cells. Further investigations on *MYCN*-ABCC3 axis could reveal key druggable pathways correlated with metastatic behaviour of neuroblastoma cells.

## **P185 Comparative Genomic Analysis of High Risk Neuroblastoma at Diagnosis and Relapse in Beat Childhood Cancer Consortium Trials**

*Nagulapally, A.B.<sup>2</sup>, DYKEMA, K.<sup>3</sup>, Lewis, E.<sup>4</sup>, Ferguson, W.<sup>5</sup>, M. Kraveka, J.<sup>6</sup>, Sholler, G.<sup>7</sup>*

SPECTRUM HEALTH, PEDIATRIC ONCOLOGY, Grand Rapids, USA <sup>2</sup>, SPECTRUM HEALTH, PEDIATRIC ONCOLOGY, Grand Rapids, USA<sup>3</sup>, SPECTRUM HEALTH, PEDIATRIC ONCOLOGY, Grand Rapids, USA<sup>4</sup>, Saint Louis University, PEDIATRIC ONCOLOGY, St.Louis, Saint Louis University<sup>5</sup>, Medical university of south carolina, Pediatric oncology, Charleston, USA<sup>6</sup>, Spectrum health, Pediatric oncology, Grand Rapids, USA<sup>7</sup>

### **Background/Introduction:**

Despite recent advances, high risk neuroblastoma (NB) accounts for 15% of all pediatric cancer deaths. Many patients will attain remission but approximately half of all patients relapse within five years of diagnosis. Those who relapse often transiently respond to additional therapy but have a high rate of subsequent relapse. Further genomic understanding and novel therapies are needed.

### **Aims:**

Compare genomic analysis of high-risk NB at diagnosis with those at relapse. Characterize differences in DNA and transcriptomes and evaluation of novel subgroups.

### **Methods/Materials:**

Genomic Analysis was performed on patients enrolled on Beat Childhood Cancer Research Consortium trials, Molecular Guided Therapy NMTRC008/009 study “Feasibility Trial Using Molecular Guided Therapy for the Treatment of Patients with Relapse and Refractory Childhood Cancer” or “PEDI-PLAN – Pediatric Precision Laboratory Advanced Neuroblastoma Therapy”. Whole-exome sequencing (WES) and RNA-Seq were performed at the Translational Genomics Research Institute (TGen) on the Illumina HiSeq. RNA expression levels were compared to a normal whole-body reference composed of 22 normal tissues as well as a pediatric cancer reference. Differential expression data was interpreted in the context of systems biology annotation for the purpose of identifying activated cellular processes targetable by drugs.

### **Results:**

Tumor WES was performed on 116 patients (69 diagnosis, 47 relapse) and RNA-Seq was performed on 127 patients (69 diagnosis, 58 relapse). DNA analysis exhibited fewer mutations in primary diagnosis compared to relapse (median 53[26-78] coding mutations per subject to 164[28-5000]). Most common mutations or copy number alterations (diagnosis, relapse) were seen in *MYCN* (29%, 34%), *ATRX* (9%,30%), *ALK* (17%,11%), *CDKN2A* (0%,13%), *PPM1D* (0%,15%). RNA expression analysis identified subsets of patients with high/low expression of *SSX1*, *SSX2* or *HOXD13* genes. *SSX1* and *SSX2* genes were overexpressed in relapse (43%, 34%) and mutually exclusive when compared to diagnosis patients (20%,14%). We also identified two expression subtypes within *MYCN* amplified tumors. The first was characterized by low *SSX1*, *SSX2*/high *MYCN* expression (*SSX1*/*SSX2* was low in 95% of *MYCN* amplified tumors vs 56% of *MYCN* negative tumors). The second was characterized by high *HOXD13*/high *MYCN* expression (*HOXD13* was overexpressed in 65% of *MYCN* amplified tumors vs 28% *MYCN* non-amplified tumors).

### **Summary/Conclusions:**

Genomic sequencing for precision therapy was feasible both at diagnosis and relapse. RNA analysis may identify new subtypes which could be used to stratify patients and develop further understanding of relapse. Validation of this approach in preclinical models may lead to novel therapeutic strategies. In order to improve outcome and decrease long term toxicities of current chemotherapy, further study of genomic guided treatments at diagnosis is warranted.



## P186 A novel cre-conditional *MYCN*-driven neuroblastoma mouse model

*Hertwig, F.*<sup>2</sup>, *McGearey, A.*<sup>3</sup>, *Ahrens, K.*<sup>4</sup>, *Witthauer, M.J.*<sup>5</sup>, *Szymansky, A.*<sup>6</sup>, *Toedling, J.*<sup>7</sup>, *Künkele, A.*<sup>8</sup>, *Henssen, A.G.*<sup>9</sup>, *Eggert, A.*<sup>10</sup>, *Schulte, J.H.*<sup>11</sup>

Charité - Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology, Berlin, Germany<sup>2</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology, Berlin, Germany<sup>3</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology, Berlin, Germany<sup>4</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology, Berlin, Germany<sup>5</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology, Berlin, Germany<sup>6</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology, Berlin, Germany<sup>7</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology, Berlin, Germany<sup>8</sup>, Experimental and Clinical Research Center (ECRC) of the Charité and MDC, Clinical Research, Berlin, Germany<sup>9</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology, Berlin, Germany<sup>10</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology, Berlin, Germany<sup>11</sup>

### Background/Introduction:

Genetically engineered mouse models for neuroblastoma enable fine-tuned mechanistic investigation of this neural crest-derived malignancy and provide a suitable *in vivo* platform for preclinical drug testing. Our group previously developed a conditional *MYCN*-driven mouse model for neuroblastoma (*Dbh-iCre;LSL-MYCN-luc*) in which Cre recombinase expression is restricted to dopamine  $\beta$ -hydroxylase-expressing cells hence enabling and confining high-level expression of human *MYCN* to those cells and their progeny. This model closely recapitulated human disease and overcame several limitations of previous mouse models for neuroblastoma. Neuroblastic tumors initially arose at a high incidence (75%) regardless of strain background. Tumors displayed immature, undifferentiated histology and were predominantly derived from adrenal glands and superior cervical ganglia. However, genetic drift and the BAC-transgenic design of the Cre donor strain used resulted in vastly diminished tumor incidence in later generations.

### Aims:

We sought to establish a novel Cre recombinase conditional mouse model for neuroblastoma employing and evaluating alternative Cre recombinase donor strains.

### Methods/Materials:

We acquired four transgenic models as alternative Cre recombinase donors, with Cre expression under the control of murine *Wnt1*, *Sox10*, *Phox2b* or the *Th* promoter activity. These models were cross-bred with the previously generated *LSL-MYCN-luc* mice to investigate potential tumor incidence, latency, localization, and histopathology in the double-transgenic offspring and compare these properties to those in the *Dbh-iCre;LSL-MYCN-luc* mouse initially.

### Results:

Cross-bred Cre donor, *LSL-MYCN* double-transgenic offspring were monitored for 40 weeks. *Sox10-cre x LSL-MYCN* crosses yielded far fewer double-transgenic progeny than expected based on Mendelian ratios, indicating embryonic lethality. No tumor development was observed in 10 *Wnt1-Cre;LSL-MYCN*, 10 *Phox2b-IRES-Cre;LSL-MYCN* and the surviving three *Sox10-Cre;LSL-MYCN* mice. On the other hand, 100% of the 10 *Th-IRES-Cre;LSL-MYCN* mice died prematurely at an average age of 220 days due to tumor development. Tumor tissue was recovered from 70% of these mice, and tumors were often localized to the mesentery, and less frequently, the liver or adrenal, indicating neoplasms derive predominantly from the celiac ganglia in this model. Tumor histopathology, evaluated by an expert pathologist, confirmed a neuroblastic phenotype of a mixed differentiation grade. We immunohistochemically detected neuronal and neuroblastoma marker proteins, markers of active proliferation and human *MYCN* protein in tumor sections. Molecular tumor

characterization by transcriptome and exome sequencing is currently ongoing and will also assess secondary genetic alterations acquired during oncogenesis.

Summary/Conclusions:

Considering four neural crest-specific Cre donor models, we established *Th-IRES-Cre;LSL-MYCN* as a new mouse model yielding neuroblastic tumors at an extremely high incidence. Histologically, tumors resemble human neuroblastomas with a mixed degree of differentiation, in contrast to our previous *Dbh*-based *MYCN*-driven model, in which tumors predominantly presented with an undifferentiated histology. This model may prove especially suitable to investigate mechanisms of high-risk neuroblastoma biology, such as research focusing on *MYCN* co-drivers and their impact on tumor onset, differentiation and drug response.

## **P187 Germline cancer predisposition gene mutations in sporadic neuroblastoma patients are inherited**

*Diskin, S.J.,<sup>1</sup> Vaksman, Z.<sup>2</sup>, Vaksman, Z.<sup>3</sup>, Blauel, E.<sup>4</sup>, Lee, A.<sup>5</sup>, Kaufman, R.<sup>6</sup>, Egolf, L. E.<sup>7</sup>, Basta, B. V.<sup>8</sup>, Olshan, A. F.<sup>9</sup>, Maris, J. M.<sup>10</sup>, Diskin, S. J.<sup>11</sup>*

Children's Hospital of Philadelphia, Oncology, Philadelphia, USA<sup>1</sup>, Children's Hospital of Philadelphia, Oncology, Philadelphia, USA<sup>2</sup>, Children's Hospital of Philadelphia, Oncology, Philadelphia, USA<sup>3</sup>, Children's Hospital of Philadelphia, Oncology, Philadelphia, USA<sup>4</sup>, Children's Hospital of Philadelphia, Oncology, Philadelphia, USA<sup>5</sup>, Children's Hospital of Philadelphia, Oncology, Philadelphia, USA<sup>6</sup>, Children's Hospital of Philadelphia, Oncology, Philadelphia, USA<sup>7</sup>, University of North Carolina, Epidemiology, Chapel Hill, USA<sup>8</sup>, University of North Carolina, Epidemiology, Chapel Hill, USA<sup>9</sup>, Children's Hospital of Philadelphia, Oncology, Philadelphia, USA<sup>10</sup>, Children's Hospital of Philadelphia, Oncology, Philadelphia, USA<sup>11</sup>

### **Background/Introduction:**

Only 1-2% of neuroblastoma cases present with a family history of the disease. The vast majority of neuroblastomas appear to arise sporadically. Through a genome-wide association study (GWAS) of sporadic neuroblastoma, we have identified over two dozen loci with common variants associated with susceptibility and tumor aggressiveness. More recently, sequencing studies by us and others have estimated 8-10% of sporadic neuroblastoma cases harbor pathogenic (P) or likely pathogenic (LP) germline variants in known cancer predisposition genes (CPGs). However, the heritability and clinical significance of P/LP variants remains unknown due to lack of parental DNA.

### **Aims:**

(1) Identify and assess heritability of rare pathogenic germline variants in sporadic neuroblastoma triads and dyads; and (2) Perform integrative tumor-normal analyses to elucidate functional relevance of genetic risk factors.

### **Methods/Materials:**

Through the Gabriella Miller Kids First (GMKF) program, we performed whole genome sequencing (WGS) at 50x on robustly annotated patient-parent triads (n=493) and dyads (n=100) along with matched tumor DNA (n=366). In addition, total RNA-sequencing (n=228) and exome sequencing (100x) on matched tumor DNA (n=366) was generated to assess relevance to tumorigenesis. Rare variants (allele frequency < 0.01% in ExAC excluding TCGA) in a predefined set of CPGs (n=198) were extensively annotated and pathogenicity was assessed using ClinVar, the American College of Medical Genetics (ACMG) guidelines as implemented in InterVar, and manual review. Heritability was assessed using family-based variant calling in n=493 trios. Aggregate gene and pathway-based enrichment testing of P/LP variants was performed using Fisher's Exact test against control cohorts (1000 Genomes, gnomAD, and others). Tumor DNA sequencing was analyzed for somatic SNV, indels, structural variants (SVs), copy number, and mutational signatures to assess "second hits" and functional relevance of germline variants. RNA sequencing data was used to assess preferential allelic imbalance/mono-allelic expression, expression quantitative trait loci (eQTL), and gene fusions.

### **Results:**

We observed 66 P/LP germline variants in CPGs involving 65 out of 653 (11.5%) neuroblastoma probands. Trio data was available for 61 of these probands, and in all cases confirmed the P/LP variants were inherited (53% paternal, 47% maternal). All P/LP variants were also observed in the matched tumor DNA. We observed one canonical *ALK* mutation (R1275Q), but no *PHOX2B* mutations. Three *BARD1* loss of function variants were detected. Multiple CPGs showed enrichment of P/LP variants, including *ERCC2* (p=2.0x10<sup>-4</sup>; odds ratio: 50.19, 95% C.I. 2.77-909.11), *ATM* (1.1x10<sup>-3</sup>, odds ratio: 40.99, 95% C.I. 2.20-762.55), *FANCA* (1.1x10<sup>-3</sup>; odds ratio: 40.99, 95% C.I. 2.20-762.55), *GBA* (p=0.006; odds ratio: 31.83, 95% C.I. 1.64-617.08), *COL7A1* (p=0.006; odds ratio: 31.83, 95% C.I. 1.64-617.08) and *BARD1* (p=0.006; odds ratio: 31.83, 95% C.I. 1.64-617.08).

#### Summary/Conclusions:

Approximately 11.5% of sporadic neuroblastoma probands harbor P/LP germline variants in CPGs. In this case series, all P/LP variants were inherited. Work is ongoing to further incorporate tumor sequencing data, extend these efforts beyond CPGs, and investigate the non-coding genome.

## **P188 Difluoromethylornithine Modulates Sphingolipid Metabolism in Human Neuroblastoma Cells**

*Kraveka, J.*<sup>2</sup>, *Rahmaniyan, M.*<sup>3</sup>, *Li, L.*<sup>4</sup>

Medical University of South Carolina, Pediatrics, Charleston, USA <sup>2</sup>, Medical University of South Carolina, Pediatrics, Charleston, USA<sup>3</sup>, Medical University of South Carolina, Pediatrics, Charleston, USA<sup>4</sup>

### Background/Introduction:

Ornithine decarboxylase (ODC) is the rate limiting enzyme in biosynthesis of polyamines. Polyamines have important role in cell growth, differentiation and apoptosis. Difluoromethylornithine (DFMO) is an irreversible inhibitor of ODC and is current trials for neuroblastoma. Sphingolipids have a crucial role in determining cell fate. It is well known that many chemotherapeutic agents exert their cytotoxic effects via altering the activity of t enzymes in SPL metabolism. Ceramide is the central building block in sphingolipid metabolism and it is well known for its role in cell growth arrest and apoptosis.

### Aims:

In this study we investigated the effects of DFMO on endogenous sphingolipid and sphingolipid enzymes in SMS-KCNR human neuroblastoma cells.

### Methods/Materials:

SMS-KCNR cells were treated with 50mM DFMO for 48 Hours. Measurement of endogenous sphingolipids was performed by LC/MS. Sphingolipid pathway enzyme activities were also determined utilizing *in vitro* assays

### Results:

Ceramide levels were increased ~9.2 fold and dihydroceramide levels were increased ~5.9 fold. Analysis of individual ceramide species showed increases in all species, with the greatest increases in C24:1 ceramides. All dihydroceramide species showed increases in all species, with the greatest increases in dhC16 ceramides. Since ceramide can be generated by several pathways, including the *de novo* pathway, salvage pathway, or by sphingomyelin (SM) hydrolysis, each enzyme in sphingolipid metabolism was individually investigated. Our assays found that for ceramide generation, Neutral sphingomyelinase 2 (NSMase 2) activity was increased 2.5 fold; while Neutral ceramidase activity was inhibited by 40%. In terms of dihydroceramide metabolism, DFMO inhibited the activity of dihydroceramide desaturase (DES1).

### Summary/Conclusions:

These data show for the first time new insight into mechanism of DFMO-induced cytotoxicity in neuroblastoma which may be modulated by alteration in SPL metabolism. Further investigation is required in order to determine the downstream targets of ceramide and the role of increased dihydroceramide in DFMO treatment.



## **P189 Activating Transcription Factor 5 (ATF5) Induces Anoikis Resistance by regulation of BCL2-modifying factor (BMF)**

*Yamashiro, D.*<sup>2</sup>, *Banerjee, D.*<sup>3</sup>, *Greene, L.*<sup>4</sup>, *Connolly, E.*<sup>5</sup>, , <sup>6</sup>

Columbia University, Pediatrics, New York, USA <sup>2</sup>, Columbia University, Pediatrics, New York, USA<sup>3</sup>, Columbia University, Pathology & Cell Biology, New York, USA<sup>4</sup>, Columbia University, Radiation Oncology, New York, Columbia University<sup>5</sup>, , , <sup>6</sup>

### Background/Introduction:

Resistance to anoikis is a pre-requisite step in metastasis and allows tumor cells to survive in systemic circulation and distant organs. We have previously demonstrated that activating transcription factor 5 (ATF5), a member of the ATF/CREB family, is highly expressed in Stage 4 *MYCN*-amplified tumors and that ATF5 acts as a promoter of neuroblastoma metastasis by inducing resistance to anoikis.

### Aims:

The aim of our current study was to determine the mechanism by which ATF5 promotes anoikis resistance.

### Methods/Materials:

To determine the molecular mechanism of induction of anoikis, we analyzed the expression of anti-apoptotic BCL-2 family members BCL-2, BCL-XL, MCL-1 and pro-apoptotic BMF, BIM, BAD, BAX, PUMA protein in adherent and suspension BE(2)-C and SK-N-DZ neuroblastoma cell lines, as well as *in vivo* after intracardiac injection.

### Results:

Pro-apoptotic BMF expression was upregulated following ATF5 depletion in BE(2)-C and SK-N-DZ under suspension conditions but not in adherent cells. Next, we asked whether ATF5 silencing induces BMF in circulating tumor cell (CTC) *in vivo*. Circulatory BE(2)-C cells with ATF5-depleted were isolated from blood of mice 12 hr after intracardiac injection exhibited an induction of *BMF* mRNA. Ectopic expression of BMF markedly reduced anchorage-independent viabilities of BE(2)-C (65% decrease,  $P < 0.001$ ), and SK-N-DZ (60% decrease,  $P < 0.01$ ), as well increasing anoikis in BE(2)-C (100%;  $P < 0.001$ ) and SK-N-DZ (80%,  $P < 0.01$ ). BMF knockdown by two different siRNAs, on the contrary, increased detached BE(2)-C and SK-N-DZ viabilities by an average of 30% and 25% and reduced anoikis by 30% and 20%. We further addressed if induction of anoikis following ATF5 depletion was mediated by endogenous BMF function. We treated detached BE(2)-C and SK-N-DZ cells, expressing shATF5-1 or shATF5-2, with siBMF-1 or siBMF-2 and siGFP (control). *BMF* knockdown, resulted in the significant inhibition of anoikis induction in ATF5-silenced cells. We next investigated how ATF5 suppresses BMF in suspension settings. BMF has been shown to be a target of transcription factor FOXO3. ATF5 knockdown increased FOXO3 levels in BE(2)-C and SK-N-DZ suspension cells. Additionally, FOXO3 silencing, by two different siRNAs, abated the induction of BMF following ATF5 depletion and reduced ATF5 silencing-induced anoikis. Next, we targeted ATF5 with a dominant negative peptide inhibitor CP-d/n-ATF5 which is a novel cell penetrating (CP) peptidomimetic drug consisting of a cell penetrating domain fused with a dominant negative ATF5 (CP-d/n-ATF5) construct. CP-d/n-ATF5 reduced anchorage-independent viabilities and increased anoikis of BE(2)-C and SK-N-DZ. CP-d/n-ATF5 treatment induced BMF only in suspension culture. Lastly, we examined the effect of CP-d/n-ATF5 *in vivo*. BE(2)-C or SK-N-DZ cells were intracardially injected into nude mice and treated with CP-d/n-ATF5 or vehicle for 12hr. CP-d/n-ATF5 treatment reduced number of BE(2)-C ( $P < 0.001$ ) and SK-N-DZ ( $P < 0.01$ ) CTCs and increase anoikis in BE(2)-C and 2-fold SK-N-DZ CTCs.

### Summary/Conclusions:

Our studies demonstrate that ATF5 induces anoikis resistance of tumor cells *in vitro* and of CTC *in vivo*, increasing their survival thereby facilitating metastasis. Mechanistically, ATF5 induces anoikis resistance by suppressing BMF. Therapeutically targeting ATF5, using a dominant negative peptide

inhibitor, CP-d/n-ATF5, increases anoikis sensitivity by increasing BMF. CP-d/n-ATF5 has the potential to be an anti-metastatic therapy and thus improve outcomes for metastatic neuroblastoma.

## **P190 Roles of Src family kinases and their substrates in the biology of neuroblastoma**

*Sakai, R.*<sup>2</sup>, *Shirakihara, T.*<sup>3</sup>, *Tomiyama, A.*<sup>4</sup>, *Hikage, S.*<sup>5</sup>

Kitasato University School of Medicine, Department of Biochemistry, Kanagawa, Japan <sup>2</sup>, Kitasato University School of Medicine, Department of Biochemistry, Kanagawa, Japan<sup>3</sup>, National Defense Medical College, Department of Neurosurgery, Saitama, Japan<sup>4</sup>, Kitasato University School of Medicine, Department of Biochemistry, Kanagawa, Kitasato University School of Medicine<sup>5</sup>

### Background/Introduction:

Recent studies suggested the implication of activated anaplastic lymphoma kinase (ALK) in malignant aspects of wide range of neuroblastoma regardless to the presence of genetic alteration of this kinase. During the analysis of phosphotyrosine-containing proteins associated with activated ALK in neuroblastoma cells, we have noticed that several known substrates of Src family kinases (SFKs) such as cortactin and delta-catenin are involved in the complex. It is also reported that inhibition of SFKs cause suppressive effect in the growth of neuroblastoma, while it is not clear how the Src signaling modulates oncogenic potential of neuroblastoma.

### Aims:

SFKs are known to be activated in various types of cancers and involved especially in the progression of the disease. The objective of this study is to elucidate the role of SFKs and their substrates in the progression of neuroblastoma.

### Methods/Materials:

Neuroblastoma cell lines with activated ALK such as NB39-nu are treated with several Src inhibitors along with ALK inhibitors and changes in the phosphotyrosine-containing proteins are analyzed by immunoblotting and compared. Dose-dependent effects of these inhibitors on cell proliferation and cell migration were measured by WST-1 assay and Transwell assay, respectively. As for several identified substrates of SFKs affected by Src inhibitors, effects of knockdown by siRNA treatment was analyzed.

### Results:

SFK inhibitors such as saracatinib, bosutinib and dasatinib generally caused more significant suppressive effects in cell migration than in cell proliferation in NB39-nu cells. Among SFK inhibitors, dasatinib showed outstanding inhibition of cell migration at the concentration as low as 10 nM. At this concentration, dasatinib caused inhibition of the phosphorylation of several substrates of SFKs such as paxillin and p130Cas. On the other hand, siRNA inhibition of these substrates did not show significant effect on the migration of NB39-nu.

### Summary/Conclusions:

Since the siRNA inhibition of paxillin or p130Cas caused enhanced tyrosine phosphorylation of these substrates possibly by some feedback mechanism, the complete knock out of these proteins might be required to see the involvement of these proteins. Identification of the key substrates of SFKs for migration of neuroblastoma which are sensitive to dasatinib should be performed for further understanding of Src-mediated regulation of neuroblastoma. This approach will lead to the development of novel therapy targeting the Src-mediated signals in advanced stages of neuroblastoma.

## **P191 Chick embryo model of neuroblastoma metastasis identifies novel genes associated with poor patient outcome**

Sée, V.<sup>2</sup>, Herrmann, A.<sup>3</sup>, Clarke, K.<sup>4</sup>, Losty, P.<sup>5</sup>, Pizer, B.<sup>6</sup>

University of Liverpool, Institute of Integrative Biology, Liverpool, United Kingdom <sup>2</sup>, University of Liverpool, Institute of Integrative Biology, Liverpool, United Kingdom<sup>3</sup>, University of Liverpool, Institute of Integrative Biology, Liverpool, United Kingdom<sup>4</sup>, Alder Hey Children's hospital, Department of Paediatric Oncology, Liverpool, Alder Hey Children's hospital<sup>5</sup>, Alder Hey Children's hospital, Department of Paediatric Oncology, Liverpool, United Kingdom<sup>6</sup>

### Background/Introduction:

Neuroblastoma is the second most common cancer in childhood but still has one of the lowest survival rates of all childhood malignancies. More than 60% of neuroblastoma cases are invasive and despite treatment advances, metastasis is still one major obstacle to overcome. We have established the chick embryo as a powerful and convenient preclinical *in vivo* model to study neuroblastoma aggressiveness and demonstrated that preconditioning neuroblastoma cells in hypoxia triggers their metastasis, and the regulation of genes supportive of tumour dissemination.

### Aims:

Our aim was to elucidate new molecular markers of neuroblastoma metastasis and to identify drugs that can revert this phenotype.

### Methods/Materials:

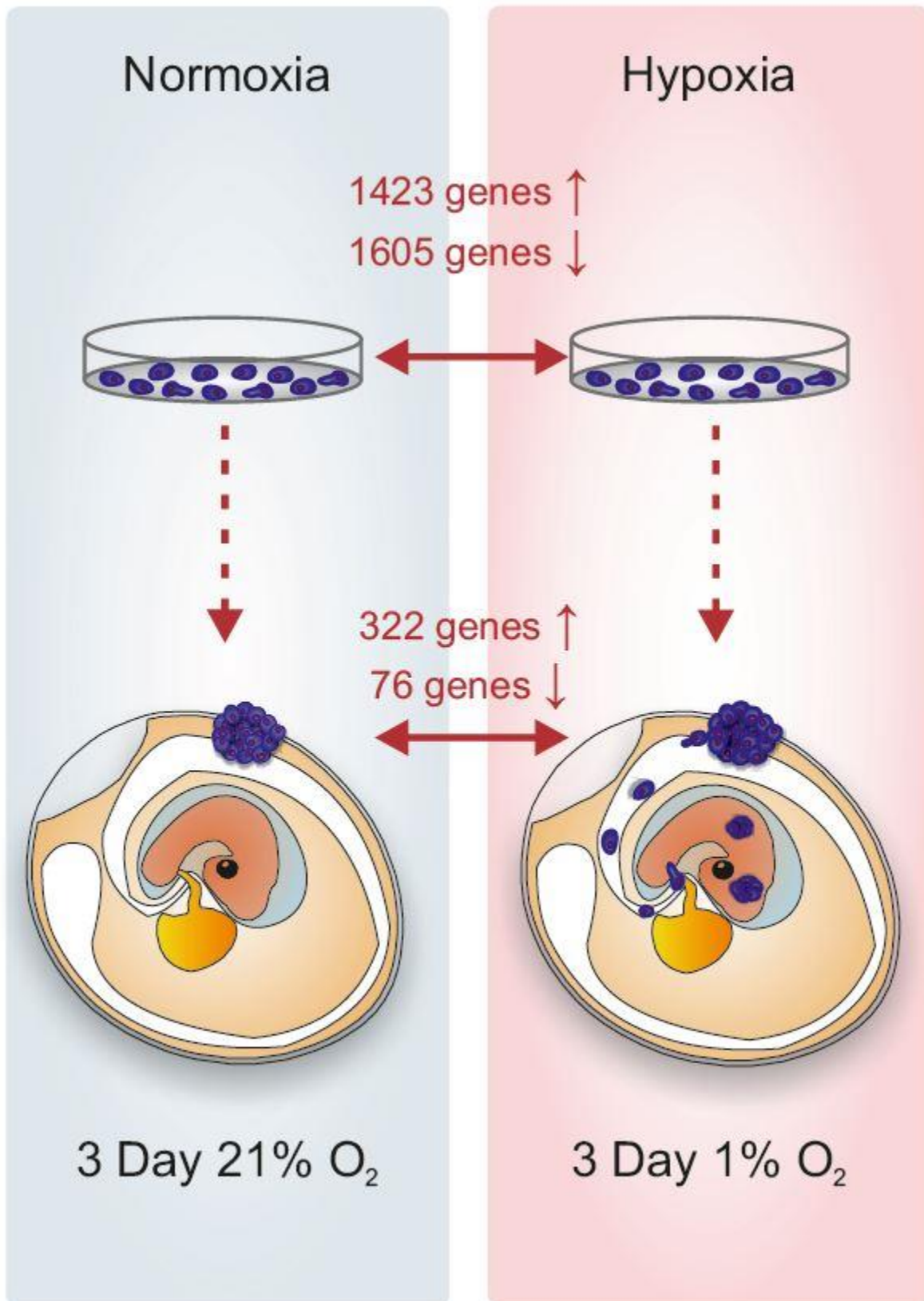
Using our established chick embryo model for neuroblastoma tumour growth and controlled metastasis, together with microarray analysis we measured the transcriptomic profile of non-metastatic and metastatic (hypoxic preconditioned) neuroblastoma primary tumours formed by SK-N-AS cells.

### Results:

We identified the significant ( $p < 0.01$ ) regulation of 398 genes in neuroblastoma tumours formed by hypoxic preconditioned cells. Out of these, only 93 genes were in common with the hypoxic gene signature observed in cells *in vitro*. To test the prognostic power of the genes identified by the hypoxic preconditioning experiment we performed a survival analysis using two independent human neuroblastoma gene expression studies (TARGET and SEQC). We discovered that 59 out of the 398 genes were putative predictors of survival. Upon series of selection criteria to search for genes with prognostic power, two genes matched the strictest filtering: ZDHHC23 and UCP3. We confirmed their regulation at protein level in tumours formed by hypoxic cells, making them potential new biomarkers for use in a diagnostic workflow. In addition, through computational analysis, we identified a number of drugs that are potentially capable of reverting the hypoxic gene signature.

### Summary/Conclusions:

We identified novel biomarkers that are indicative of poor prognosis in NB patients and could aid disease stratification especially in the non-*MYCN* amplified patient cohort. Our approach also identified specific drugs indicative of reversing the metastatic phenotype. Together this work paves the way for new neuroblastoma metastatic molecular markers and future therapeutic strategies for high-risk metastatic neuroblastoma patients.



Caption 1: Gene expression analysis of metastatic and non-metastatic neuroblastoma tumours grown in the chick embryo.

## **P192 Whole genome sequencing of recurrent neuroblastoma reveals somatic mutations that affect key players in cancer progression and telomere maintenance**

*Fransson, S.M.<sup>2</sup>, Martinez-Monleon, A.<sup>3</sup>, Johansson, M.<sup>4</sup>, Sjöberg, R.M.<sup>5</sup>, Björklund, C.<sup>6</sup>, Ljungman, G.<sup>7</sup>, Ek, T.<sup>8</sup>, Kogner, P.<sup>9</sup>, Martinsson, T.<sup>10</sup>*

University of Gothenburg, Laboratory medicine, Göteborg, Sweden <sup>2</sup>, University of Gothenburg, Laboratory medicine, Göteborg, Sweden<sup>3</sup>, University of Gothenburg, The Science for Life laboratory, Clinical Genomics, Göteborg, Sweden<sup>4</sup>, University of Gothenburg, Laboratory medicine, Göteborg, University of Gothenburg<sup>5</sup>, Umeå University Hospital, Pediatrics, Umeå, Sweden<sup>6</sup>, University of Uppsala, Women's and Children's Health, Uppsala, Sweden<sup>7</sup>, Sahlgrenska University Hospital, Childrens Cancer Center, Queen Silvia Children's Hospital, Gothenburg, Sweden<sup>8</sup>, Karolinska Institutet, Women's and Children's Health, Stockholm, Sweden<sup>9</sup>, University of Gothenburg, Laboratory medicine, Göteborg, Sweden<sup>10</sup>

### Background/Introduction:

Despite recent improvements in multimodal treatment, survival for children diagnosed with high-risk neuroblastoma (NB) remains below 50% at 5 years from treatment. Although an initial response to treatment is seen in a majority of patients, a significant portion will subsequently relapse with lesions resistant to standard therapy, and for NB patients with relapsed or refractory metastatic disease, survival is less than 10% despite recent advances in treatment regimen.

### Aims:

The aim of this study is to investigate and compare molecular and genetic aberrations in relapsed NB tumors with tumor material from time of diagnosis in order to find mechanisms associated to refractory disease.

### Methods/Materials:

In order to study genomic alterations seen in association with tumor relapse and therapy resistance, whole genome sequencing was performed on diagnostic and relapsed/refractory lesions together with constitutional DNA from seven neuroblastoma patients. Sequencing was performed using Illumina instrumentation for an average coverage of at least 60X and 30X for tumor samples and normal tissue respectively. Mapping and variant calling was done using TNscope from Sentieon, copy number calling through the Canvas tool while structural variants were called using the software Manta.

### Results:

Sequencing of the patients with relapse show somatic alterations in a diverse set of genes that includes genes involved in RAS-MAPK signaling, that promote cell cycle progression or function in telomere maintenance. Among recurrent alterations *CCND1*-gain, *TERT* rearrangements and point mutations in *POLR2A*, *CDK5RAP* and *MUC16* was seen in at least two individuals. In our cohort we also find examples of converging genomic alterations in primary-relapse tumor pair indicating dependencies related to specific genetic lesions. Furthermore, we also detect rare genetic germline variants in DNA repair genes, e.g. *BARD1*, *BRCA2*, *CHEK2* and *WRN*, which might cooperate with somatically acquired variants in this patient group with highly aggressive recurrent neuroblastoma. The number of somatic mutations at time of diagnosis respective time of relapse/progression shows correlation with the patients' age at time of sampling although one patient appears to deviate with a pronounced increase in mutation rate as compared to the other samples.

### Summary/Conclusions:

In this study of recurrent NB we identify alterations that affects genes promoting cell cycle progression, activating ALK-RAS-MAPK signaling or that is important for telomere maintenance and immortalization. Our findings in combination with previous studies of relapsed NB provide a rationale for that comprehensive genomic characterization of recurrent neuroblastoma is required in order to

optimize treatment and prolonged survival. This could be used as an incitement for novel combination therapies with compounds abrogating MAPK signaling, cell cycle progression and telomere activity.

## **P193 Biological and clinical significances of DNA methylation profiles in aggressive neuroblastomas and their application to treatments targeting tumor metabolism**

*Watanabe, K.*<sup>2</sup>, *Kimura, S.*<sup>3</sup>, *Seki, M.*<sup>4</sup>, *Isobe, T.*<sup>5</sup>, *Hiwatari, M.*<sup>6</sup>, *Kawai, T.*<sup>7</sup>, *Suzuki, H.*<sup>8</sup>, *Yoshida, K.*<sup>9</sup>, *Fujii, Y.*<sup>10</sup>, *Sato, Y.*<sup>11</sup>, *Kataoka, K.*<sup>12</sup>, *Shiraishi, Y.*<sup>13</sup>, *Chiba, K.*<sup>14</sup>, *Tanaka, H.*<sup>15</sup>, *Oka, A.*<sup>16</sup>, *Koh, K.*<sup>17</sup>, *Hata, K.*<sup>18</sup>, *Miyano, S.*<sup>19</sup>, *Ogawa, S.*<sup>20</sup>, *Takita, J.*<sup>21</sup>

The University of Tokyo, Department of Pediatrics, Graduate School of Medicine, Tokyo, Japan <sup>2</sup>, Hiroshima University, Department of Pediatrics, Hiroshima, Japan<sup>3</sup>, The University of Tokyo, Department of Pediatrics, Graduate School of Medicine, Tokyo, Japan<sup>4</sup>, The University of Tokyo, Department of Pediatrics, Graduate School of Medicine, Tokyo, The University of Tokyo<sup>5</sup>, The University of Tokyo, Department of Pediatrics, Graduate School of Medicine, Tokyo, Japan<sup>6</sup>, National Research Institute for Child Health and Development, Department of Maternal-Fetal Biology, Tokyo, Japan<sup>7</sup>, Kyoto University, Department of Pathology and Tumor Biology, Kyoto, Japan<sup>8</sup>, Kyoto University, Department of Pathology and Tumor Biology, Kyoto, Japan<sup>9</sup>, Kyoto University, Department of Pathology and Tumor Biology, Kyoto, Japan<sup>10</sup>, The University of Tokyo, Department of Urology, Tokyo, Japan<sup>11</sup>, National Cancer Center Research Institute, Division of Molecular Oncology, Tokyo, Japan, <sup>12</sup>, National Cancer Center Research Institute, Section of Genome Analysis Platform, Center for Cancer Genomics and Advanced, Tokyo, Japan <sup>13</sup>, National Cancer Center Research Institute, Section of Genome Analysis Platform, Center for Cancer Genomics and Advanced, Tokyo, Japan <sup>14</sup>, The Institute of Medical Science, The University of Tokyo, Laboratory of DNA Information Analysis, Human Genome Center, Tokyo, Japan <sup>15</sup>, The University of Tokyo, Department of Pediatrics, Graduate School of Medicine, Tokyo, Japan <sup>16</sup>, Saitama Children's Medical Center, Department of Hematology/Oncology, Saitama, Japan <sup>17</sup>, National Research Institute for Child Health and Development, Department of Maternal-Fetal Biology, Tokyo, Japan <sup>18</sup>, The Institute of Medical Science, The University of Tokyo, Laboratory of DNA Information Analysis, Human Genome Center, Tokyo, Japan <sup>19</sup>, Kyoto University, Department of Pathology and Tumor Biology, Graduate School of Medicine, Kyoto, Japan <sup>20</sup>, Kyoto University, Department of Pediatrics, Graduate School of Medicine, Kyoto, Japan <sup>21</sup>

### Background/Introduction:

It is widely accepted that since somatically acquired gene mutations are relatively less frequent, other mechanisms such as epigenetic alterations and/or metabolomic changes would play important roles in tumorigenesis as well as progression of neuroblastoma. Previously, DNA methylation profiling has been reported to have a relation with *MYCN* amplifications, but epigenetic mechanisms involved in neuroblastoma biology and clinical behavior have yet to be fully elucidated. Except for association with *MYCN* amplification and oxidative and glycolytic metabolisms, the knowledge of metabolomic alterations is very limited in neuroblastoma.

### Aims:

We aimed to elucidate DNA methylation characteristics and metabolomic alterations, which involve in aggressive phenotype of neuroblastoma and get better insight into molecular basis of neuroblastoma. We also tried to identify novel therapeutic targets for intractable neuroblastoma.

### Methods/Materials:

We conducted DNA methylation array analysis, RNA sequencing and target capture sequencing on 34 advanced neuroblastoma specimens. Publicly available data including TARGET were used for validation. For functional evaluation, siRNA-mediated gene silencing analysis and metabolomic screening in neuroblastoma-derived cell lines were also performed.

### Results:

Based on the DNA methylation profiles, we obtained two distinct methylation clusters among the cases with 11q loss of heterozygosity (11qLOH), cluster A and cluster B. These clusters were well correlate with genetic alterations and clinical outcomes. Cluster A was characterized by frequent 4p



deletion and ATRX alterations, while these alterations were less common in cluster B. Cluster B was associated with significantly poor prognosis. Because metabolomic alterations were thought to be correlated with aggressive phenotype and drug resistance in many cancers, we explored metabolomic targets in cluster B showing dismal prognosis. In comparison to cluster A, much higher expression of serine biosynthesis related gene, PHGDH, was detected in cluster B. Intriguingly, this upregulation was also found in all cases with *MYCN* amplifications. Our methylation clustering and gene expression pattern of PHGDH were confirmed in available open data with a large number of cases. Inhibition of PHGDH with siRNA or inhibitors resulted in significant decrease in cell proliferation of neuroblastoma cells. On the other hand, we also observed a similar expression pattern of ASS1, an arginine metabolism regulator, to PHGDH, and both inhibition of PHGDH and arginine metabolism in ASS1-defective neuroblastoma cells with low PHGDH expression showed synergistic cytotoxic effect. In RNA sequencing, SLC7A11, a membrane translocation of amino acids and NQO1, a polyamine metabolism related gene, were upregulated by treatment of PHGDH inhibitor alone or combination of PHGDH and arginine metabolism inhibitors, suggesting that these genes would be also promising targets. Furthermore, metabolite set enrichment analysis showed that inhibition of PHGDH upregulated several pathways including Warburg effect, and inhibition of arginine metabolism upregulated glycine and serine metabolism. Integrated network analysis visualized the complex effects of inhibition of arginine metabolism towards genes and metabolites which affect serine and glycine metabolism.

#### Summary/Conclusions:

Our results demonstrate a unique methylation subgroup with characteristic genetic features and very dismal prognosis and provide important clues for development of serine/arginine metabolisms targeting therapies in aggressive neuroblastomas.

## **P194 Prospective germline next-generation sequencing in pediatric patients with neuroblastoma identifies frequent alterations in genes involved in DNA damage repair**

*Cohen-Gogo, S.<sup>2</sup>, Langenberg-Ververgaert, K.<sup>3</sup>, Villani, A.<sup>4</sup>, Lo, W.<sup>5</sup>, Kanwar, N.<sup>6</sup>, Davidson, S.<sup>7</sup>, Layeghifard, M.<sup>8</sup>, Harripaul, R.<sup>9</sup>, Anderson, N.<sup>10</sup>, Gallinger, B.<sup>11</sup>, Young, T.<sup>12</sup>, Waldman, L.<sup>13</sup>, Morgenstern, D.A.<sup>14</sup>, Brunga, L.<sup>15</sup>, Meyn, S.<sup>16</sup>, Malkin, D.<sup>17</sup>, Shlien, A.<sup>18</sup>, Irwin, M.S.<sup>19</sup>*

The Hospital for Sick Children, Division of Hematology/Oncology, Toronto, France <sup>2</sup>, Princess Maxima, Department of Translational Research, Utrecht, Netherlands<sup>3</sup>, The Hospital for Sick Children, Division of Hematology/Oncology, Toronto, France<sup>4</sup>, The Hospital for Sick Children, Program in Genetics and Genome Biology, Toronto, The Hospital for Sick Children<sup>5</sup>, The Hospital for Sick Children, Program in Genetics and Genome Biology, Toronto, France<sup>6</sup>, The Hospital for Sick Children, Program in Genetics and Genome Biology, Toronto, France<sup>7</sup>, The Hospital for Sick Children, Program in Genetics and Genome Biology, Toronto, France<sup>8</sup>, The Hospital for Sick Children, Program in Genetics and Genome Biology, Toronto, France<sup>9</sup>, The Hospital for Sick Children, Program in Genetics and Genome Biology, Toronto, France<sup>10</sup>, The Hospital for Sick Children, Program in Genetics and Genome Biology, Toronto, France<sup>11</sup>, The Hospital for Sick Children, Program in Genetics and Genome Biology, Toronto, France, <sup>12</sup>, The Hospital for Sick Children, Program in Genetics and Genome Biology, Toronto, France <sup>13</sup>, The Hospital for Sick Children, Division of Hematology/Oncology, Toronto, France <sup>14</sup>, The Hospital for Sick Children, Program in Genetics and Genome Biology, Toronto, France <sup>15</sup>, University of Wisconsin, Department of Pediatrics, Madison, USA <sup>16</sup>, The Hospital for Sick Children, Division of Hematology/Oncology, Toronto, France <sup>17</sup>, The Hospital for Sick Children, Program in Genetics and Genome Biology, Toronto, France <sup>18</sup>, The Hospital for Sick Children, Division of Hematology/Oncology, Toronto, France <sup>19</sup>

### Background/Introduction:

The role of germline *ALK* and *PHOX2B* variants in predisposition to neuroblastoma (NB) is well-established. Alterations in genes associated with other syndromes, including RASopathies, Fanconi anemia and Li-Fraumeni, have also been detected. Emerging data suggests potential roles for DNA damage repair (DDR) pathway genes in NB predisposition. The use of Next Generation Sequencing (NGS) facilitates the unbiased detection of both known and novel variants as well as the identification of tumour DNA mutational signatures. Mutational signature 3 has been strongly associated with defects in homologous recombination repair of DNA double strand breaks.

### Aims:

Report our single centre experience of comprehensive sequencing of germline and tumour DNA in patients with neuroblastoma, including some with features of genetic predisposition.

### Methods/Materials:

Patients with newly diagnosed and relapsed malignancies were referred to our institutional pediatric oncology NGS (SickKids Cancer Sequencing (KiCS)) program. Germline DNA was sequenced using a custom pediatric cancer panel targeting 15,000 exons across 864 genes (1000X coverage). Variant pathogenicity was classified according to ACMG criteria and evaluated by our multi-disciplinary molecular tumor board. Rare variants of uncertain significance (VUS) with supportive corresponding somatic data, patient phenotypes, literature and/or in-silico functional analyses were termed "VUS with limited evidence for pathogenicity" (VUS-LEP). The same custom exon panel and whole genome sequencing (WGS) was performed on tumour DNA whenever available. Mutational signatures were assessed through WGS for the presence of SBS3 (single base substitution signature 3).

### Results:

Analyses have been completed for 45 NB patients. Patient history was retrospectively categorized as "high genetic predisposition risk" (HGPR) in 15/45 patients based on  $\geq 1$  of the following criteria: family history of NB and/or significant family history of other cancer(s); patient with NB and another malignancy; NB with congenital abnormalities; multifocal NB. Germline pathogenic (P), likely

pathogenic (LP) variants or VUS-LEP in a known cancer predisposition gene were identified in 7/15 HGPR patients (46.6%), predominantly in DDR-related genes (*PALB2*, *BRCA1/CHEK2*, *CHEK2/PALB2*, *NF1*, *DICER1*, *MITF*, *EZH2/SMARCA4*). No *ALK* or *PHOX2B* germline variants were identified for the five patients with NB family history. For the 30 non-HGPR pts, germline P, LP variants or VUS-LEP in DDR genes were most common and detected in 8/30 pts (26.6%), in genes including *BAP1*, *BARD1* (n=2), *BLM*, *BRCA2*, *CHEK2*, *RAD51*, and *RAD51D/NBN*. In this non-HGPR population, we also identified a germline TP53 P variant (n=1), a MUTYH P variant (n=1), a *FH* LP variant (n=1) as well as germline VUS-LEPs in *EZH2* (n=1) and *ERCC2* (n=1). Preliminary analyses of mutational signatures on tumour DNA (n=26) reveal the presence of SBS3 in a high proportion of tumours (15/26), with an enrichment of samples carrying germline variants in DDR genes.

Summary/Conclusions:

Prospective sequencing identified frequent germline variants in NB pts, predominantly in genes involved in DDR, regardless of classification as HGPR. We report a high incidence of SBS3 in neuroblastoma tumour samples. Further studies are needed to correlate genomic variants and mutational signature patterns. Our findings have implications for treatment recommendations as well as for appropriate genetic counselling.

## **P195 The prognostic impact of chromosome 11q loss in stage 4S neuroblastoma**

*Theissen, J.<sup>2</sup>, Plum, G.<sup>3</sup>, Lieberz, D.<sup>4</sup>, Lorenz, W.<sup>5</sup>, Hemstedt, N.<sup>6</sup>, Ortmann, M.<sup>7</sup>, Simon, T.<sup>8</sup>, Berthold, F.<sup>9</sup>, Hero, B.<sup>10</sup>, Fischer, M.<sup>11</sup>*

University Children's Hospital of Cologne, Experimental Pediatric Oncology, Cologne, Germany <sup>2</sup>, University Children's Hospital of Cologne, Pediatric Oncology and Hematology, Cologne, Germany<sup>3</sup>, University Children's Hospital of Cologne, Pediatric Oncology and Hematology, Cologne, Germany<sup>4</sup>, University Children's Hospital of Cologne, Experimental Pediatric Oncology, Cologne, University Children's Hospital of Cologne<sup>5</sup>, University Children's Hospital of Cologne, Experimental Pediatric Oncology, Cologne, Germany<sup>6</sup>, University Hospital of Cologne, Department of Pathology, Cologne, Germany<sup>7</sup>, University Children's Hospital of Cologne, Pediatric Oncology and Hematology, Cologne, Germany<sup>8</sup>, University Children's Hospital of Cologne, Pediatric Oncology and Hematology, Cologne, Germany<sup>9</sup>, University Children's Hospital of Cologne, Pediatric Oncology and Hematology, Cologne, Germany<sup>10</sup>, University Children's Hospital of Cologne, Experimental Pediatric Oncology, Cologne, Germany<sup>11</sup>

### **Background/Introduction:**

Genomic loss of chromosome 11q is known to be associated with poor outcome in neuroblastoma. According to the INRG risk classification, stage 4S patients harboring loss of chromosome 11q are considered to be at high risk, however, this classification was developed on few patients only.

### **Aims:**

We here aimed to assess the prognostic impact of 11q loss in a large cohort of patients with stage 4S.

### **Methods/Materials:**

Patients with stage 4S disease and lacking *MYCN* amplification who had been registered in the German neuroblastoma trials NB95-S, NB97 and NB2004 between 1995 and 2016 were included in the analysis. The copy number status of chromosome 11q at diagnosis was investigated by interphase fluorescence in situ hybridization (FISH) analysis in a dual color procedure, with DNA probes covering the region of 11q22.3 and centromere as control. At least 50 cells per slide were analyzed. Loss of 11q (deletion or imbalance) was defined according to the international consensus criteria.

### **Results:**

Tumor samples of 165 neuroblastoma patients with stage 4S disease were analyzed for 11q copy number status. The median follow-up of patients was 9.6 years (0.15-25 years). Loss of chromosome 11q was found in 10 of the 165 tumors (6.1%; deletion, n=5; imbalance, n=4; deletion and imbalance, n=1). Median age at diagnosis was 5.3 months for patients with 11q aberrations (range, 0 – 10.6 months) and 3.2 months for patients without 11q aberrations (range, 0 – 11.2 months; p=0.098). Metastatic pattern did not differ between the groups. Tumor progression was observed in 4/10 patients with 11q loss (40%), and in 27/155 patients with normal 11q (17%). Five-year event-free survival was  $0.70 \pm 0.15$  in patients with 11q loss and  $0.83 \pm 0.03$  in patients with normal 11q (p=0.065). Five-year overall survival was high in both groups (11q loss,  $0.90 \pm 0.10$ ; 11q normal,  $0.93 \pm 0.02$ ; p=0.26). Interestingly, metastatic progression to stage 4 disease was observed for all four patients with 11q loss who had an event. By contrast, only 10 of the patients with normal 11q had metastatic progression into stage 4 disease (p=0.005). Accordingly, metastasis-free survival differed significantly between the groups ( $0.70 \pm 0.15$  vs.  $0.93 \pm 0.02$ ; p<0.001).

### **Summary/Conclusions:**

Loss of chromosome 11q is rare in stage 4S neuroblastoma and does not define high-risk disease *per se*. The relatively high frequency of metastatic progression into stage 4 disease in this subgroup, however, suggest that additional factors associated with 11q loss may drive the clinical course of the disease.



## **P196 Identification of deregulated ECM and Axon guidance pathways in ALK- overexpressing Neuroblastoma xenografts**

*Vivancos Stalin, L.*<sup>2</sup>, *Balmas Bourlout, K.*<sup>3</sup>, *Praz, V.*<sup>4</sup>, *Riggi, N.*<sup>5</sup>, *Joseph, J.*<sup>6</sup>, *Jaquier, N.*<sup>7</sup>, *Renella, R.*<sup>8</sup>, *Mühlethaler-Mottet, A.*<sup>9</sup>

Lausanne University Hospital and University of Lausanne, Woman-Mother-Child Department, Lausanne, Switzerland <sup>2</sup>, Lausanne University Hospital and University of Lausanne, Woman-Mother-Child Department, Lausanne, Switzerland<sup>3</sup>, Lausanne University Hospital and University of Lausanne, Woman-Mother-Child Department, Lausanne, Switzerland<sup>4</sup>, Lausanne University Hospital and University of Lausanne, Experimental Pathology, Lausanne, Lausanne University Hospital and University of Lausanne<sup>5</sup>, Lausanne University Hospital and University of Lausanne, Woman-Mother-Child Department, Lausanne, Switzerland<sup>6</sup>, Lausanne University Hospital and University of Lausanne, Woman-Mother-Child Department, Lausanne, Switzerland<sup>7</sup>, Lausanne University Hospital and University of Lausanne, Woman-Mother-Child Department, Lausanne, Switzerland<sup>8</sup>, Lausanne University Hospital and University of Lausanne, Woman-Mother-Child Department, Lausanne, Switzerland<sup>9</sup>, ,

### Background/Introduction:

Neuroblastoma (NB) tumors are composed of both adrenergic (ADR) and mesenchymal (MES) cell types displaying plasticity. The Anaplastic Lymphoma Kinase (ALK) gene is frequently altered in NB through copy number changes and mutations, with ALK<sup>-F1174L</sup> and ALK<sup>-R1275Q</sup> being the most potent activating mutations. ALK thus represents a bona fide target for therapy.

### Aims:

Here we aim to identify common and ALK-status specific downstream target genes and signaling pathways activated by ALK-wt, ALK<sup>-F1174L</sup> and ALK<sup>-R1275Q</sup> activating mutations *in vivo*.

### Methods/Materials:

ALK-wt, ALK<sup>-F1174L</sup> and ALK<sup>-R1275Q</sup> were overexpressed in the human NB cell lines SK-N-Be2c (adrenergic and tumorigenic) and SH-EP (mesenchymal and weakly tumorigenic) using a lentiviral vector (pLIV). Transduced cells were further injected orthotopically (SK-N-Be2c) in the adrenal gland of athymic Swiss nude mice or subcutaneously (SH-EP) in NOD-SCID- mice. Tumor growth was followed by echography. Histologic analysis were performed on hematoxylin and eosin stained tumors. Transcriptomic analyses were performed by RNA-seq (Illumina).

### Results:

The tumor growth capacity of SH-EP cells was highly increased by ALK<sup>-F1174L</sup>, and to a lesser extent by ALK-wt overexpression. Histologic analysis revealed that ALK expression in SH-EP cells altered xenograft histology. Indeed, tumor cells of the SH-EP-control group (pLIV) displayed a MES-like phenotype, while ALK<sup>-R1275Q</sup> and ALK-wt tumors presented heterogeneous regions with either MES-like cells or differentiating neuroblasts, and ALK<sup>-F1174L</sup> expressing tumors were mainly composed of undifferentiated/poorly differentiated neuroblasts. In contrast, ALK expression had no impact on the *in vivo* growth properties of SK-N-Be2c cells and on tumor cell histology. RNAseq analyses revealed numerous differentially expressed genes ( $|\log_2(\text{FC})| \geq 1$ ; adjusted pvalue  $\leq 0.05$ ) in SH-EP and SK-N-Be2c ALK<sup>-F1174L</sup> (n=1371 and n= 963, respectively), ALK<sup>-R1275Q</sup> (n=1049 and n=102) and ALK-wt tumors (n=1639 and n=282) relative to controls. In addition, Gene Set Enrichment Analysis (GSEA) of the ADR and MES signature showed an enrichment of the adrenergic signature in SH-EP ALK<sup>-F1174L</sup> and ALK-wt tumors. Moreover, shared deregulated genes and pathways were identified in SH-EP and SK-N-Be2c ALK-overexpressing tumors. First, gene ontology (GO) term enrichment analysis revealed the deregulation of MAP kinase (MAPK/ERK) related genes in ALK-overexpressing tumors. Second, several deregulated pathways associated to the ECM and to axon guidance/neuronal differentiation in ALK-overexpressing tumors were identified. Finally, among these pathways, we observed specifically deregulated genes belonging to the collagen family, the semaphorin/plexin complex and to the ROBO

family. Moreover, STRING analysis (interaction score: highest confidence 0.900) revealed high interactions across ECM and axon guidance/neuronal differentiation pathways.

#### Summary/Conclusions:

These transcriptomic results highlight a possible role for ALK in downregulating biological pathways related to ECM and neuronal differentiation that are known to be involved in NB pathogenesis. Selected genes identified in these pathways will be further investigated in order to validate their implication in ALK-overexpressing NB tumors. The validation of new genes/signaling pathways would bring new information about the pathways activated through ALK deregulated signaling and highlight potential new targets for NB therapy.

## **P197 Naxitamab, an antibody with distinct complementary determining regions and high binding affinity to disialoganglioside GD2**

*Liebenberg, N.*<sup>2</sup>, *Lisby, S.*<sup>3</sup>, *BuKrinski, J.*<sup>4</sup>, *Søderby, P.*<sup>5</sup>, *Mahiuddin, A.*<sup>6</sup>, *Lund-Hansen, T.*<sup>7</sup>

Y-mAbs Inc., Technical Operations, Hoersholm, Denmark <sup>2</sup>, Y-mAbs Inc., Medical Department, Hoersholm, Denmark<sup>3</sup>, CMC Assist Aps, CMC support, Copenhagen, Denmark<sup>4</sup>, CMC Assist Aps, CMC support, Copenhagen, CMC Assist Aps<sup>5</sup>, Y-mAbs Inc., Research, Hoersholm, Denmark<sup>6</sup>, Y-mAbs Inc., Technical Operations, Hoersholm, Denmark<sup>7</sup>

### Background/Introduction:

Monoclonal antibodies (mAbs) that target disialoganglioside 2 (GD2) have shown clinical efficacy in the treatment of GD2 expressing tumours and are being used as part of the new standard of care for the treatment of high risk neuroblastoma. Two chimeric antibodies already have received regulatory approval for pediatric neuroblastoma: dinutuximab (Unituxin) and dinutuximab beta (Qarziba). Here we present detailed structural characterizations of naxitamab, a humanized form of mu3F8 antibody targeting GD2, which has been in clinical development since 2011.

### Aims:

The three-dimensional structural models of the GD2-targeting complementary determining regions (CDRs) of dinutuximab beta and naxitamab were compared and their *in vitro* GD2 binding kinetics and affinity were evaluated side-by-side. The fragment crystallizable region (Fc) structure and function of the two mAbs were also compared in terms of their N-glycan profiles and *in vitro* antibody-dependent cellular phagocytosis (ADCP) and complement-dependent cytotoxicity (CDC) activities.

### Methods/Materials:

The position of the CDRs in naxitamab and dinutuximab beta were determined using Kabat numbering and visualized in 3D using the known X-ray structures of m14G2a, m14G2a in complex with GD2, and m3F8. Surface plasmon resonance (SPR) and enzyme-linked immunosorbent assay (ELISA) were used to characterize GD2 binding. ADCP activity was assessed using an in-vitro cell-based reporter assay based on a modified human Jurkat cell line transfected to express CD32 receptors. CDC activity was measured using an *in vitro* cell-based assay in which binding of the mAb Fc region to C1q activates the complement cascade and induces killing of GD2-expressing target cells. The N-glycan profiles of the mAbs were characterized by releasing the N-glycans from the mAbs, labelling with a fluorescent marker and quantification using high-pressure liquid chromatography with fluorescence detection (HPLC-FLD).

### Results:

Clear differences in the CDR regions of naxitamab and dinutuximab beta were observed with only 23% similarity. The significance of this is substantiated by the structural analysis showing that the three-dimensional structure of the GD2-targeting CDRs of dinutuximab beta and naxitamab are distinct. Furthermore, a difference in GD2 binding properties resulting in an approximately ten-fold higher affinity for naxitamab to GD2 compared to dinutuximab beta was demonstrated. The higher affinity predominantly was due to its slower off-rate.

### Summary/Conclusions:

Naxitamab is a humanized anti-GD2 antibody which has been in clinical development for more than 8 years. The prominent differences in the sequence and three-dimensional structure of the GD2-targeting CDRs indicate a differentiated binding capability. This is supported by an approximate 10-fold higher affinity for GD2, predominantly due to a slower off-rate. These properties together with optimized clinical dosing schedules suggest that naxitamab could represent a new approach to target GD2 in paediatric oncology.





## P198 DHODH impact on Neuroblastoma biology

*Dyberg, C.*<sup>2</sup>, *Olsen, T.*<sup>3</sup>, *Otte, J.*<sup>4</sup>, *Alchahin, A.*<sup>5</sup>, *Westerhout, E.*<sup>6</sup>, *Koster, J.*<sup>7</sup>, *Versteeg, R.*<sup>8</sup>, *Kogner, P.*<sup>9</sup>, *Johnsen, J.I.*<sup>10</sup>, *Sykes, D.*<sup>11</sup>, *Baryawno, N.*<sup>12</sup>

Karolinska Institutet, Women's and children's health, Stockholm, Sweden<sup>2</sup>, Karolinska Institutet, Women's and Children's health, Stockholm, Sweden<sup>3</sup>, Karolinska Institutet, Women's and Children's health, Stockholm, Sweden<sup>4</sup>, Karolinska Institutet, Women's and children's health, Stockholm, Karolinska Institutet<sup>5</sup>, Amsterdam UMC University of Amsterdam, Department of Oncogenomics, Amsterdam, Netherlands<sup>6</sup>, Amsterdam UMC University of Amsterdam, Department of Oncogenomics, Amsterdam, Netherlands<sup>7</sup>, Amsterdam UMC University of Amsterdam, Department of Oncogenomics, Amsterdam, Netherlands<sup>8</sup>, Karolinska Institutet, Women's and Children's Health, Stockholm, Sweden<sup>9</sup>, Karolinska Institutet, Women's and Children's Health, Stockholm, Sweden<sup>10</sup>, Massachusetts General Hospital, Center for Regenerative Medicine, Boston, USA<sup>11</sup>, Karolinska Institutet, Women's and Children's Health, Stockholm, Sweden,<sup>12</sup>

### Background/Introduction:

Despite intensive therapy, the cure rate for children diagnosed with high-risk neuroblastoma is still too low, accentuating the need for more effective therapies. Inhibition of DHODH is effective as differentiation therapy in myeloid leukemia (Sykes et al., Cell, 2016), but the mechanisms on how DHODH inhibition causes differentiation in cancer is unknown. DHODH is an enzyme that converts dihydroorotate (DHO) to orotate (OA) in the only redox step in the *de novo* synthesis of pyrimidines.

### Aims:

We analyzed the potential therapeutic role of DHODH in high-risk neuroblastoma and evaluate the mechanism through which DHODH inhibition promotes differentiation.

### Methods/Materials:

Survival and gene expression analyses (Kaplan-Meier analysis and multivariate Cox regression) were performed using available TARGET and SEQC-498 datasets (n=647). The activity of DHODH inhibition using brequinar was studied in neuroblastoma cell lines, transgenic TH-*MYCN* mice and the human neuroblastoma cell lines SK-N-AS and SK-N-BE(2). TH-*MYCN* tumors, SK-N-AS and SK-N-BE(2) xenografts were analyzed using RNA-seq and/or ChIP-seq. Mechanistic insights were based on RNA-sequencing and ChIP-sequencing analysis of transgenic neuroblastoma tumors treated *in vivo* with brequinar or vehicle control.

### Results:

Data from two independent neuroblastoma patient cohorts demonstrated that *DHODH* expression is an independent risk factor for aggressive disease; higher *DHODH* levels were correlated to worse overall and event-free survival. Strikingly, we identified a subset of high-risk patients in Stage 4 disease with very high expression of *DHODH* that correlated with a dismal prognosis (5 year survival less than 10%). Furthermore, increased *DHODH* expression was significantly correlated to advanced stages of neuroblastoma disease and *MYCN* amplification.

We show *in vitro* that several human neuroblastoma cell lines are sensitive to DHODH inhibition (IC<sub>50</sub> values ~ 5 nM), and that brequinar sensitivity is positively correlated to *MYCN* gene expression. In TH-*MYCN* mice, we demonstrate a significant reduction in tumor size after only 72 hours of brequinar treatment and a dramatic prolongation of survival (mean survival of controls: 39 days; mean survival of brequinar-treated: 173 days). Following discontinuation of brequinar after 120 days of treatment, mice relapsed with the reappearance of tumors (median survival 12 days).

Brequinar significantly reduced the growth of established human

neuroblastoma xenografts (SK-N-BE (2) in NMRI nu/nu mice.). ChIP and/or RNA-seq analysis of mouse tumors showed a shift towards a more mesenchymal phenotype after brequinar treatment, whereas tumors at relapse had re-gained their adrenergic phenotype.

Summary/Conclusions:

We conclude that DHODH is a critical metabolic mediator of neuroblastoma growth and that specific DHODH inhibition is a highly effective therapy in neuroblastoma. Targeting DHODH as a cancer-specific metabolic dependency may benefit high-risk patients, in particular the *MYCN*-amplified subset, and should be further tested in clinical studies.

## **P199 Detecting alternative lengthening of telomeres in personalized oncology**

*Hartlieb, S.*<sup>2</sup>, *Claveras Cabezudo, A.*<sup>3</sup>, *Sieverling, L.*<sup>4</sup>, *Toprak, U.*<sup>5</sup>, *Fischer, M.*<sup>6</sup>, *Jones, D.*<sup>7</sup>, *Pfaff, E.*<sup>8</sup>, *Pfister, S.*<sup>9</sup>, *Brors, B.*<sup>10</sup>, *Feuerbach, L.*<sup>11</sup>, *Westermann, F.*<sup>12</sup>

Hopp Children`s Cancer Center (KiTZ) & German Cancer Research Center (DKFZ), Neuroblastoma Genomics, Heidelberg, Germany<sup>2</sup>, German Cancer Research Center (DKFZ), Applied Bioinformatics, Heidelberg, Germany<sup>3</sup>, German Cancer Research Center (DKFZ), Translational Medical Oncology, Heidelberg, Germany<sup>4</sup>, Hopp Children`s Cancer Center (KiTZ) & German Cancer Research Center (DKFZ), Neuroblastoma Genomics, Heidelberg, Hopp Children`s Cancer Center (KiTZ) & German Cancer Research Center (DKFZ)<sup>5</sup>, University Children's Hospital Cologne, Experimental Pediatric Oncology, Cologne, Germany<sup>6</sup>, Hopp Children`s Cancer Center (KiTZ) & German Cancer Research Center (DKFZ), Pediatric Neurooncology, Heidelberg, Germany<sup>7</sup>, Hopp Children`s Cancer Center (KiTZ) & German Cancer Research Center (DKFZ), Pediatric Neurooncology, Heidelberg, Germany<sup>8</sup>, Hopp Children`s Cancer Center (KiTZ) & German Cancer Research Center (DKFZ), Pediatric Neurooncology, Heidelberg, Germany<sup>9</sup>, German Cancer Research Center (DKFZ), Applied Bioinformatics, Heidelberg, Germany<sup>10</sup>, German Cancer Research Center (DKFZ), Applied Bioinformatics, Heidelberg, Germany<sup>11</sup>, Hopp Children`s Cancer Center (KiTZ) & German Cancer Research Center (DKFZ), Neuroblastoma Genomics, Heidelberg, Germany<sup>12</sup>

### **Background/Introduction:**

Telomere maintenance, which is achieved either by *MYCN/MYC* activation resulting in telomerase activation or alternative lengthening of telomeres (ALT), is associated with poor clinical outcome in neuroblastoma (NB). Clinically and biologically, ALT-positive NBs were found to form a distinct subgroup with different disease progression and potentially different response to standard therapies. Thus, ALT is an important marker in NB risk assessment.

### **Aims:**

In the presented study, we aimed to assess ALT frequency in an unbiased relapse cohort of the registry trial INFORM. INFORM profiles the genetic features of individual tumors in order to provide a personalized treatment. We intended to identify ALT-activity using only the omics data generated in the INFORM pipeline without using classical ALT markers like C-Circles or APBs to enable ALT scoring in the routine analysis.

### **Methods/Materials:**

Using exome sequencing, low coverage whole genome sequencing, methylation array and RNA sequencing data generated via the INFORM workflow from 99 relapsed NBs, the presence of an active TMM (ALT, TERT or *MYCN* activation) was analyzed.

### **Results:**

Criteria to detect ALT activity were a higher telomere content in the tumor compared to the control, increased TERRA expression and the presence of a 1q42.2-1qter deletion. Due to the almost mutual exclusivity of ALT, amplified *MYCN* and *TERT* activation, absence of amplified *MYCN* and lack of *TERT* expression were used as additional parameters. Our preliminary dataset of 40 relapsed tumors with known C-Circle status was used as a training set (n=40) for the applied criteria and subsequently the classification was done for 59 additional relapsed NBs.

In total, ALT activity was identified in about 40% of the relapsed NB cases indicating an enrichment of ALT in relapsed disease.

Taken together, for the majority of cases it was possible to detect ALT activity or to identify another TMM being *MYCN* or TERT activation. However, for about 10% of the tumors this was not possible using the available data. The lack of available RNA sequencing data for some cases was thereby an important limitation. Detection of *ATRX* mutations, which are found in about half of the ALT-positive

cases based on our previous data, was very challenging using the INFORM pipeline due to the lack of high coverage whole genome sequencing data.

**Summary/Conclusions:**

In summary, the assessment of ALT activation is possible using the routine INFORM pipeline and can be used for accurate risk assessment. However, targeted therapies for this subgroup of patients being enriched in the relapse are still missing, but are urgently needed and should therefore be an extensive area of research.

## **P200 Telomere maintenance versus gene expression-based classification for risk assessment of neuroblastoma patients**

*Ackermann, S.<sup>2</sup>, Rosswog, C.<sup>3</sup>, Kahlert, Y.<sup>4</sup>, Lieberz, D.<sup>5</sup>, Fischer, J.<sup>6</sup>, Berthold, F.<sup>7</sup>, Simon, T.<sup>8</sup>, Hero, B.<sup>9</sup>, Fischer, M.<sup>10</sup>*

University Hospital of Cologne, Experimental Pediatric Oncology, Cologne, Germany <sup>2</sup>, University Hospital of Cologne, Experimental Pediatric Oncology, Cologne, Germany<sup>3</sup>, University Hospital of Cologne, Experimental Pediatric Oncology, Cologne, Germany<sup>4</sup>, University Hospital of Cologne, Pediatric Oncology and Hematology, Cologne, University Hospital of Cologne<sup>5</sup>, University Hospital of Cologne, Experimental Pediatric Oncology, Cologne, Germany<sup>6</sup>, University Hospital of Cologne, Pediatric Oncology and Hematology, Cologne, Germany<sup>7</sup>, University Hospital of Cologne, Pediatric Oncology and Hematology, Cologne, Germany<sup>8</sup>, University Hospital of Cologne, Pediatric Oncology and Hematology, Cologne, Germany<sup>9</sup>, University Hospital of Cologne, Experimental Pediatric Oncology, Cologne, Germany<sup>10</sup>

### **Background/Introduction:**

Gene expression-based classification is a promising strategy for risk stratification of neuroblastoma patients. Previous studies showed improved outcome prediction when a gene expression-based classifier (SVM-th10) was integrated into current risk stratification systems. Recent studies, however, suggested that the individual risk of neuroblastoma patients may be also precisely assessed by a mechanistic classification that takes telomere maintenance (TM) mechanisms into account, including activation of telomerase and alternative lengthening of telomeres (ALT).

### **Aims:**

We here aimed to evaluate and compare the performance of two distinct molecular risk assessment systems, i.e., gene expression-based classification vs. telomere maintenance, for outcome prediction of neuroblastoma patients.

### **Methods/Materials:**

Microarray-based gene expression profiles and TM were determined in diagnostic tumor samples of 201 neuroblastoma patients covering the entire spectrum of the disease. Patients were classified into favorable or unfavorable subgroups using the previously described SVM-th10 gene expression classifier. Tumor samples were considered as TM-positive if they harbored *TERT* rearrangements or *MYCN* amplification (as determined by FISH), elevated *TERT* expression (as determined by microarrays), or ALT-associated PML nuclear bodies (as determined by combined immunofluorescence/FISH).

### **Results:**

TM was found in 99/201 tumors (49.3%), and unfavorable gene expression profiles in 82 patients (40.8%). Unfavorable gene expression was associated with TM. 75/99 patients (75.8%) with TM and 7/102 patients without TM had an unfavorable gene expression classification ( $p < 0.001$ ). Both TM and unfavorable classification were associated with stage 4, *MYCN* amplification, age  $\geq 18$  months and high-risk disease ( $p < 0.001$  each). Both TM and unfavorable classification were also associated with poor outcome: Three-year event-free survival (EFS) of patients with TM-positive tumors was  $0.43 \pm 0.05$  compared to  $0.91 \pm 0.03$  for patients with TM-negative tumors ( $p < 0.001$ ; 3-year overall survival (OS),  $0.72 \pm 0.05$  vs.  $1.0$ ,  $p < 0.001$ ). Three-year EFS of patients with unfavorable gene expression classification was  $0.47 \pm 0.06$  compared to  $0.81 \pm 0.04$  for patients with favorable classification ( $p < 0.001$ ; 3-year OS,  $0.74 \pm 0.05$  vs.  $0.95 \pm 0.02$ ;  $p < 0.001$ ). For the whole cohort, multivariable analysis for EFS revealed TM (hazard ratio: 15.6, CI 7.0 – 34.6,  $p < 0.001$ ), but not unfavorable gene expression profile ( $p = 0.71$ ) as significant. Similar results were found for non-high-risk patients ( $n = 119$ ; TM: hazard ratio: 14.1, CI 6.2 – 31.8,  $p < 0.001$ ; SVMth10:

$p = 0.325$ ). Separate multivariable analysis for high-risk patients was not possible because only one high-risk patient was TM negative.

**Summary/Conclusions:**

Assessment of TM appears to be superior to gene expression-based classification in outcome predication of neuroblastoma patients. The accuracy of TM in neuroblastoma risk assessment should thus be evaluated in large prospective studies.

## **P201 The H2B deubiquitinase USP44 promotes aggressive behavior, restrains differentiation, and is associated with poor outcomes in neuroblastoma**

Hussain, S.,<sup>1</sup> Galardy, P.<sup>2</sup>, Bedekovics, T.<sup>3</sup>, Ali, A.<sup>4</sup>, Zhu, S.<sup>5</sup>, Galardy, P.<sup>6</sup>

Mayo Clinic, Pediatric and Adolescent Medicine, Rochester, USA<sup>1</sup>, Mayo Clinic, Pediatric and Adolescent Medicine, Rochester, USA<sup>2</sup>, Mayo Clinic, Pediatric and Adolescent Medicine, Rochester, USA<sup>3</sup>, Mayo Clinic, Pediatric and Adolescent Medicine, Rochester, USA<sup>4</sup>, Mayo Clinic, Biochemistry and Molecular Biology, Rochester, Mayo Clinic<sup>5</sup>, Mayo Clinic, Pediatric and Adolescent Medicine, Rochester, USA<sup>6</sup>

### Background/Introduction:

Recent work has implicated epigenetic regulation as having an important role in the biology of neuroblastoma. The details of which epigenetic readers and writers drive the pathology and aggression are largely unknown. Deubiquitinating enzymes (DUBs) are emerging drug targets and important gatekeepers of the ubiquitin code, including several that regulate histone mono-ubiquitination.

### Aims:

To identify new potential drug targets and to better understand the biology of neuroblastoma.

### Methods/Materials:

We analyzed existing clinically-annotated gene expression datasets using the R2 Genomics Analysis and Visualization Platform (<http://r2.amc.nl>) and the PREDiction of Clinical Outcomes using Genomic profiles (PRECOG; <https://precog.stanford.edu/>). USP44 expression was manipulated in human neuroblastoma cell lines (SH-SY5Y, IMR-32, LAN6, NGP) using RNAinterference, CRISPR gene editing, and lentiviral expression. We used an IncuCyte system to analyze cell proliferation, the transwell system to study cell migration and invasion, and image processing to analyze retinoic acid induced differentiation. We overexpressed USP44 under control of the dopamine- $\beta$ -hydroxylase ( $d\beta h$ ) promoter (Tg( $d\beta h:Usp44-2A-mCherry$ )). The construct was injected into embryos from stable transgenic fish expressing EGFP-MYCN (Tg( $d\beta h:EGFP-MYCN$ )) and tumor onset was monitored by detection of GFP in live fish.

### Results:

To identify new potential drug targets, we performed an unbiased family-wide study of the relationship between DUBs and patient survival in neuroblastoma. Using the PRECOG tool, we determined the z-scores for survival association for 95 DUBs in neuroblastoma. This revealed that the enzyme *USP44* had the strongest association with poor survival with a z-score of 8.33 (*MYCN* z-score = 8.18). Using the R2 platform, *USP44* expression was significantly higher in tumor samples from patients with high-risk tumors compared with non-high risk. In a large clinically annotated dataset, high *USP44* was significantly associated with poor survival, metastatic disease, and unfavorable histology. The impact of *USP44* on survival was restricted to patients with *MYCN* non-amplified tumors and was also significant in non-high-risk cases. We identified cell lines with differential expression of *USP44* and found a significant acceleration of proliferation when *USP44* was introduced, and slowing when it was depleted. Consistent with its association with metastatic disease, we found a significant increase in migration and invasion in cells transduced to express *USP44*, whereas those with depleted expression had reductions in both. As *USP44* removes ubiquitin from histone H2B – an epigenetic mark associated with differentiation – we hypothesized that *USP44* may restrain differentiation. Supporting this notion, we observed a significant reduction in retinoic acid induced neurite extension and growth impairment in SH-SY5Y cells expressing *USP44*. To determine the in vivo relevance of our data, we generated compound *USP44-MYCN* transgenic zebrafish and found a significant acceleration of tumor onset compared with *MYCN* alone.



#### Summary/Conclusions:

Combining results from human datasets with our *in vitro* and *in vivo* experimental evidence, we conclude that *USP44* is a novel negative prognostic marker in *MYCN* non-amplified neuroblastoma. The expression of this epigenetic regulator promotes aggressive tumor behavior and restrains differentiation. Our work suggests that *USP44* may be an attractive therapeutic target in this disease.

## **P202 Anti-tumor immunity in neuroblastomas associated with opsoclonus myoclonus ataxia syndrome (OMAS) is mediated by B lymphocytes**

*Rosenberg, M.I.<sup>2</sup>, Greenstein, E.<sup>3</sup>, Buchkovich, M.<sup>4</sup>, Timmor, R.<sup>5</sup>, Mikl, M.<sup>6</sup>, Reshef, D.<sup>7</sup>, Santoni-Rugio, E.<sup>8</sup>, Salovin, A.<sup>9</sup>, Ulitsky, I.<sup>10</sup>, De Alarcon, P.<sup>11</sup>, Naranjo, A.<sup>12</sup>, Irwin, M.<sup>13</sup>, Lynch, D.<sup>14</sup>, Weigman, V.<sup>15</sup>, Yaari, G.<sup>16</sup>, Friedman, N.<sup>17</sup>, Panzer, J.A.<sup>18</sup>, Maris, J.M.<sup>19</sup>*

Hebrew University of Jerusalem, EEB, Jerusalem, Israel <sup>2</sup>, Weizmann Institute of Science, Immunology, Rehovot, Israel<sup>3</sup>, Q2 Solutions, Translational Genomics, Morrisville, Nc, USA<sup>4</sup>, Bar Ilan University, Engineering, Ramat Gan, Bar Ilan University<sup>5</sup>, Weizmann Institute of Science, Department of Molecular Cell Biology, Department of Applied Mathematics, Rehovot, Israel<sup>6</sup>, Weizmann Institute of Science, Immunology, Rehovot, Israel<sup>7</sup>, Rigshospitalet, Pathology, Copenhagen, Denmark<sup>8</sup>, Children's Hospital of Philadelphia, Neurology, Philadelphia, USA<sup>9</sup>, Weizmann Institute of Science, Biological Regulation, Rehovot, Israel<sup>10</sup>, University of Illinois College of Medicine, Pediatrics, Peoria, USA<sup>11</sup>, Children's Oncology Group, Biostatistics, Gainesville, FL, USA, <sup>12</sup>, SickKids Research Institute, Hospital for Sick Children, Pediatrics, Toronto, Canada <sup>13</sup>, Children's Hospital of Philadelphia, Neurology, Philadelphia, USA <sup>14</sup>, Q2 Solutions, Translational Genomics, Morrisville, Nc, USA <sup>15</sup>, Bar Ilan University, Engineering, Ramat Gan, Israel <sup>16</sup>, Weizmann Institute of Science, Immunology, Rehovot, Israel <sup>17</sup>, Children's Hospital of Philadelphia, Neurology, Philadelphia, USA <sup>18</sup>, Children's Hospital of Philadelphia, Oncology, Philadelphia, USA <sup>19</sup>

### Background/Introduction:

OMAS is a paraneoplastic disorder that affects about 2% of children with neuroblastoma. It is associated with excellent tumor-related outcomes, but devastating neurological sequelae impacting cerebellar and brain stem functions.

### Aims:

To discover the molecular basis of OMAS in order to understand adaptive immunologic recognition of neuroblastoma as a window into novel therapeutic strategies for both diseases.

### Methods/Materials:

We retrospectively procured all primary tumor samples (N=38) available from the COG ANBL00P3 clinical trial testing the efficacy of IVIg in neuroblastoma patients with OMAS (de Alarcon, 2018) and 13 low- and 13 high-risk tumor samples from neuroblastoma patients without OMAS as a comparator. We first performed immunohistochemical analyses with anti-CD3, anti-CD20, and anti-Ki67 antibodies. Next, all samples were subjected to bulk RNA sequencing (Illumina RNA Access platform), as well as T and B cell receptor repertoire analyses using the Adaptive Immunoseq platform. Finally, we carried out an association study to identify HLA alleles enriched in neuroblastoma patients with OMAS compared to neuroblastoma patients without OMAS symptoms.

### Results:

OMAS-associated tumors were enriched for germinal center-like structures with maturing B lymphocytes, as well as neuronal localization of both B cells and T cells. RNAseq showed B and T cell infiltration and activity and interferon gamma signaling as the dominant distinguishing features of OMAS tumors. We observe striking diversity of both T and B cell repertoires from OMAS-associated tumors, with no detectable clonal enrichment of any T or B cell receptors. We confirm and extend prior observations of particular MHC Class II alleles being significantly associated with OMAS (DOB\*01:01; DRB1\*13:02; DRB1\*01:01), implicating B cells and CD4+ helper T cells in OMAS neuropathology and tumor immunity.

### Summary/Conclusions:

The absence of dominant T or B cell clones in OMAS tumors suggests that no single antigen is responsible for driving an anti-tumor immune response. Instead, the essential nature of B cells in OMAS neuroimmunity, and the observations of enriched MHC Class II allele expression, and

increased B cell infiltration and activation in OMAS tumors, support a central role for B cells in OMAS anti-tumor immunity. We propose a model in which B cells acting as antigen presenting cells promote improved and sustained T cell activation via antigen presentation by their MHC, acting as potentiators of already broad T cell specificity that accompanies OMAS autoimmunity.

## **P203 The degree of chromosomal instability in circulating cell-free DNA is associated with clinical features in neuroblastoma**

*Ma, Y.<sup>2</sup>, Cho, H.<sup>3</sup>, Ju, H.Y.<sup>4</sup>, Lee, J.W.<sup>5</sup>, Cho, E.H.<sup>6</sup>, Yoo, K.H.<sup>7</sup>, Sung, K.W.<sup>8</sup>, Koo, H.H.<sup>9</sup>*

Seoul National University Bundang Hospital, Pediatrics, Seongnam, South Korea <sup>2</sup>, Samsung Medical Center, Pediatrics, Seoul, South Korea<sup>3</sup>, Samsung Medical Center, Pediatrics, Seoul, South Korea<sup>4</sup>, Samsung Medical Center, Pediatrics, Seoul, Samsung Medical Center<sup>5</sup>, Green Cross Genome, Genome Research Center, Yongin, South Korea<sup>6</sup>, Samsung Medical Center, Pediatrics, Seoul, South Korea<sup>7</sup>, Samsung Medical Center, Pediatrics, Seoul, South Korea<sup>8</sup>, Samsung Medical Center, Pediatrics, Seoul, South Korea<sup>9</sup>, ,

### Background/Introduction:

Chromosomal instability (CIN) is thought to take part in neuroblastoma development and aggressiveness. Circulating cell-free DNA (cfDNA), a promising biomarker for various cancers, is detectable in most patients with neuroblastoma.

### Aims:

The present study investigated the degree of CIN in cfDNA of neuroblastoma patients and its association with clinical features.

### Methods/Materials:

Among the patients diagnosed with neuroblastoma at Samsung Medical Center between November 2016 and August 2019, patients who had stored serum samples at diagnosis were enrolled. Low depth whole-genome sequencing of cfDNA was performed to identify genome-wide copy number alteration, and the I-score was used to evaluate CIN. The I-score was defined as the sum of absolute Z-scores of sequenced reads on each chromosome.

### Results:

A total of 48 patients (22 girls and 26 boys) were evaluated for CIN using serum at diagnosis. Whole-genome sequencing of cfDNA was successful in all patients, with a median I-score of 8396 (range, 0 - 28260). Abdominal primary site, advanced stage, less differentiated pathology (poorly differentiated, undifferentiated), bone marrow involvement by neuroblastoma, high-risk tumor, 1p deletion, 11q deletion and higher NSE values were associated with a higher I-score. Three patients experienced relapse/progression, the I-scores of these patients were 9203, 26510, and 14047.

### Summary/Conclusions:

A higher I-score was associated with high-risk features at diagnosis of neuroblastoma. We demonstrated that the I-score using cfDNA is a potential biomarker predicting the aggressiveness of neuroblastoma.

## **P204 Cross-talk between neuroblastoma cells and their microenvironment in the metastatic bone marrow niche**

*Timmerman, I.*<sup>2</sup>, *Van Zogchel, L.M.J.*<sup>3</sup>, *Kleijer, M.*<sup>4</sup>, *Kuijk, C.*<sup>5</sup>, *Tol, S.*<sup>6</sup>, *Van der Schoot, C.E.*<sup>7</sup>, *Voermans, C.*<sup>8</sup>, *Tytgat, G.A.M.*<sup>9</sup>

Sanquin Research and Landsteiner Laboratory, Amsterdam UMC, Hematopoiesis, Amsterdam, Netherlands<sup>2</sup>, Princess Maxima Center for Pediatric Oncology, Princess Maxima Center for Pediatric Oncology, Utrecht, Netherlands<sup>3</sup>, Sanquin Research and Landsteiner Laboratory, Amsterdam UMC, Hematopoiesis, Amsterdam, Netherlands<sup>4</sup>, Sanquin Research and Landsteiner Laboratory, Amsterdam UMC, Hematopoiesis, Amsterdam, Sanquin Research and Landsteiner Laboratory, Amsterdam UMC<sup>5</sup>, Sanquin Research and Landsteiner Laboratory, Amsterdam UMC, Molecular and Cellular Hemostasis, Amsterdam, Netherlands<sup>6</sup>, Sanquin Research and Landsteiner Laboratory, Amsterdam UMC, Experimental Immunohematology, Amsterdam, Netherlands<sup>7</sup>, Sanquin Research and Landsteiner Laboratory, Amsterdam UMC, Hematopoiesis, Amsterdam, Netherlands<sup>8</sup>, Princess Maxima Center for Pediatric Oncology, Princess Maxima Center for Pediatric Oncology, Utrecht, Netherlands<sup>9</sup>, ,

### **Background/Introduction:**

Neuroblastoma (NB) is a childhood malignancy originating from the sympathetic nervous system, prone to metastasize to bone marrow (BM). Persistence of micrometastases in BM is a likely key factor in relapse, which is unacceptably high for NB. The BM microenvironment appears to possess unique features that protect tumor cells. BM-residing Mesenchymal Stromal Cells (MSCs), for example, induce therapy resistance and promote tumor cell survival in several cancer types. MSCs can also exert pro-tumorigenic actions on NB-cells, as demonstrated by *in vitro* studies. We hypothesize that MSCs in BM are modified by NB-cells and involved in bidirectional interactions; thus, supporting NB-cells. Targeting MSCs within the BM microenvironment may offer a new avenue to combat metastatic-NB; hence, a better understanding of this milieu is of great importance.

### **Aims:**

To unravel NB-induced effects on MSCs within the BM microenvironment by isolating and characterizing MSCs from BM of NB-patients with and without metastases.

### **Methods/Materials:**

Diagnostic BM aspirates from patients with metastatic-NB were compared to BM from patients with localized-NB. The extent of BM infiltration was determined by reverse transcriptase quantitative PCR (RT-qPCR), using NB mRNA markers as previously described (Stutterheim, 2009). We analyzed fresh diagnostic BM of a cohort of 48 patients, and included adult and fetal BM as controls. We also examined follow-up BM samples of metastatic-NB patients: during and after treatment and at relapse. For phenotypic characterization flow cytometry was performed, using the classical MSC marker panel: CD34<sup>-</sup>, CD45<sup>-</sup>, CD90<sup>+</sup> and CD105<sup>+</sup>, with addition of CD146 and CD271 to explore subpopulations. Colony-forming unit-fibroblast (CFU-F) assays were used as a functional method to quantify the frequency of stromal progenitors in BM samples. Moreover, MSCs were isolated from BM and their differentiation capacity towards osteogenic and adipogenic lineages was examined.

### **Results:**

We found that BM with metastases contains a higher number of MSC. We identified a MSC subtype that is present in BM of NB-patients with metastases, but not in BM of patients with localized-NB or ganglioneuroma. Interestingly, the MSC subtype disappeared after treatment, when no detectable amounts of tumor cells are present, and was found again at relapse. This subtype was also present in fetal BM, suggesting that it might represent an immature MSC population. qPCR testing on FACS-sorted MSC from BM of metastatic-NB patients proved the absence of tumor cells in the MSC population. MSCs isolated from BM of metastatic-NB patients were more prone to differentiate

towards osteoblasts compared to localized-NB-MSc; emphasizing that MSCs from metastatic-NB patients not only differ phenotypically, but also functionally.

#### Summary/Conclusions:

We found that the MSC compartment in BM of NB-patients is modified when NB-cells are present. MSCs of patients suffering from metastatic-NB differ from 'normal' MSCs. Together, our findings point toward the existence of an active crosstalk between NB and MSC. Ultimately, increasing our understanding of MSC-NB crosstalk could provide targets with exciting potential for a promising novel approach to eradicate incurable metastatic disease: targeting the BM microenvironment.

## **P205 Defects in 8-oxo-Guanine repair pathway cause high frequency of C>A substitutions in neuroblastoma**

*Van den Boogaard, M.L.<sup>2</sup>, Oka, R.<sup>3</sup>, Hakkert, A.<sup>4</sup>, Schild, L.<sup>5</sup>, Ebus, M.E.<sup>6</sup>, Zwijnenburg, D.A.<sup>7</sup>, Hoyng, L.L.<sup>8</sup>, Dolman, M.E.M.<sup>9</sup>, Essing, A.H.W.<sup>10</sup>, Koopmans, B.<sup>11</sup>, Helleday, T.<sup>12</sup>, Drost, J.<sup>13</sup>, Van Boxtel, R.<sup>14</sup>, Versteeg, R.<sup>15</sup>, Koster, J.<sup>16</sup>, Molenaar, J.J.<sup>17</sup>*

Princess Máxima Center, Research, Utrecht, Netherlands <sup>2</sup>, Princess Máxima Center, Research, Utrecht, Netherlands<sup>3</sup>, Princess Máxima Center, Research, Utrecht, Netherlands<sup>4</sup>, Princess Máxima Center, Research, Utrecht, Princess Máxima Center<sup>5</sup>, Princess Máxima Center, Research, Utrecht, Netherlands<sup>6</sup>, Academic Medical Center, Oncogenomics, Amsterdam, Netherlands<sup>7</sup>, Academic Medical Center, Oncogenomics, Amsterdam, Netherlands<sup>8</sup>, Princess Máxima Center, Research, Utrecht, Netherlands<sup>9</sup>, Princess Máxima Center, Research, Utrecht, Netherlands<sup>10</sup>, Princess Máxima Center, Research, Utrecht, Netherlands<sup>11</sup>, Karolinska Institutet, Science for Life Laboratory, Department of Oncology-Pathology, Stockholm, Sweden, <sup>12</sup>, Princess Máxima Center, Research, Utrecht, Netherlands <sup>13</sup>, Princess Máxima Center, Research, Utrecht, Netherlands <sup>14</sup>, Academic Medical Center, Oncogenomics, Amsterdam, Netherlands <sup>15</sup>, Academic Medical Center, Oncogenomics, Amsterdam, Netherlands <sup>16</sup>, Princess Máxima Center, Research, Utrecht, Netherlands <sup>17</sup>

### Background/Introduction:

In neuroblastoma tumors C>A substitutions are the most common substitution type. C>A substitutions might be a result of defects in the 8-oxo-Guanine (8-OxoG) repair pathway. 8-oxoG is one of the most abundant DNA lesions that is generated by reactive oxygen species. During replication, DNA polymerases can insert an incorrect Adenine opposite of 8-oxoG. In the next round of replication, a Thymine will be inserted opposite the Adenine, finally resulting in a C>A substitution. 8-oxoG repair is initiated by excision of 8-oxoG by OGG1 or excision of the mis-inserted A, opposite 8-oxo-Guanine by MUTYH. *OGG1* and *MUTYH* are located on chromosome 3p and 1p, respectively, which are recurrently lost in neuroblastoma. This suggests that defects in *OGG1* or *MUTYH* might contribute to the high levels of C>A substitutions in neuroblastoma.

### Aims:

Investigate whether defects in 8-OxoG repair cause high levels of C>A substitutions in neuroblastoma

### Methods/Materials:

We used whole genome sequencing (WGS) data to investigate the frequency of C>A substitutions in a cohort of neuroblastoma tumors and correlated these C>A frequencies to INSS stage and survival. We also determined *MUTYH* or *OGG1* copy number loss in this cohort and correlated this to C>A substitution frequencies. In addition, we created CRISPR CAS9 knockout clones of *MUTYH* or *OGG1* in a neuroblastoma cell line (CHP134) and performed WGS to investigate mutational patterns. We performed mutational signature analysis for the *MUTYH* or *OGG1* knockout clones and the neuroblastoma tumors. Finally, we performed compound screens on the knockout clones and evaluated a previously published dataset of primary and relapse neuroblastoma tumors for C>A substitutions in the RAS MAPK pathway.

### Results:

We identified high levels of somatic C>A substitutions in our cohort of neuroblastoma tumors, which was associated with a poor survival prognosis. In addition, we identified a strong association between increased levels of C>A substitutions and copy number loss of *OGG1* or *MUTYH*. In the *OGG1* or *MUTYH* single cell knockout clones we identified an increased accumulation of C>A substitutions. Mutational signature analysis of *OGG1* or *MUTYH* knockout clones revealed an enrichment for signatures 18 and 36, respectively. These clones cluster together with neuroblastoma tumors with *OGG1* or *MUTYH* copy number loss, which also show a strong contribution of signature 18 or signature 36. In a compound screen we did not identify differential sensitivities between the *OGG1* or

*MUTYH* knockout clones compared to the mother line. Evaluation of a previously published dataset of primary and relapse neuroblastoma tumors indicates that in this small dataset 47% of the tumor evolution events in the RAS MAPK pathway are C>A substitutions.

Summary/Conclusions:

Defects in the 8-oxoG repair pathway result in high frequencies of C>A substitutions in neuroblastoma cell line models and tumors. These high levels of C>A substitutions correlate with a poor survival prognosis, which might be related to an increased adaptive capacity of these tumors.

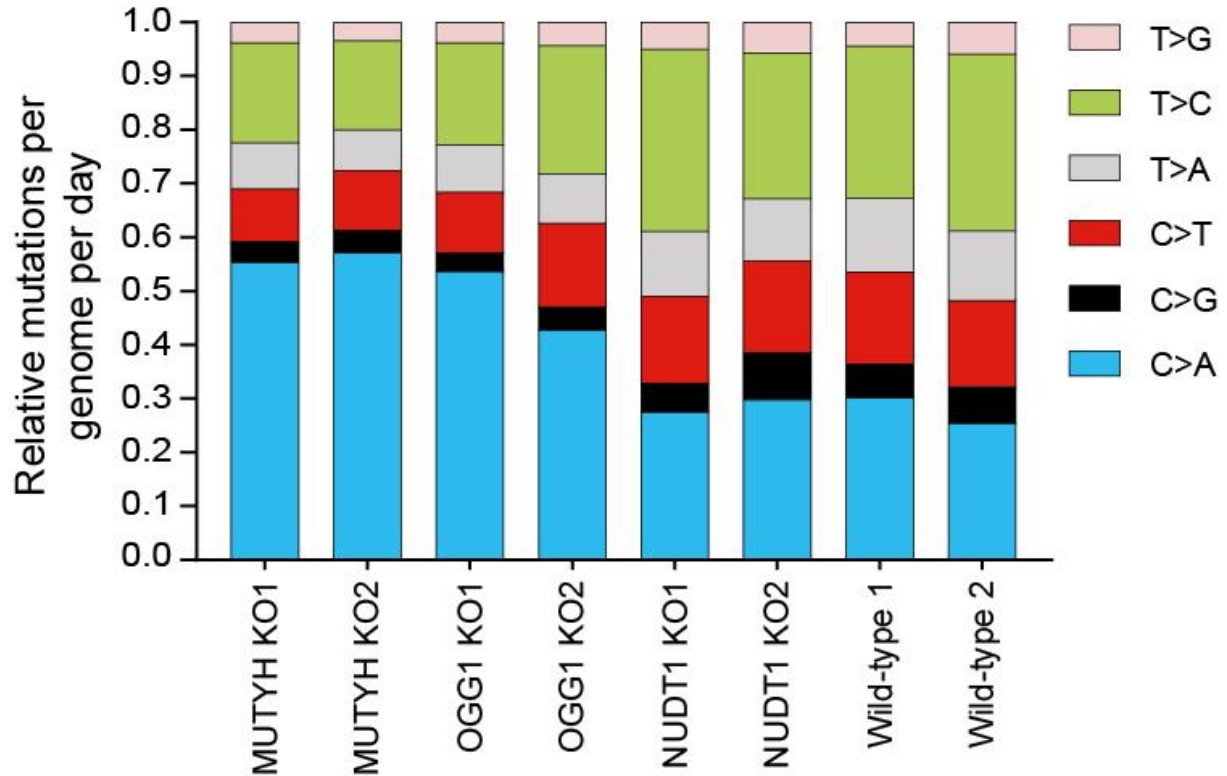


Figure 1. *MUTYH* or *OGG1* knockout results in accumulation of C>A substitutions in neuroblastoma cell line. Relative substitutions per genome per day that accumulated in *MUTYH* knockout, *OGG1* knockout, *NUDT1* knockout and wild-type CHP134 clones. Substitution types are indicated with colors.



Caption 1: MUTYH or OGG1 knockout results in accumulation of C>A substitutions in neuroblastoma cell line.

## **P206 The m6A RNA modification sustains neuroblastoma tumour aggressiveness and is a promising new pharmacological target**

*Montuori, G.<sup>2</sup>, Micaelli, M.<sup>3</sup>, Pasini, L.<sup>4</sup>, Dassi, E.<sup>5</sup>, Destefanis, E.<sup>6</sup>, Ricci, G.<sup>7</sup>, Broso, F.<sup>8</sup>, Toolan-Kerr, P.<sup>9</sup>, Capitanich, C.<sup>10</sup>, Ule, J.<sup>11</sup>, Provenzani, A.<sup>12</sup>, Quattrone, A.<sup>13</sup>*

University of Trento, Department of Cellular, Computational and Integrative Biology, Trento, Italy <sup>2</sup>, University of Trento, Department of Cellular, Computational and Integrative Biology, Trento, Italy<sup>3</sup>, IRST - Scientific Institute of Romagna for the Study and Treatment of Cancer, Department of Medical Oncology, Forlì, Italy<sup>4</sup>, University of Trento, Department of Cellular, Computational and Integrative Biology, Trento, University of Trento<sup>5</sup>, University of Trento, Department of Cellular, Computational and Integrative Biology, Trento, Italy<sup>6</sup>, University of Trento, Department of Cellular, Computational and Integrative Biology, Trento, Italy<sup>7</sup>, University of Trento, Department of Cellular, Computational and Integrative Biology, Trento, Italy<sup>8</sup>, The Francis Crick Institute, Molecular Neuroscience Department, London, United Kingdom<sup>9</sup>, The Francis Crick Institute, Molecular Neuroscience Department, London, United Kingdom<sup>10</sup>, The Francis Crick Institute, Molecular Neuroscience Department, London, United Kingdom<sup>11</sup>, University of Trento, Department of Cellular, Computational and Integrative Biology, Trento, Italy, <sup>12</sup>, University of Trento, Department of Cellular, Computational and Integrative Biology, Trento, Italy <sup>13</sup>

### Background/Introduction:

Neuroblastoma (NB) is a neuroendocrine tumour of the developing sympathoadrenal lineage that arises from the transformation of neural crest cells (NCCs). NB presents few somatic mutations, and it is mostly characterized by dramatic genomic copy number abnormalities. Therefore, a quantitative rather than a qualitative transcriptome imbalance should be at the heart of this disease. Moreover, the ability of some NBs which are metastatic at diagnosis to spontaneously regress suggests the involvement of reversible alterations. m<sup>6</sup>A is the most pervasive post-transcriptional modification in the transcriptome, controlling almost every aspect of mRNA biology. Strong evidence shows now consistent links between alteration in the m<sup>6</sup>A content and cancer progression, and the m<sup>6</sup>A modification has been also demonstrated to influence embryonic development. From the analysis of public datasets, we discovered that some m<sup>6</sup>A key regulators (METTL14, ALKBH5 and YTHDF1) are aberrantly expressed in high-risk neuroblastoma patients.

### Aims:

We aim to propose an innovative target for NB treatment, the m<sup>6</sup>A mRNA methylation, by proving the association between increase disease aggressiveness in patients and the causality for increased aggressiveness *in vitro* and *in vivo*.

### Methods/Materials:

NB cell lines were genetically engineered to overexpress METTL14, ALKBH5 or YTHDF1 and cellular and *in vivo* experiments were performed to demonstrate a role of the m<sup>6</sup>A in neuroblastoma aggressiveness and progression. We also analysed the methylome, the translome and the transcriptome of NB cells and integrated all the data to find those target that are most affected by m<sup>6</sup>A methylation, and that therefore could play a role in the neuroblastoma tumor phenotype.

### Results:

We find that overexpression of the methyltransferase METTL14 and the RNA-binding protein YTHDF1 increase neuroblastoma cell clonal potential and invasion both in adherent and 3D growing cells. Tumour growth *in vivo* is also enhanced. The restoration of the demethylase ALKBH5 acts in the opposite fashion, by strongly decreasing neuroblastoma progression. Molecularly, METTL14 positively controls neuroblastoma cell translation, while ALKBH5 play a role in the regulation of transcript stability. We finally provide proof-of-principle of the feasibility of pharmacological intervention on NB by interfering with the transduction of the m<sup>6</sup>A signal after a screening-based selection of a small molecule.

#### Summary/Conclusions:

NB is currently an urgent medical need in paediatric oncology. We discovered a new potential drug target for NB, the m6A RNA modification. We show that NB aggressiveness is positively correlated with the m6A-induced signal transduction in NB cells, leading to the effect of increased tumour fitness evident both in cultured *in vitro* conditions and in nude mice, and due to distinct actions of writers and erasers on mRNA. In the last 40 years, only few therapeutics solution have been added to the clinician toolkit to high-risk NB apart from the classic, heavy and highly toxic therapeutic approach of myeloablative chemotherapy followed by autologous stem cell transplantation. We propose that the suppression of m6A transduction is a new, promising drug target for high-risk NB.

## **P207 Evaluation of V600E BRAF mutation in neuroblastic tumors – an immunohistochemical study**

*Ash, S.<sup>2</sup>, Tzafiriri, O.<sup>3</sup>, Feinmesser, M.<sup>4</sup>*

Schneider Children's Medical Center of Israel, The Rina Zaizov Pediatric Hematology Oncology Division, Petach Tikva, Israel <sup>2</sup>, Shamir Medical Center, Pathology department, Rishon Lezion, Israel<sup>3</sup>, Rabin Medical Center, Department of pathology, Petach Tikva, Israel<sup>4</sup>

### Background/Introduction:

BRAF is a serine/threonine kinase immediately downstream of KRAS in the MAPK signaling pathway. The mutation most commonly expressed in human neoplasia, V600E, is expressed in a variety of common malignancies, among them are colon cancer, melanoma, thyroid cancer, glioma and more. Previous studies demonstrated that in some tumors this mutation is a prognostic marker and can be utilized for targeted therapy. In general, few studies investigated the presence of this mutation in pediatric tumors, and in particular, the evaluation of neuroblastoma, has been minimal.

### Aims:

The aim of this study was to investigate the expression of the most common BRAF mutation - V600E, in a variety of neuroblastic tumors.

### Methods/Materials:

We used the antibody Mouse anti-human BRAF V600E monoclonal antibody (clone VE1). Demographic as well as clinical and pathologic data was collected for these patients including age, gender, stage and outcome. The immunohistochemical stains were evaluated using a 3-tiered semiquantitative method.

### Results:

The study included tumor samples from sixty four patients: 55 Neuroblastomas, 7 Ganglioneuroblastomas and 2 Ganglioneuromas. The immune stains from all samples were negative for the BRAF. The controls were all positive.

### Summary/Conclusions:

These results cannot support the existence of BRAF mutation (V600E) in neuroblastic tumors. Therefore, this mutation cannot be considered as a target for treatment of neuroblastic tumors.

## **P208 Inhibiting PHGDH counteracts chemotherapeutic efficacy against *MYCN*-amplified neuroblastoma**

*Artl, B.*<sup>2</sup>, *Zasada, C.*<sup>3</sup>, *Wünschel, J.*<sup>4</sup>, *Winkler, A.*<sup>5</sup>, *Lodrin, M.*<sup>6</sup>, *Gürgen, D.*<sup>7</sup>, *Hoffmann, J.*<sup>8</sup>, *Baum, K.*<sup>9</sup>, *Wolf, J.*<sup>10</sup>, *Eggert, A.*<sup>11</sup>, *Kempa, S.*<sup>12</sup>, *Deubzer, H.E.*<sup>13</sup>

Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin, Germany<sup>2</sup>, Max-Delbrück Center for Molecular Medicine in the Helmholtz Association, Integrative Proteomics & Metabolomics, BIMSB, Berlin, Germany<sup>3</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin, Germany<sup>4</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin, Germany<sup>5</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin, Germany<sup>6</sup>, Experimental Pharmacology and Oncology Berlin-Buch GmbH, EPO, Berlin, Germany<sup>7</sup>, Experimental Pharmacology and Oncology Berlin-Buch GmbH, EPO, Berlin, Germany<sup>8</sup>, Max-Delbrück Center for Molecular Medicine in the Helmholtz Association, Mathematical Modelling of Cellular Processes, Berlin, Germany<sup>9</sup>, Max-Delbrück Center for Molecular Medicine in the Helmholtz Association, Mathematical Modelling of Cellular Processes, Berlin, Germany<sup>10</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin, Germany<sup>11</sup>, Max-Delbrück Center for Molecular Medicine in the Helmholtz Association, Integrative Proteomics & Metabolomics, BIMSB, Berlin, Germany, <sup>12</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin, Germany <sup>13</sup>

### Background/Introduction:

While directly targeting the *MYCN* oncogene remains a challenge, indirect approaches that target its binding partners or downstream effectors yield encouraging results.

### Aims:

We set out to identify metabolic alterations specifically associated with amplified *MYCN*.

### Methods/Materials:

We performed liquid chromatography mass spectrometry-based proteomics and pulsed stable isotope-resolved metabolomics (pSIRM) after labeling with <sup>13</sup>C-glucose. The *CRISPR/Cas9* technology was employed to generate single cell-derived PHGDH knockout clones. Functional studies were performed in cell culture and patient-derived neuroblastoma xenografts in mice.

### Results:

We analyzed the proteome of a representative cohort of 49 primary neuroblastoma biopsies, 13 neuroblastoma cell lines and the genetically engineered clone SH-EP-Tet21/N with adjustable *MYCN* levels. For each cell type, ~2.000-2.300 proteins were quantified. In total, 230 proteins significantly correlated with high-level *MYCN* expression in the biopsies and 73 in the cell lines. Altogether, 9 proteins consistently correlated with *MYCN* in both models, among them phosphoglycerate dehydrogenase (PHGDH), the rate-limiting enzyme in *de novo* serine synthesis (tumor cohort, Spearman correlation coefficient  $r=0.86$ ; cell line panel,  $r=0.71$ ). High-level *PHGDH* mRNA expression in primary neuroblastomas correlated in two independent patient cohorts (Kocak cohort,  $n=476$ ; Versteeg cohort,  $n=122$ ) with unfavorable patient survival and established clinical and molecular markers for unfavorable tumor biology. We analyzed changes in PHGDH expression on transcriptional and translational levels occurring in the synthetic *MYCN*-inducible system, SH-EP-Tet21/N, and found that conditional *MYCN* expression induced PHGDH expression on mRNA and protein level. We performed ChIP qRT-PCR to test whether *MYCN* associated with the *PHGDH* promoter and found an enrichment to two regions previously shown to be enriched for PHGDH in existing ChIP Seq data, altogether arguing for a role of *MYCN* in the transcriptional regulation of *PHGDH* in neuroblastoma cells. pSIRM and absolute quantitative GC-MS analysis demonstrated a higher <sup>13</sup>C-glucose incorporation in *de novo* serine synthesis in *MYCN*-amplified cells compared to normal *MYCN* diploid cells. While serine and glycine starvation did not affect proliferation and nucleotide pools of *MYCN*-

amplified cells, proliferation of normal *MYCN* diploid cells was attenuated and nucleotide pools decreased, altogether pointing towards an independence of *MYCN*-amplified cells from exogenous serine and glycine supply. Proliferation of 5 single-cell derived CRISPR/Cas9-mediated PHGDH knockout clones was attenuated compared to that of *MYCN*-amplified control cells. Treatment of *MYCN*-amplified neuroblastoma cells with two PHGDH small molecule inhibitors likewise arrested proliferation without affecting cell viability. Treatment of NMRI-Foxn1<sup>nu/nu</sup> mice carrying subcutaneous patient-derived *MYCN*-amplified neuroblastoma xenografts with PHGDH inhibitors as single-agent therapy slowed down tumor growth. The 2-drug combinations PHGDH inhibitor/cisplatin revealed an antagonizing effect on chemotherapy efficacy *in vivo*.

#### Summary/Conclusions:

Our results indicate a strong correlation between amplified *MYCN* and high-level PHGDH expression in neuroblastoma cells. PHGDH knockout and inhibition by small molecules consistently slows proliferation, but stops short of killing the cells, which then establish resistance to classical chemotherapy. Although PHGDH inhibition with small molecules has produced encouraging results in other preclinical cancer models, this approach must be considered with caution in neuroblastoma. Studies investigating potential metabolic rerouting by PHGDH inhibition are ongoing.

## **P209 Novel metabolic vulnerabilities: Inhibition of cysteine uptake as a therapeutic concept for MYCN/MYC-driven tumors**

Garbowicz, K.A.<sup>2</sup>, Brückner, L.M.<sup>3</sup>, Gogolin, S.<sup>4</sup>, Alborzinia, H.<sup>5</sup>, Flórez, A.F.<sup>6</sup>, Höfer, T.<sup>7</sup>, Westermann, F.<sup>8</sup>

Hopp-Children's Comprehensive Cancer Center at the NCT (KITZ), Neuroblastoma Genomics, Heidelberg, Germany<sup>2</sup>, Hopp-Children's Comprehensive Cancer Center at the NCT (KITZ), Neuroblastoma Genomics, Heidelberg, Germany<sup>3</sup>, Hopp-Children's Comprehensive Cancer Center at the NCT (KITZ), Neuroblastoma Genomics, Heidelberg, Germany<sup>4</sup>, Heidelberg University, Institute of Pharmacy and Molecular Biotechnology, Heidelberg, Heidelberg University<sup>5</sup>, German Cancer Research Center (DKFZ), Theoretical Systems Biology, Heidelberg, Germany<sup>6</sup>, German Cancer Research Center (DKFZ), Theoretical Systems Biology, Heidelberg, Germany<sup>7</sup>, Hopp-Children's Comprehensive Cancer Center at the NCT (KITZ), Neuroblastoma Genomics, Heidelberg, Germany<sup>8</sup>

### Background/Introduction:

Aberrant *MYCN* oncogene expression determines a subset of highly aggressive neuroblastomas (NB) with poor clinical outcome, mainly due to resistance to conventional treatment. Recent findings have established a novel functional link between oncogenic *MYCN* and ferroptosis - a regulated, iron-dependent form of cell death, characterized by accumulation of ROS-mediated lipid hydroperoxides dependent on the availability of the amino acid rate-limiting for glutathione synthesis, cysteine.

### Aims:

Our aim is to develop a precise mechanistic model of cysteine usage in ferroptosis sensitive/resistant cells and to validate this novel metabolic vulnerability in various preclinical models. We focused on understanding the impact of cyst(e)ine uptake, cysteine synthesis via methionine-to-cysteine conversion (transsulfuration) and cysteine catabolism on the glutathione metabolism, particularly glutathione peroxidase 4 (GPX4)-detoxifying activity controlling lipid hydroperoxide formation.

### Methods/Materials:

To understand the drivers of cysteine addiction of NB cells we performed cystine titration experiments that allowed us to define a panel of ferroptosis sensitive and resistant neuroblastoma cell lines. These models are currently tested in flux experiments using stable isotope labelled cysteine/cystine, glutamine, and methionine (<sup>13</sup>C, <sup>34</sup>S) to quantify the distribution and flux into different synthetic processes (glutathione, protein and CoA cofactor synthesis). Moreover, multi-omics profiling of NB cell lines and primary NBs were used to define genetic markers that predict sensitivity of NB cells to ferroptosis induction. NB xenograft mouse models were used to validate this metabolic vulnerability *in vivo*.

### Results:

Titration experiment show that *MYCN*-amplified NB cells are strongly addicted to cyst(e)ine supply, whereas cells lacking *MYCN* or *MYC* expression are largely resistant to limiting cystine supply. More importantly, downregulation of selected ferroptosis-related proteins increased sensitivity of *MYCN*-amplified NB cells to cystine deprivation as well as ferroptosis inducers. Flux experiments using stable isotope labelled amino acids revealed striking differences in distribution and flux into different metabolic processes when comparing both cell types. Of note, activation of the transsulfuration pathway due to epigenetic remodeling of genes controlling this process is a novel oncogenic function of *MYCN*, which provides a critical additional cysteine source in a subset of high-risk NBs.

### Summary/Conclusions:

In summary, our data show that ferroptosis execution emerges as a novel tumor suppressor function that needs to be inactivated during tumor formation and malignant progression. Conversely, *MYCN*-amplified tumors acquire metabolic activities to overcome ferroptosis sensitivity. Nevertheless, *MYCN*-

amplified cells are highly sensitive to ferroptosis induction, disclosing a yet unknown metabolic vulnerability that can be exploited therapeutically.



## **P210 The role of the neurotrophin receptors, TrkA/NTRK1 and TrkB/NTRK2, in modulation of radiation responses in neuroblastoma.**

*Hassiepen, C.H.<sup>2</sup>, Aashish, A.S.<sup>3</sup>, Ali, A.S.<sup>4</sup>, Iliakis, G.I.<sup>5</sup>, Schramm, A.S.<sup>6</sup>*

Universitätsklinikum Essen/ Innere Klinik, Molecular Oncologie, Essen, Germany <sup>2</sup>, University Hospital Essen / Medical Radiation Biology, Medical Radiation Biology, Essen, Germany<sup>3</sup>, University Hospital Essen /Radiotherapy, Radiotherapy, Essen, Germany<sup>4</sup>, University Hospital Essen / Medical Radiation Biology, Medical Radiation Biology, Essen, University Hospital Essen / Medical Radiation Biology<sup>5</sup>, University Hospital Essen / Inner clinic ( tumor research), Molecular Oncology, Essen, Germany<sup>6</sup>

### Background/Introduction:

Radiotherapy is an integral part of treatment in many cancer types including neuroblastoma (NB). High expression of TrkA/NTRK1 has been found to correlate with excellent prognosis of NB, while high TrkB/NTRK2 expression is associated with unfavorable outcome. Both, TrkA/NTRK1 and TrkB/NTRK2 are members of the neurotrophin receptor family regulating cell fate decisions to favour survival, differentiation or cell death, but their role in modulating radiation resistance is not well characterized.

### Aims:

The aim of the project how TrkA/NTRK1 and TrkB/NTRK2 and their downstream targets are involved in radioresistance of(NB).

### Methods/Materials:

We previously established inducible TrkA/NTRK1 or TrkB/NTRK2 overexpression in the human neuroblastoma cell lines, SH-SY5Y and SK-N-AS. To investigate the effects of TrkA/NTRK1 and TrkB/NTRK2 expression and activation in response to irradiation (IR), we monitored changes in cell-cycle distribution by PI-staining and H3pS10-assay. The effects of TrkA/NTRK1 and TrkB/NTRK2 overexpression and activation in combination with different irradiation intensities were analyzed by MTT and colony formation assay.

### Results:

Induction and activation of both NTRKs, TrkA/NTRK1 and TrkB/NTRK2 was unaffected by irradiation. Phenotypic and molecular characterization of the radiation response indicated a defective G2/M checkpoint in TrkA/NTRK1 -overexpressing cells, indicated by a higher mitotic index upon ionizing radiation (IR). This effect could be rescued by addition of the NTRK-inhibitor, Loxo-101, suggesting a direct impact of TrkA/NTRK1 on the response to IR. Likewise, ATM- or ATR-inhibition results in an increased mitotic index, which suggests a defect in the damage-sensing pathway. By contrast, TrkB/NTRK2 expression enhanced short-term and long-term survival upon IR. The responsible pathways were examined by inhibitor treatments and preliminary findings suggest involvement of the PI3K-pathway.

### Summary/Conclusions:

Our experimental data indicate that both, TrkA/NTRK1 and TrkB/NTRK2, are modulators of the radiation response. While increased TrkA/NTRK1 activity in SH-SY5Y cells led to DNA damage independent cell-cycle progression, increased TrkB/NTRK2 signaling was characterized by an increased short-term and also long-term survival upon irradiation. Both findings are in line with clinical data, which correlated high TrkA/NTRK1 expression with a favorable and high TrkB/NTRK2 expression with an unfavorable prognosis. However, further investigation of the molecular downstream mechanisms of these receptors is important to understand underlying principles and to identify potential therapeutic targets.

## **P211 Universal targeting of neuroblastoma via adapter chimeric antigen receptor (AdCAR)-modified NK-92 cells**

*Jung, G.*<sup>2</sup>, *Grote, S.*<sup>3</sup>, *Buchner, M.*<sup>4</sup>, *Baden, C.*<sup>5</sup>, *Mittelstaet, J.*<sup>6</sup>, *Kaiser, A.*<sup>7</sup>, *Handgretinger, R.*<sup>8</sup>, *Schleicher, S.*<sup>9</sup>

University Children's Hospital, Hematology and Oncology, Tuebingen, Germany <sup>2</sup>, University Children's Hospital, Hematology and Oncology, Tuebingen, Germany<sup>3</sup>, University Children's Hospital, Hematology and Oncology, Tuebingen, Germany<sup>4</sup>, University Children's Hospital, Hematology and Oncology, Tuebingen, Germany<sup>5</sup>, Miltenyi Biotec GmbH, Biotec, Bergisch Gladbach, Germany<sup>6</sup>, Miltenyi Biotec GmbH, Biotec, Bergisch Gladbach, Germany<sup>7</sup>, University Children's Hospital, Hematology and Oncology, Tuebingen, Germany<sup>8</sup>, University Children's Hospital, Hematology and Oncology, Tuebingen, Germany<sup>9</sup>, ,

### **Background/Introduction:**

Neuroblastoma emerges from immature neural crest cells of the sympathetic nervous system. Representing 7% of all pediatric cancers neuroblastoma is the most common extracranial tumor affecting children and accounts for about 15% of overall childhood cancer mortality. Neuroblastoma is a complex and heterogeneous disease with nearly 50% of patients having a high-risk phenotype characterized by widespread dissemination and poor long-term survival despite intensive multimodal therapy. As such, there is an urgent need for new and effective neuroblastoma treatment options.

### **Aims:**

Preclinical evaluation of universal adapter CAR (AdCAR)-engineered NK-92 cells as a treatment option for high risk neuroblastoma.

### **Methods/Materials:**

In the context of this project, the FDA approved human NK cell line NK-92 was genetically modified by lentiviral transduction to express a universal adapter chimeric antigen receptor construct. AdCARs are based on the unique properties of a novel single-chain variable fragment (scFv) targeting a "neo"-epitope-like structure derived from the endogenous vitamin biotin, linked to a monoclonal antibody which serves as adapter molecule. Using single cell sorting, AdCAR NK-92 clones with the highest CAR expression were selected and tested in combination with biotinylated therapeutic or clinical phase I/II antibodies targeting a variety of antigens expressed on different neuroblastoma cell lines. Calcein AM-based cytotoxicity assays, real-time impedance measurements and flow cytometry analysis were used to evaluate the specificity and activity of AdCAR NK-92 cells *in vitro*.

### **Results:**

AdCAR-engineered NK-92 cells in contrast to parental unmodified NK-92 cells induced specific and potent neuroblastoma cell lysis in an antibody and antigen dose-dependent manner. The selective cytotoxicity of AdCAR NK-92 cells stringently relied on the presence of biotin-conjugated antibodies, verifying the specificity of the AdCAR system.

Irradiation of AdCAR NK-92 cells, as required in all active clinical trials, had no immediate effect on target cell lysis or chemokine receptor expression, but led to a gradual decline in cytotoxicity and viability over several days post radiation exposure.

### **Summary/Conclusions:**

AdCAR NK-92 cell effector functions can be redirected against one or multiple antigens allowing universal and flexible targeting of neuroblastoma cells, thus counteracting antigen loss and immune escape. Moreover, AdCAR NK-92 cells can be manufactured as an "off-the-shelf on demand" standardized cellular product improving the practicality of CAR NK cell therapy combined with the possibility of tailoring an antibody platform for patient-individualized neuroblastoma treatment.



## **P212 Preliminary results of Phase I study of T-cell Engaging Anti-GD2 Bispecific Antibody Hu3F8-BsAb (NCT03860207): T-cell activation and Clinical Toxicities.**

*Modak, S.*<sup>2</sup>, *Suzuki, M.*<sup>3</sup>, *Childers, A.*<sup>4</sup>, *Mauguen, A.*<sup>5</sup>, *Cheung, N.K.*<sup>6</sup>

Memorial Sloan Kettering Cancer Center, Pediatrics, New York, USA <sup>2</sup>, Memorial Sloan Kettering Cancer Center, Pediatrics, New York, USA<sup>3</sup>, Memorial Sloan Kettering Cancer Center, Pediatrics, New York, USA<sup>4</sup>, Memorial Sloan Kettering Cancer Center, Pediatrics, New York, Memorial Sloan Kettering Cancer Center<sup>5</sup>, Memorial Sloan Kettering Cancer Center, Pediatrics, New York, USA<sup>6</sup>

### Background/Introduction:

T-cells have potent anti-cancer properties but their utility in pediatric malignancies is limited because of low tumor mutational burden, sparse tumor infiltration, chemotherapy-related immunosuppression and an immunosuppressive tumor microenvironment. In leukemia, chimeric antigen receptors and T-cell engaging bispecific antibodies (BsAb) have successfully redirected polyclonal T cells for anti-cancer activity. Built on the IgG(L)-scFv format, the first fully humanized BsAb targeting the carbohydrate antigen GD2, hu3f8bsAb showed high potency *in vitro* and *in vivo* against GD2(+) xenografts without neurotoxicity (Cancer Immunol Res 3:266, 2015). A phase I/II study was initiated in 2019 to test the toxicity and efficacy of hu3F8-BsAb.

### Aims:

The primary objectives of the phase I part of this study are to evaluate the toxicity of hu3F8-BsAb in patients with GD2-expressing tumors and to establish a recommended phase II dose. Secondary objectives include investigating pharmacokinetics, immune modulation, immunogenicity (by human anti-bispecific antibody response [HABA]) and anti-tumor activity.

### Methods/Materials:

Salient eligibility criteria include presence of measurable or evaluable high-risk disease resistant to standard therapy, absolute lymphocyte count >500/ul, lack of human anti-human antibody if previously exposed to monoclonal antibody. After premedication with acetaminophen, anti-histamines and dexamethasone hu3F8-BsAb is administered on days 1 and 7 with a dose-limiting toxicity observation period of 28 days. The first dose is administered in-patient with monitoring for at least 48 hours. Subsequent doses may be administered out-patient. Cohorts of one patient per dose level were planned with 3-10-fold dose-escalation between cohorts until a grade 2 related toxicity was encountered. Further dose-escalation follows a continuous reassessment method. Toxicity is assessed using CTCAE version 4.03. HABA and pharmacokinetics are measured using ELISA. Immune effects are evaluated by immunophenotyping of lymphocyte subsets and by serum cytokine levels.

### Results:

Six patients (5 with neuroblastoma and one with osteosarcoma) have thus far received 10 cycles of hu3F8-BsAb. Dose escalation commenced at 0.009 mcg/kg per cycle. At dose level 4 (2.6mcg/kg/cycle), grade 2 cytokine release syndrome (CRS) characterized by hypotension, hypoxia, myalgia, lymphopenia and thrombocytopenia was encountered. A subsequent patient was enrolled at that dose level and after no >grade 2 toxicities were encountered, dose was escalated to dose level 5 (4.5mcg/kg/cycle) with a fixed first dose of 1.3mcg/kg dose and a second dose of 3.2mcg/kg. Grade 3 CRS that had resolved to grade 1 within 48 hours was observed in this latter patient. Management of CRS included rescue doses of dexamethasone, tocilizumab, fluid boluses and supplemental oxygen. Onset of CRS occurred 2-4 hours after the end of hu3F8-BsAb infusion and resolved within 24-48 hours. CRS was most marked after the first dose, with mild to no symptoms with subsequent doses. Neither other related >grade 2 toxicities (including neurotoxicity) nor HABA were seen even after 4 cycles. Dose-limiting toxicity has not yet been encountered. CRS was characterized by log-fold, though transient elevations of IL-6, IL-10, IL-13, IL-2, IL-8, IFN- $\gamma$  and TNF- $\alpha$ .

Summary/Conclusions:

Hu3F8-BsAb activates T-cells with associated cytokine release syndrome. Dose-escalation is ongoing, and phase II will commence after recommended dose is established.

## **P213 Enhancement of anti-tumor immunity by T cells engineered to restore HLA expression in neuroblastoma**

*Iglesias, F.*<sup>2</sup>, *Weston, S.*<sup>3</sup>, *Atanackovic, D.*<sup>4</sup>, *Luetkens, T.*<sup>5</sup>

University of Utah/Huntsman Cancer Institute, Pediatric Hematology-Oncology, Salt Lake City, USA <sup>2</sup>, University of Utah/Huntsman Cancer Institute, Hematology and Hematologic Malignancies, Salt Lake City, USA<sup>3</sup>, University of Utah/Huntsman Cancer Institute, Hematology and Hematologic Malignancies, Salt Lake City, USA<sup>4</sup>, University of Utah/Huntsman Cancer Institute, Hematology and Hematologic Malignancies, Salt Lake City, University of Utah/Huntsman Cancer Institute<sup>5</sup>

### **Background/Introduction:**

T cells can target cancer cells by recognizing tumor-specific peptides presented by HLA proteins. HLA downregulation is one of the most common neuroblastoma (NB) immune escape mechanisms, and it is associated with an adverse prognosis. Interferon-gamma (IFN $\gamma$ ) has been shown to rescue HLA expression on NB cells; however, no strategies exist for the targeted delivery of IFN $\gamma$  to upregulate HLA on tumor cells.

### **Aims:**

To present a novel approach to induce HLA expression in NB cells using targeted cellular therapy.

### **Methods/Materials:**

We genetically engineered GD2-specific T cells expressing synthetic-Notch (synNotch) receptors. In our receptors, an internal Notch domain is cleaved upon binding of the target antigen GD2, which releases a transcription factor driving expression of IFN $\gamma$  (IFN $\gamma$  synNotch). We analyzed co-cultures of IFN $\gamma$  -synNotch T cells and human NB cell lines by flow cytometry, enzyme-linked immunosorbent assay, enzyme-linked immunospot assay, and luminescence-based cytotoxicity assays.

### **Results:**

Treatment with recombinant IFN $\gamma$  led to a strong induction of HLA expression in 6/6 NB cell lines. Next, we generated 13 synNotch receptors targeting NB-specific antigen GD2, and one CD19-specific synNotch receptor. One of the 13 GD2-targeting synNotch receptors showed strong surface expression and no basal IFN $\gamma$  expression. T cells expressing this construct (GD2-IFN $\gamma$ -synNotch T cells) produced IFN $\gamma$  in the presence of GD2-positive NB cells and not GD2-negative cells. *In vitro*, GD2- IFN $\gamma$  -synNotch T cells efficiently upregulated HLA on NB cell lines SK-N-DZ and NB-1643. HLA upregulation was maintained for seven days after removing GD2- IFN $\gamma$  -synNotch T cells from the co-culture. In contrast, treatment with GD2- IFN $\gamma$  -synNotch T cells did not increase PD-L1 on NB cells or PD-1 on T cells. *In vivo*, NOD.Cg-PrkdcscidIl2rgtm1Wjl/SzJ (NSG) mice were engrafted with the human NB cell line Kelly. Once tumors reached a diameter of 5 mm, GD2-IFN $\gamma$ -synNotch T cells were injected intratumorally. Three days after treatment, mice were euthanized, and their tumors were harvested and analyzed by immunohistochemistry for HLA induction. Mice treated with GD2- IFN $\gamma$  -synNotch T cells, but not CD19- IFN $\gamma$  -synNotch T cells, showed HLA upregulation in their tumors. Finally, GD2- IFN $\gamma$ - synNotch T cells significantly enhanced the killing of HLA-A\*02+ NB cell lines by T cell receptor (TCR) transgenic T cells targeting the tumor antigens NY-ESO-1 and PRAME.

### **Summary/Conclusions:**

Induction of HLA on tumor cells using engineered IFN $\gamma$  synNotch T cells is feasible and increases T cell-mediated tumor cell killing. Treatment with GD2-IFN $\gamma$ -synNotch T cells alone or in combination with TCR-transgenic T cells represents a promising approach to improve anti-tumor immunity in solid tumors.

## **P214 Functional analysis of natural antibody and NK cells against neuroblastoma**

*Kawakubo, N.*<sup>2</sup>, *Harada, Y.*<sup>3</sup>, *Ishii, M.*<sup>4</sup>, *Souzaki, R.*<sup>5</sup>, *Tajiri, T.*<sup>6</sup>, *Taguchi, T.*<sup>7</sup>, *Yonemitsu, Y.*<sup>8</sup>

Faculty of Medical Sciences, Kyushu University, Department of Pediatric Surgery, Fukuoka, Japan <sup>2</sup>, Graduate School of Pharmaceutical Sciences, Kyushu University, R&D Laboratory for Innovative Biotherapeutics, Fukuoka, Japan<sup>3</sup>, Faculty of Medical Sciences, Kyushu University, Department of Pediatric Surgery, Fukuoka, Japan<sup>4</sup>, Faculty of Medical Sciences, Kyushu University, Department of Pediatric Surgery, Fukuoka, Faculty of Medical Sciences, Kyushu University<sup>5</sup>, Kyoto Prefecture University of Medicine, Department of Pediatric Surgery, Kyoto, Japan<sup>6</sup>, Faculty of Medical Sciences, Kyushu University, Department of Pediatric Surgery, Fukuoka, Japan<sup>7</sup>, Graduate School of Pharmaceutical Sciences, Kyushu University, R&D Laboratory for Innovative Biotherapeutics, Fukuoka, Japan<sup>8</sup>

### **Background/Introduction:**

The mechanism underlying the spontaneous regression of neuroblastoma is unclear. Although it was hypothesized that this regression occurs via an immunological mechanism, there is no clinical evidence, and no animal models have been developed to investigate the involvement of immune systems, especially natural antibodies and Natural Killer (NK) cells, against neuroblastoma.

### **Aims:**

We hypothesized that immunological systems correlate to spontaneous regression of neuroblastoma, and investigated immunological function of natural antibody and NK cells against neuroblastoma.

### **Methods/Materials:**

**Mouse model:** We performed an immunological analysis of homo- and heterozygous TH-*MYCN* transgenic mice in which MHC-Class1 is null expression. The tumor cells obtained from TH-*MYCN* mice were incubated with plasma obtained from TH-*MYCN* mice and stained by fluorescence-labeled anti-mouse IgG/IgM, then the fluorescence was detected by flow cytometry. To analyze the function of anti-tumor antibody, complement-dependent cytotoxicity (CDC) assay and antibody-dependent cellular cytotoxicity (ADCC) assay were conducted. Depletion experiments *in vivo* (NK, CD4, CD8 and B) were performed by administration of anti-asialo GM1 antibody, anti-CD4 antibody (clone GK1.5), anti-CD8 antibody (clone 53-6.72) and anti-CD20 antibody (clone 5D2), respectively.

**Human model:** Highly activated NK cells (GAIA-102) were generated as previously described (Saito S, et al. *Hum Gene Ther* 2013, AdoptCell-NK Kit: Cat. no. 16030400). Briefly, CD3-depleted PBMCs were cultured under the condition of adhesion culture for 14 days. A spheroid of IMR32 (neuroblastoma cell line) was co-cultured with fluorescence-labeled GAIA-102, and invasion of the effector cells into spheroid was observed. Spheroids were then manually disrupted, and the number of live tumor cells were counted by flow cytometry.

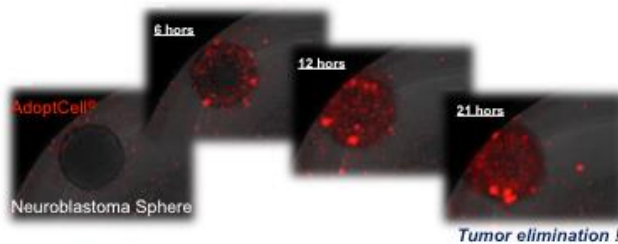
### **Results:**

**Mouse model:** Mice with no or small (<5 mm) tumors showed higher antibody titers in plasma than mice with large (>5 mm) tumors. A significant negative correlation was observed between the tumor diameter and the titer of antitumor antibody. This antibody had CDC but not ADCC against neuroblastoma cells. Moreover, B-cell depletion had no effect on the tumor incidence *in vivo*. On the other hand, NK-cell depletion showed tendency of increase of tumor incidence. **Human model:** In 3D cytotoxicity assay, GAIA-102 efficiently killed IMR32 spheroid as compared to primary NK cells, anti-GD2 antibody or combination of both. Live imaging demonstrated GAIA-102 actively migrate, infiltrate and alter spheroid morphology subsequently mediate tumor cell killing in IMR32 spheroid. In contrast, primary NK cells could not invade into the spheroid.

### **Summary/Conclusions:**

We revealed that TH-MYCN transgenic mice have a natural antibody against neuroblastoma that correlate with tumor size. However, this antibody does not correlate with the spontaneous regression of neuroblastoma. Thus, the function of the natural antibody is limited. From our depletion study of NK cells and *in vitro* ADCC assay, it appears that NK cells may be involved in the spontaneous regression. In human model, GAIA-102 exhibits high cytotoxicity and invasion activity against neuroblastoma spheroid. GAIA-102 offers a highly promising approach for the treatment of solid, bulky lesion of advanced neuroblastoma.

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- ✓ Can infiltrate and eliminate solid tumors
- ✓ Not be affected by MDSC/Treg/TGF-β
- ✓ Can achieve total tumor cell killing





## **P215 KETAMINE BASED MANAGEMENT OF NAXITAMAB (Hu3F8) INDUCED PAIN IN THE OUTPATIENT SETTING AT HSJD.**

*Mora, J.<sup>2</sup>, Chamizo, A.<sup>3</sup>, Lazaro, J.J.<sup>4</sup>, Chamorro, S.<sup>5</sup>, Lopez-Miralles, S.<sup>6</sup>, Castañeda, A.<sup>7</sup>, Flores, M.A.<sup>8</sup>, Simao, M.<sup>9</sup>, Santamaria, V.<sup>10</sup>, Garraus, M.<sup>11</sup>, Gorostegui, M.<sup>12</sup>*

PCCB, Oncology, Barcelona, Spain<sup>2</sup>, PCCB, Anesthesiology, Barcelona, Spain<sup>3</sup>, PCCB, Anesthesiology, Barcelona, Spain<sup>4</sup>, PCCB, Oncology, Barcelona, PCCB<sup>5</sup>, PCCB, Oncology, Barcelona, Spain<sup>6</sup>, PCCB, Oncology, Barcelona, Spain<sup>7</sup>, PCCB, Oncology, Barcelona, Spain<sup>8</sup>, PCCB, Oncology, Barcelona, Spain<sup>9</sup>, PCCB, Oncology, Barcelona, Spain<sup>10</sup>, PCCB, Oncology, Barcelona, Spain<sup>11</sup>, PCCB, Oncology, Barcelona, Spain,<sup>12</sup>

### **Background/Introduction:**

Immunotherapy with anti-GD2 MoAbs has improved outcomes (EFS and OS) in patients with high-risk NB. Generalized neuropathic pain is a dose-limiting toxicity of 3F8 anti-GD2 MoAbs. The pain associated with anti-GD2 therapy is similar to other neuropathic pain syndromes and is relatively opioid-resistant; nevertheless, opioids are routinely utilized for pain control during anti-GD2 infusions. Neuropathic pain is mediated mainly through the N-methyl-D-aspartate (NMDA) receptor and Ketamine is an NMDA-R antagonist. It releases nitric oxide and binds to mu receptors to increase the effectiveness of opioid-induced signaling. These multifactorial actions help in producing effective analgesia.

### **Aims:**

We investigated the management of Naxitamab (hu3F8) induced acute neuropathic pain with ketamine.

### **Methods/Materials:**

The same outpatient clinic personnel treated all patients and a physician was involved in all bedside decisions including when to give rescue doses of analgesics to control pain. Standard supportive therapy included Gabapentin (10mg/kg q8h po) from day -5; before the infusion paracetamol (15 mg/kg po), cetirizine (2.5-10 mg po), ranitidine (2mg/kg po), ondansetron (5 mg/m<sup>2</sup> po) and Morphine Chloride 25-100 mcg/Kg iv. Patients were selected to ketamine management if standard support therapy was ineffective to control reactions during infusion. They received treatment in the procedure room under the supervision of anesthesiologist. Same premedication but instead of Morphine before hu3F8 infusion received Midazolam (0.05 mg/Kg iv bolus), Lidocaine (2 mg/Kg iv bolus), Ketamine (2 mg/Kg iv bolus), and Atropine (0.005 mg/Kg iv if normal heart rate). Middle way through hu3F8 infusion (minute 15) a second dose of Lidocaine (2 mg/Kg iv bolus) and Ketamine (2 mg/Kg iv bolus; up to a total of 4 mg/kg) is provided.

### **Results:**

Since June 2017, 115 patients received a total of 659 Naxitamab cycles (range 1-20). 21 (18%) pts were selected for ketamine protocol: four because of complex allergic reactions potentially enhanced by the use of opioids; six because of apnea; four because of refractory hypotension; and seven because of unmanageable pain. 19 of 21 patients successfully completed planned treatment with Ketamine and were all managed outpatient. The recovery time and resume of normal daily activities is faster (3-5 days) compared to standard management (10-15 days). Two very young patients developed apnea in the first few minutes of infusion also with ketamine based protocol and were taken off study.

### **Summary/Conclusions:**

Ketamine management of neuropathic pain related to Naxitamab is safe and effective in the outpatient setting.



## **P216 NAXITAMAB (Hu3F8) plus GM-CSF FOR HIGH-RISK NEUROBLASTOMA IN COMPLETE REMISSION PATIENTS: RESULTS OF A PATIENT RESTRICTED USE PROGRAM (YMABS) AT HSJD.**

*Mora, J.*<sup>2</sup>, *Castañeda, A.*<sup>3</sup>, *Flores, M.A.*<sup>4</sup>, *Gorostegui, M.*<sup>5</sup>, *Simao, M.*<sup>6</sup>, *Santamaria, V.*<sup>7</sup>, *Garraus, M.*<sup>8</sup>, *Chamorro, S.*<sup>9</sup>, *Lopez-Miralles, S.*<sup>10</sup>, *Chamizo, A.*<sup>11</sup>, *Lazaro, J.J.*<sup>12</sup>

PCCB, Oncology, Barcelona, Spain<sup>2</sup>, PCCB, Oncology, Barcelona, Spain<sup>3</sup>, PCCB, Oncology, Barcelona, Spain<sup>4</sup>, PCCB, Oncology, Barcelona, PCCB<sup>5</sup>, PCCB, Oncology, Barcelona, Spain<sup>6</sup>, PCCB, Oncology, Barcelona, Spain<sup>7</sup>, PCCB, Oncology, Barcelona, Spain<sup>8</sup>, PCCB, Oncology, Barcelona, Spain<sup>9</sup>, PCCB, Oncology, Barcelona, Spain<sup>10</sup>, PCCB, Anesthesiology, Barcelona, Spain<sup>11</sup>, PCCB, Anesthesiology, Barcelona, Spain,<sup>12</sup>

### Background/Introduction:

Immunotherapy with chimeric anti-GD2 MoAbs (m3F8; Dinutuximab/beta) has improved outcomes (EFS and OS) in patients with high-risk NB in complete remission (CR) after induction therapy and autologous bone marrow transplantation (ABMT). Anti-GD2 antibodies differ in their *in vitro* and *in vivo* properties.

### Aims:

We investigated the humanized anti-GD2 MoAb Naxitamab (previously termed Hu3F8) in combination with GM-CSF (granulocyte macrophage colony stimulating factor) in patients achieving CR after standard induction therapy.

### Methods/Materials:

Patients were treated on a patient restricted program based on an ongoing phase 2 trial of the same agents (NCT 03363373). Eligibility criteria included non-evidence of disease by bone marrow studies (4 aspirates), MIBG-SPECT/FDG-PET and whole body MRI. Prior anti-GD2 MoAbs was permitted. Each cycle comprised sc GM-CSF at 250 µg/m<sup>2</sup>/d on days -4 to 0 (Wednesday through Sunday), followed by 500 µg/m<sup>2</sup>/d on days 1 to 5 (Monday through Friday); and hu3F8 iv for 30 minutes on days 1, 3, and 5 (3 doses/cycle) at 3 mg/kg/day (total 9 mg/kg or 270 mg/m<sup>2</sup> per cycle). Treatment consisted of 5 cycles, all outpatient. Toxicity was measured by CTCAE v4.0.

### Results:

Since June 2017, 49 patients (34 in first CR and 15 in 2nd CR) received a total of 180 cycles. Six patients had prior isolated CNS relapse; 8 had systemic relapse; and one had both. 11 (22%) patients had MYCN amplified NB. 7 (20%) of 34 in first CR and 9 (60%) of 15 in 2nd CR had prior ABMT. Sixteen (32%) had MRD based on Phox2B qRT-PCR. Toxicities included pain and hypertension expected with Naxitamab. No other >grade 2 related toxicities occurred. 36 completed all planned cycles. 4 and 9 patients could not complete 5 cycles because of toxicity and PD, respectively. With a median F/up of 13.5 months, 7 (20%) of 34 pts in 1st CR and 6 (40%) of 15 pts in 2nd CR have relapsed. Three (6%) patients have died of disease progression. 2-year EFS and OS for all patients is 65.3%, 95% CI = (49.4%; 86.3%) and 87.2%, 95% CI = (73.1%; 100%), respectively. 2-year EFS and OS for 1st CR patients with no prior ABMT (n=27) is 62.2%, 95% CI = (38.4%; 100%) and 80.2%, 95% CI = (55.7%; 100%).

### Summary/Conclusions:

Naxitamab-based immunotherapy is effective in consolidating both first and second remission in high-risk neuroblastoma.

**P217 Naxitamab-based Chemoimmunotherapy for Resistant High-Risk Neuroblastoma: Preliminary results of HITS treatment through compassionate use at HSJD.**

*Mora, J.<sup>2</sup>, Castañeda, A.<sup>3</sup>, Flores, M.A.<sup>4</sup>, Gorostegui, M.<sup>5</sup>, Simao, M.<sup>6</sup>, Santamaria, V.<sup>7</sup>, Chamizo, A.<sup>8</sup>, Lopez-Miralles, S.<sup>9</sup>, Garraus, M.<sup>10</sup>, Chamorro, S.<sup>11</sup>, Lazaro, J.J.<sup>12</sup>*

PCCB, Oncology, Barcelona, Spain<sup>2</sup>, PCCB, Oncology, Barcelona, Spain<sup>3</sup>, PCCB, Oncology, Barcelona, Spain<sup>4</sup>, PCCB, Oncology, Barcelona, PCCB<sup>5</sup>, PCCB, Oncology, Barcelona, Spain<sup>6</sup>, PCCB, Oncology, Barcelona, Spain<sup>7</sup>, PCCB, Anesthesiology, Barcelona, Spain<sup>8</sup>, PCCB, Oncology, Barcelona, Spain<sup>9</sup>, PCCB, Oncology, Barcelona, Spain<sup>10</sup>, PCCB, Oncology, Barcelona, Spain<sup>11</sup>, PCCB, Anesthesiology, Barcelona, Spain,<sup>12</sup>

**Background/Introduction:**

Chemoresistant and relapsed disease are major obstacles to curing high-risk neuroblastoma (HR-NB). Anti-GD2 monoclonal antibody (MoAb) is effective in preventing relapse after remission but responses in relapsed or progressive disease (PD) are rare.

**Aims:**

We investigated the combination of humanized anti-GD2 MoAb naxitamab, (previously termed H u3F8), irinotecan, temozolomide and argatroban (GM-CSF): a HITS patient restricted use against resistant HR-NB following the phase II study (NCT03189706).

**Methods/Materials:**

Salient eligibility criteria included evaluable or measurable chemoresistant disease. Prior anti-GD2 MoAb and/or irinotecan/temozolomide (I/T) therapy was permitted. Each cycle comprised of irinotecan 50 mg/m<sup>2</sup>/day intravenously (IV) plus temozolomide 150 mg/m<sup>2</sup>/day IV or orally (days 1-5); naxitamab 2.25 mg/kg/day IV over 30 minutes, days 2, 4, 8 and 10 (total 9 mg/kg or 270 mg/m<sup>2</sup> per cycle), and GM-CSF 250 mg/m<sup>2</sup>/day subcutaneously, days 6-10. Toxicity was measured by CTCAE v4.0 and responses by modified International Neuroblastoma Response Criteria.

**Results:**

Forty-four (36 evaluable and 8 non-evaluable) heavily prior-treated patients (median age at enrollment: 6 years; median number of prior relapses: 2) have received 210 (median 6; range 1-14) cycles to date. 16 patients had HR-NB refractory to induction chemotherapy while 28 had prior relapse. Toxicities included myelosuppression and diarrhea expected with I/T, and pain and hypertension expected with naxitamab. No other >grade 2 related toxicities occurred; treatment was outpatient. Early responses, assessed after 2 cycles, were documented in 17 (47%) of 36 evaluable patients and were complete (n=11), and partial (n=6); 10 patients had stable disease. Nine (25%) patients progressed before or at cycle 2 evaluation. 1-year progression-free survival is 37.0%, 95% CI (21.8%, 62.9%), median time to progression 9.2 months. 1-year Overall Survival is 55%, CI (39.4%, 76.9%).

**Summary/Conclusions:**

High-dose naxitamab-based chemoimmunotherapy is safe and effective against chemoresistant HR-NB.

## **P218 A novel neuroblastoma-based model to study the effects of suppressive monocytes on CART cell therapy**

*Frosch, J.<sup>2</sup>, Gargiulo, L.<sup>3</sup>, Wallace, R.<sup>4</sup>, Hanif-Muflahah, C.<sup>5</sup>, Straathof, K.<sup>6</sup>, Anderson, J.<sup>7</sup>*

UCL Institute of Child Health, DBC Cancer, London, United Kingdom <sup>2</sup>, UCL Institute of Child Health, DBC Cancer, London, United Kingdom<sup>3</sup>, UCL Institute of Child Health, DBC Cancer, London, United Kingdom<sup>4</sup>, UCL Institute of Child Health, DBC Cancer, London, UCL Institute of Child Health<sup>5</sup>, UCL Institute of Child Health, DBC Cancer, London, United Kingdom<sup>6</sup>, UCL Institute of Child Health, DBC Cancer, London, United Kingdom<sup>7</sup>

### **Background/Introduction:**

Neuroblastoma is a paediatric solid tumour which presents with poor survival rates among patients within the high-risk group. Despite advances in cancer therapy, clinical outcomes remain poor, and novel approaches are needed. Chimeric antigen receptor (CAR) T cells have been demonstrated to be an effective cell-based treatment in blood cancers, where genetically engineered T cells are re-directed to detect highly expressed surface antigens of the tumour cells. However, the immunosuppressive microenvironment that is formed by solid tumours and the surrounding tumour-conditioned immune cells heavily affects the efficacy of CART cell therapy due to inhibition of proliferation and effector functions. Myeloid derived suppressor cells (MDSC) are a key group among tumour-induced immune cells and have been shown to correlate with cancer stage, disease progression, and resistance to standard therapies.

### **Aims:**

To successfully overcome local inhibition of CART cells, the effects of the microenvironment of solid tumours need to be studied more closely. Even though they are a key player, the detailed interactions with MDSC are not yet fully understood. The aim of this work is to provide a neuroblastoma-based model to study and overcome interactions between CART cells and suppressive myeloid cells.

### **Methods/Materials:**

In an *in vitro* polarisation assay, healthy monocytes were grown in neuroblastoma-conditioned media to mimic the conditions of the tumour environment. The generated neuroblastoma-conditioned monocytes (NbM) were characterised for changes in their phenotype and their suppressive effect on T cells in co-cultures, using flow cytometry analysis and ELISA.

### **Results:**

Phenotype analysis of NbM showed an enrichment of a subtype reflecting mononuclear MDSC (M-MDSC; CD14<sup>+</sup> HLA-DR<sup>low</sup>), as well as early-stage MDSC (eMDSC; CD14<sup>-</sup> CD15<sup>-</sup> HLA- DR<sup>low</sup>). In co-cultures, polarised monocytes significantly inhibit T cell proliferation and activation (interferon gamma release). Sunitinib malate, a receptor tyrosine kinase inhibitor, can partially recover T cell functions through inhibition of NbM. Scaling to 96 well format shows the level of reproducibility necessary for high-throughput drug library screens for inhibitors of NbM suppression function using interferon gamma secretion as a read out.

### **Summary/Conclusions:**

Here we present a neuroblastoma-based model to screen for strategies to overcome myeloid cell-mediated T cell suppression in the tumour environment. The generated NbM reflect phenotype and suppressive effect of M-MDSC, which can be found in the neuroblastoma microenvironment. A 96 well plate inhibition of suppression assay is now being used to identify retargetable agents. Thus, the established model can lay the basis for improving CART cell immunotherapy by reducing MDSC-based suppression.



## **P219 Pilot clinical trial of DNA vaccination against neuroblastoma**

Meleshko, A.N.<sup>2</sup>, Proleskovskaya, I.V.<sup>3</sup>

Belarussian Research Center for Pediatric Oncology, Hematology and Immunology, Genetic biotechnology, Minsk, Belarus <sup>2</sup>, Belarussian Research Center for Pediatric Oncology, Hematology and Immunology, Clinical administration, Minsk, Belarus<sup>3</sup>

### Background/Introduction:

Effective treatment of high risk neuroblastoma (NB) remains a serious problem for pediatric oncology. A promising therapy option for this group is immunotherapy, including antitumor vaccination. We launched a pilot clinical trial of combined DNA vaccine, preceded by a long period of preclinical *in vitro* and *in vivo* studies in the animal model.

### Aims:

to check the safety and immunogenicity of combined DNA vaccination against neuroblastoma

### Methods/Materials:

DNA vaccination requires complete immune recovery, the absence of a bulky tumor, the time for the induction of an immune response, and the absence of immunosuppressive immunotherapy during the vaccination period. Considering this, we decided to include patients in remission 2-4 months after the completion of relapse chemotherapy. Each patient receives the vaccine against three NB-associated antigens of 6 candidates: tyrosine hydroxylase, Phox2B, Survivin, MAGE-A1, MAGE-A3, PRAME. IHC and RQ-PCR confirmation determines the choice of antigen for each patient. The genetic design of the vaccine consists of fusion of antigen and immune-enhancer - the potato virus X coat protein (PVXCP) gene.

The vaccine includes two ways of administration. An injectable vaccine based on the DNAPolyethyleneimine conjugate (PEI) is administered intramuscularly. Linear PEI-20kDa was chosen as optimal for *in vivo* vaccination according to the results of our preclinical tests. Conjugation is carried out by mixing a DNA and PEI solutions in a mass ratio of DNA:PEI = 1.2-1.3 (400 µg DNA/500 µg PEI).

The oral form of the vaccine is delivered by live bacteria. The attenuated strain SS2017 (*aroA*<sup>-</sup>, *guaAB*<sup>-</sup>) was obtained by further modification of the original vaccine strain *Salmonella enterica* serovar typhimurium SL7207. One dose includes 10<sup>10</sup> CFU carrying plasmid DNA vaccine in suspension with 50% glycerol in a gelatin capsule. Three vaccination courses are carried out, with three introductions of a new antigen in each.

Before and during vaccination, accompanying chemotherapy is conducted, including cyclophosphamide 300 mg/m<sup>2</sup> once three days before vaccination, lenalidomide 25 mg/day starting 7 days before the first vaccination, propranolol and celecoxib according to the metronome therapy regimen.

Monitoring includes: assessment of minimum residual disease (MRD) by RQ-PCR; determination of circulating tumor cells (CD45<sup>-</sup>, GD2<sup>+</sup>, CD81<sup>+</sup>, CD9<sup>+</sup>, CD56<sup>+</sup>); production of INF-γ by lymphocytes in the presence of PVXCP protein or peptide library of the corresponding antigen by ELISPOT.

### Results:

The protocol and ethics committee were approved on January 9, 2019. To date, 4 patients have received a full course of vaccination. Patients tolerated the vaccine well without adverse symptoms. All four are in remission with negative MRD in the bone marrow. Two of them (B1, K3) are free of tumor by MRT/MIBG scintigraphy. 6 months after vaccination, patient B1 had isolated CNS relapse. M4 - had a residual MIBG-positive site in paravertebral area. Both foci of the tumor were completely surgically removed and pronounced CD8 infiltration was observed. Immune response was negative for

patient K3, other three patients had pronounced CD4 immune response against the PVXCP protein and positive CD8 to the antigens peptide library.

Summary/Conclusions:

Combined vaccination of DNA-PEI injection and oral bacteria was safe and immunogenicity against NB antigens was proved



## **P220 Antibodies from mice cured of melanoma identify >3000 antigens; several are homologs for proteins found in HLA-clefts on human neuroblastomas**

*Sondel, P.M.<sup>2</sup>, Hoefges, A.<sup>3</sup>, Erbe, A.K.<sup>4</sup>, Ong, I.M.<sup>5</sup>, McIlwain, S.J.<sup>6</sup>, Melby, D.S.<sup>7</sup>, Xu, A.<sup>8</sup>, Mathers, N.<sup>9</sup>, Rakhmievich, A.L.<sup>10</sup>, Hank, J.A.<sup>11</sup>, Baniel, C.<sup>12</sup>, Heinze, C.<sup>13</sup>, Yarmarkovich, M.<sup>14</sup>, Maris, J.M.<sup>15</sup>, Garcia, B.<sup>16</sup>, Patel, B.<sup>17</sup>, Morris, Z.S.<sup>18</sup>*

University of WI, Pediatrics, Human Oncology, Genetics, Madison, USA <sup>2</sup>, University of WI, Human Oncology, Madison, USA<sup>3</sup>, University of WI, Human Oncology, Madison, USA<sup>4</sup>, University of WI, Biostatistics and Medical Informatics, Madison, USA<sup>5</sup>, University of WI, Biostatistics and Medical Informatics, Madison, USA<sup>6</sup>, University of WI, Human Oncology, Madison, USA<sup>7</sup>, University of WI, Human Oncology, Madison, USA<sup>8</sup>, University of WI, Human Oncology, Madison, USA<sup>9</sup>, University of WI, Human Oncology, Madison, USA<sup>10</sup>, University of WI, Human Oncology, Madison, USA<sup>11</sup>, University of WI, Human Oncology, Madison, USA, <sup>12</sup>, University of Washington, Bioengineering, Seattle, USA <sup>13</sup>, Children's Hospital of Philadelphia and University of Pennsylvania, Pediatrics, Philadelphia, USA <sup>14</sup>, Children's Hospital of Philadelphia and University of Pennsylvania, Pediatrics, Philadelphia, USA <sup>15</sup>, Nimble Therapeutics, Discovery, Madison, USA <sup>16</sup>, Nimble Therapeutics, Discovery, Madison, USA <sup>17</sup>, University of WI, Human Oncology, Madison, USA <sup>18</sup>

### **Background/Introduction:**

C57Bl/6 mice bearing the GD2<sup>pos</sup>. B78 melanoma were cured of large (~200mm<sup>3</sup>) tumors by a combination of 12-Gy radiotherapy + intratumoral anti-GD2/IL2 fusion protein (the hu14.18-IL2 immunocytokine)<sup>1,2</sup>. Cured mice showed memory by rejecting a B78 as well as a GD2<sup>neg</sup>. B16 melanoma rechallenge<sup>1,2</sup>. Sera from cured mice, showed strong binding to B16 melanoma (by flow cytometry), compared to background binding by naïve mouse sera.

### **Aims:**

We seek to comprehensively identify antigens that are recognized by antibodies induced during the immuno-therapeutically driven rejection of large neuroectodermal origin cancers in mice, and determine their relevance to human neuroblastoma antigens.

### **Methods/Materials:**

Sera from 6 naïve mice and 7 cured mice that rejected rechallenge were evaluated on a high-density peptide array, reflecting the entire murine proteome (~8.5 million 16-mer peptides, reflecting the full proteome of ~56,000 uniprot IDs, ~22,000 genes) using technology developed by Nimble Therapeutics<sup>3</sup>. This methodology should allow detection of any peptidebased epitope that is <16 amino acids.<sup>3</sup>

### **Results:**

These immune sera from individual mice show clear binding to over 20,000 separate probes, derived from ~6500 separate genes. 6777 probes were each co-recognized by 2 separate immunotherapeutically cured immune mice (corresponding to ~3000 genes). The epitopes demonstrating the most robust binding by multiple mouse sera are being identified/characterized. We focused on 162 murine proteins that are the homologs of 162 human proteins from which peptides were highly represented in the clefts of MHC molecules on human neuroblastomas (also of neuroectodermal origin, like melanoma), as identified by mass spectrometry in the lab of John Maris (see Yarmarkovich M., ANR 2020). Of the murine proteins identified by the immune mouse sera, 34 were from this group of 162 human neuroblastoma homologs, including: Igfbp1, Phox2b and Cdk1. In addition, of the unique murine genes identified by these immune mice, human homologs for 149 showed strong expression on human neuroblastoma and not normal tissues, as determined by comparison of RNA-seq data between 153 tumors from the TARGET database and 1641 normal tissues from the GTEx database. We are now focusing on the proteins showing the strongest binding,

to characterize them and determine if any new targets may be identified. Future studies will perform similar analyses with immune sera from mice immunotherapeutically cured of neuroblastoma.<sup>4</sup>

#### Summary/Conclusions:

Our data suggest that:

Antigens detected prominently by sera of cured mice may correspond to homologous human antigens that could be recognized by immunotherapy-treated patients;

Acquisition of strong antibody responses to certain antigens shared by several tumors might potentially serve as an early serum biomarker for an effective clinical immunotherapeutic response;

Antigens strongly detected by immune mouse sera may correspond to homologous human antigens, that may be considered as future targets for creation of mAb or engineered T cell receptor binders for therapeutic development.

#### References

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## **P221 Role of Clinical Trial Nurses in Humanized Bispecific Antibody (Hu3F8-BsAb) Therapy**

Childers, A.<sup>2</sup>, Modak, S.<sup>3</sup>, Cheung, N.K.<sup>4</sup>, D'Andrea, L.<sup>5</sup>, Szenes, V.<sup>6</sup>, Pacelli, C.<sup>7</sup>, Fortier, B.<sup>8</sup>, Suzuki, M.<sup>9</sup>

Memorial Sloan Kettering Cancer Center, Clinical Trials Nursing, New York, USA <sup>2</sup>, Memorial Sloan Kettering Cancer Center, Pediatrics, New York, USA<sup>3</sup>, Memorial Sloan Kettering Cancer Center, Pediatrics, New York, USA<sup>4</sup>, Memorial Sloan Kettering Cancer Center, Pediatrics, New York, Memorial Sloan Kettering Cancer Center<sup>5</sup>, Memorial Sloan Kettering Cancer Center, Clinical Trials Nursing, New York, USA<sup>6</sup>, Memorial Sloan Kettering Cancer Center, Clinical Trials Nursing, New York, USA<sup>7</sup>, Memorial Sloan Kettering Cancer Center, Clinical Trials Nursing, New York, USA<sup>8</sup>, Y-mAbs Therapeutics, Inc, Clinical Research, Horsholm, Denmark<sup>9</sup>

### Background/Introduction:

Anti-GD2 immunotherapy has improved progression-free survival rates of patients with metastatic Neuroblastoma. The International Neuroblastoma Risk Group found that the 5-year post-relapse overall survival for stage 4 Neuroblastoma was 8% (Journal of clinical oncology 2011;29:208-13). Novel treatment approaches are therefore warranted.

A Phase I/II study of humanized 3F8 bispecific antibody (Hu3F8-BsAb) in patients with relapsed/refractory neuroblastoma, osteosarcoma, and other GD2(+) solid tumors opened in 2019 (NCT03860207). Hu3F8-BsAb has two specificities: Hu3F8 which targets the cell surface disialoganglioside GD2, and huOKT3 (anti-CD3) which binds and activates T-cells. This hu3F8-BsAb has shown potent anti-tumor activity in *in vitro* and *in vivo* preclinical animal models.

### Aims:

The aim of this report is to describe the role of Clinical Trial Nurses (CTNs) in facilitating the conduct of the above study in both inpatient and outpatient settings.

### Methods/Materials:

We reviewed the role of CTNs in the care of patients enrolled on this clinical trial and in protocol education.

### Results:

CTNs play an active role in all aspects of this clinical trial, from protocol activation, patient screening and consent, treatment, toxicity management, relevant data capture, and coordination of care.

Protocol activation encompasses the development of study tools such as order sets, patient consents and other materials. Prior to and during execution of the phase I/II trial, CTNs assist in the development of education materials for clinicians for adherence to protocol requirements and to update them on toxicities. CTNs coordinate the periodic distribution of this information to nurses, nurse practitioners, and physicians caring for enrolled patients. CTNs are responsible for real-time assessment of toxicities. This includes recognition and management of adverse events, anticipated and unanticipated, specifically cytokine release syndrome and neurotoxicity. Issues related to the clinical trial are discussed in weekly clinic meetings.

Patients are screened for eligibility by the CTN and the consenting physicians of the trial. CTNs ensure that every patient and guardian have a clear understanding of the nature of clinical trials, specific study rationale, planned treatment interventions, the risks and potential benefits of the treatment, and follow-up requirements. The CTN is present at each infusion to ensure protocol compliance, help with management of adverse reactions and update relevant drug and trial information for practitioners. As part of protocol compliance, CTNs ensure accurate collection of blood and tissue samples for pharmacokinetics and biologic correlate studies which require coordination with the clinical and research laboratories. The first six patients on Hu3F8-BsAb were treated successfully without dose limiting toxicities. This supports a continuation of dose escalation in this trial.

Summary/Conclusions:

The key to the successful implementation of this Hu3F8-BsAb trial is close coordination and communication between CTNs, nurses, PIs, physicians, and the patient/family, to maximize compliance, safety, quality of clinical and research data, and adherence to institutional and FDA guidelines. For novel agents such as bispecific antibodies where toxicities in children are unknown, CTNs should provide the conduit for information transfer and data capture vital for reaching trial intended endpoints.

## **P222 Biomarkers of a Potent Allogeneic Ex Vivo Expanded Gamma Delta T Cell Product for Neuroblastoma Immunotherapy**

*Jonus, H.*<sup>2</sup>, *Burnham, R.*<sup>3</sup>, *Spencer, T.*<sup>4</sup>, *Goldsmith, K.*<sup>5</sup>

Emory University, Pediatrics, Atlanta, USA <sup>2</sup>, Emory University, Pediatrics, Atlanta, USA<sup>3</sup>, Emory University, Pediatrics, Atlanta, USA<sup>4</sup>, Emory University/Children's Healthcare of Atlanta, Pediatrics, Atlanta, Emory University/Children's Healthcare of Atlanta<sup>5</sup>

### **Background/Introduction:**

High-risk neuroblastoma (NB) patient survival significantly improved following introduction of the therapeutic anti-GD2 antibody dinutuximab, increasing interest in other immunotherapies, like adoptive transfer of cellular products. Adoptive cell therapy has focused on  $\alpha\beta$  T cells, which so far, have been unsuccessful for solid tumors.  $\gamma\delta$  T cells are an innovative and potentially superior product as they are MHC independent, directly cytotoxic to tumor cells, and lack the alloreactivity of  $\alpha\beta$  T cells. Previously, we demonstrated  $\gamma\delta$  T cells expanded from NB patient apheresis products effectively kill NB cell models. However, NB patient marrows are heavily pretreated and residual apheresis products are often depleted. We, therefore, propose expanding from healthy donors to yield a more robust cell therapy product and provide ample supply to meet the demands of our planned first-in-human Phase 1 clinical trial of expanded  $\gamma\delta$  T cells, which will be combined with temozolomide and dinutuximab for patients with recurrent NB.

### **Aims:**

This work aims to potentiate the clinical effectiveness of allogeneic *ex vivo* expanded  $\gamma\delta$  T cells by defining biomarkers for the most potent donor products.

### **Methods/Materials:**

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood of healthy donors and expanded following our GMP-certified, serum free protocol using zoledronate and IL-2. Expansion products were  $\alpha\beta$  T cell depleted and immunophenotyped for  $\gamma\delta$  T cell and Natural Killer (NK) cell content prior to assaying cytolytic potential against IMR-5 NB cells. Negative selection was performed for  $\gamma\delta$ -TCR or CD56 expression to test the cytotoxic potential of  $\gamma\delta$  T cells and NK cells alone.

### **Results:**

Healthy donor PBMC expansions yield cellular products predominantly populated with  $\gamma\delta$  T cells in addition to NK cells. The  $\gamma\delta$  T cell-to-NK cell ratio varies donor-to-donor and may depend on the NK cell content pre-expansion. This ratio remains relatively stable through one freeze-thaw cycle. All expanded  $\gamma\delta$  T cell products effectively kill NB cells *in vitro*. Notably, across 15 expansions, products with a final NK cell content >50% demonstrated the most potent anti-NB cell death (>70-80% at 5:1, effector:target). However, only 5/15 expansions reached a threshold of >50% NK, suggesting most expanders yield  $\gamma\delta$  T cell-dominant populations. Depletion of  $\gamma\delta$ -TCR+ cells from the expanded product resulted in a highly potent NK cell population, while depletion of CD56+ cells resulted in a  $\gamma\delta$  T cell population with reduced killing potential compared to the complete product. It remains unclear how CD56+  $\gamma\delta$  T cells contribute to the potency of the overall product or how different NK cell content influences dinutuximab responses, as  $\gamma\delta$ -TCR+ T cells after expansion express high CD16.

### **Summary/Conclusions:**

Not all healthy donors expand cytolytic lymphocytes equally nor are their products equally potent. CD56 expression on both NK and  $\gamma\delta$  T cells may serve as a biomarker of cytolytic potential. We are determining NB responses to CD56 enriched and depleted products combined with dinutuximab, and distinguishing if CD56 expression pre-expansion predicts potency postexpansion. Concomitantly, we will determine if CD56 may be induced *ex vivo* to derive the most potent  $\gamma\delta$  T/NK cellular therapeutic to-date.

## **P223 Anti-GD2 therapy in high-risk and relapsed/refractory neuroblastoma in Taiwanese pediatric patients: NTUCH experience**

*Lu, M.Y.<sup>2</sup>, Su, M.Y.<sup>3</sup>, Yang, Y.L.<sup>4</sup>*

National Taiwan University Hospital, Pediatrics, Taipei, Taiwan <sup>2</sup>, National Taiwan University Hospital, Pediatrics, Taipei, Taiwan<sup>3</sup>, National Taiwan University Hospital, Laboratory Medicine, Taipei, Taiwan<sup>4</sup>

### Background/Introduction:

High-risk neuroblastoma treated with anti-GD2 antibody showed better overall survival. In Taiwan, the experience of anti-GD2 therapy was limited. Here we reported the experience of anti-GD2 therapy in our hospital.

### Aims:

We reported the experience of anti-GD2 antibody therapy in our hospital.

### Methods/Materials:

We review the chart of the neuroblastoma patients treated with anti-GD2 regimen retrospectively.

### Results:

Six patients received anti-GD2 therapy in our hospital in the past 3 years. Two patients were in relapse/refractory status, one was in second complete response status and three patients were in first complete response status under maintain therapy. Two patients received anti-GD2 antibody therapy combined with chemotherapy. Five of six patients(83%) continue complete response till now, mean event free time is 21.6 months(2~38 months). One patient shows progression disease and dropped out after 3 courses of the anti-GD2 therapy. Among the side effect, fever is the most common side effect (79%). Capillary leakage syndrome (CLS) with desaturation, tachypnea, wheezing, stridor and edema was the second common (38%). Pain was controlled well by morphine. No patient died in the treatment, and they all recovered from symptomatic treatment or by slowing down the rate of medication infusion.

### Summary/Conclusions:

Anti-GD2 therapy seemed to be safe and effective to high-risk neuroblastoma and relapsed neuroblastoma. The most common effect is fever which usually happen within 48 hours after infusion. CLS is another noticeable side effect. CLS becomes worse in the last courses in our experience. The development of Human anti-mouse antibody might be considered.

## **P224 Management of Acute Toxicities of Naxitamab (Hu3F8): A Model for Infusion Nursing at the Bedside**

*Scoppetuo, L.A.<sup>2</sup>, Glincher, R.<sup>3</sup>, Alessi, K.<sup>4</sup>, Murphy, K.<sup>5</sup>, Bhattacharya, R.<sup>6</sup>, Bompensiero, N.<sup>7</sup>, Modak, S.<sup>8</sup>, Cheung, N.<sup>9</sup>, Kushner, B.<sup>10</sup>*

Memorial Sloan Kettering Cancer Center, Nursing, New York, USA <sup>2</sup>, Memorial Sloan Kettering Cancer Center, Nurse Practitioner, New York, USA<sup>3</sup>, Memorial Sloan Kettering Cancer Center, Nursing, New York, USA<sup>4</sup>, Memorial Sloan Kettering Cancer Center, Nursing, New York, Memorial Sloan Kettering Cancer Center<sup>5</sup>, Memorial Sloan Kettering Cancer Center, Nursing, New York, USA<sup>6</sup>, Memorial Sloan Kettering Cancer Center, Nursing, New York, USA<sup>7</sup>, Memorial Sloan Kettering Cancer Center, Pediatrics MD, New York, USA<sup>8</sup>, Memorial Sloan Kettering Cancer Center, Pediatrics MD, New York, USA<sup>9</sup>, Memorial Sloan Kettering Cancer Center, Pediatrics MD, New York, USA<sup>10</sup>

### **Background/Introduction:**

Anti-GD2 monoclonal antibody (MoAb) therapy is an integral part of treatment for patients with high-risk neuroblastoma. The humanized anti-GD2 MoAb (naxitamab) is given as a 30-90 minute infusion in the outpatient setting at Memorial Sloan Kettering Cancer Center (MSKCC), unlike dinutuximab (Ch 14.18), which is given inpatient over 10-20 hours. Adverse events encountered during or soon after the infusion include pain, allergic reactions, respiratory distress, lethargy, and hypotension. Treatment is administered by infusion nurses trained specifically in naxitamab therapy. Other nursing personnel involved include nurse practitioners and bedside nurses. Nursing autonomy emphasizes timely intervention within specific guidelines using best clinical judgment and medical knowledge to manage adverse events at the bedside.

### **Aims:**

The aim of this report is to describe the role of the infusion nurse in the outpatient administration of naxitamab.

### **Methods/Materials:**

Infusion nurses are required to receive formal training over a 12-week period on the management of naxitamab adverse events. They familiarize themselves with therapeutic interventions which include administration of hydromorphone, racemic epinephrine, intramuscular epinephrine, levalbuterol, naloxone, inhaled oxygen, diphenhydramine and IV fluid boluses as clinically indicated. Detailed order sets governing the use of the above medications are prepared by nurse practitioners. Infusion nurses are assigned to each patient receiving naxitamab and remain at the bedside for the duration of the treatment and immediate recovery period. Within the framework of nursing guidelines, they assess and manage adverse events using these medications throughout the infusion. A primary nurse model is utilized to develop familiarity with individual patient adverse reactions. Infusion nurses are assisted by bedside nurses who provide pre-infusion management, assist during the infusion, and provide post-management care. When the need for naloxone, epinephrine, or fluid boluses arises, infusion nurses call upon nurse practitioners or attending physicians for consultation and advice. In the absence of the above adverse events, infusion nurses exercise autonomy in the management of naxitamab infusion. In rare occasions when cardiopulmonary support is needed, patient care will transition to attending staff. Patients remain in the clinic until all adverse events have subsided and are cleared for discharge by infusion nurses, nurse practitioners or attending physicians.

### **Results:**

Up to 5 infusion nurses can treat up to 8 patients/day while maintaining safety and patient flow in the outpatient setting. More than 250 patients have been treated at MSKCC with >1000 infusions in the outpatient setting from 2011-2019. There was no drug-associated mortality, and post-treatment inpatient admission rates were <1%. Coordination, cooperation and trust have enabled infusion nurses to consistently make appropriate clinical judgements. Having dedicated trained infusion nurses at the bedside, with nurse practitioner and attending physician back-up, allows for rapid and consistent

responses to clinical changes in the patients' status, and the ability to treat multiple patients in the same day.

**Summary/Conclusions:**

Trained infusion nurses can administer naxitamab safely in the outpatient setting and may provide a viable nursing model for testing future biologics with predictable and severe acute side effects.



## **P225 A novel multimeric IL15/IL15R $\alpha$ -Fc complex to enhance neuroblastoma immunotherapy**

*Xu, H.*<sup>2</sup>, *Buhtoiarov, I.*<sup>3</sup>, *Guo, H.F.*<sup>4</sup>, *Cheung, N.K.*<sup>5</sup>

Memorial Sloan Kettering Cancer Center, Pediatrics, New York, USA <sup>2</sup>, Memorial Sloan Kettering Cancer Center, Pediatrics, New York, USA<sup>3</sup>, Memorial Sloan Kettering Cancer Center, Pediatrics, New York, USA<sup>4</sup>, Memorial Sloan Kettering Cancer Center, Pediatrics, New York, Memorial Sloan Kettering Cancer Center<sup>5</sup>

### **Background/Introduction**

The role of T cells and NK cells in controlling a wide spectrum of human cancers is proven. The success requires their continual *in vivo* persistence and their ability to traffic to tumor sites. Yet, there is no clinically approved cytokine available that will expand, maintain, and activate effector T cells or NK cells, other than IL-2 which has many undesirable toxicities. IL-15 has diverse immunologic effects, and plays an important role in the development, homeostasis and function of memory CD8<sup>+</sup> T cells, NK cells, and other immune cells. A soluble IL15/IL15R $\alpha$  complex, using the sushi domain of the IL15R $\alpha$  and the N72D mutation in IL15 (IL15-N72D/IL15R $\alpha$ -Sushi-Fc, ALT-803), has been proven effective in preclinical and clinical trials. GD2 is one of the proven neuroblastoma antigens for immunotherapy, and 3F8 mAbs and hu3F8-BsAb have shown robust anti-tumor activities against neuroblastoma and other GD2<sup>+</sup> tumors both *in vitro* and *in vivo*.

### **Aims**

To develop a novel IL15/IL15R $\alpha$  complex in order to achieve better *in vivo* efficacy than ALT-803 without the disadvantage of introducing mutations in IL15 which could potentially induce neutralizing antibodies.

### **Methods/Materials**

Instead of using Sushi domain (SU) of IL15R $\alpha$  (aa1-66) in the complex, we used full length ectodomain (FL) of IL15R $\alpha$  (aa1-175) fused to the CH2-CH3 of human IgG1 Fc region. Both WT and N72D mutant IL15 (MUT, to enhance affinity for IL15 $\beta\gamma$ c), in complex with either FL or SU domain of IL15R $\alpha$  were made for comparison. All four complexes (WT-FL, MUT-FL, WT-SU and MUT-SU) showed >90% purity and stability with storage.

### **Results**

We successfully engineered a full length IL15R $\alpha$  construct (WT-FL) that allowed the IL15/IL15R $\alpha$  complex to form spontaneous trimers of dimers (6 IL15 + 6 IL15R $\alpha$ ). The complex was able to stimulate PBMC-derived T cells and NK cells with augmented *in vitro* tumor cytotoxicity, increased expression of surface markers such as stimulatory receptors, cytotoxic proteins both intracytoplasmically and on the cell surface. Furthermore, when compared to MUT-SU (ALT-803), the WT-FL complex showed substantially longer serum half-life, with considerable enhancement of anti-neuroblastoma potency in the presence of NK + 3F8 mAbs, or T cells + hu3F8-BsAb, in multiple xenograft models.

### **Summary/Conclusions**

The increase in serum half-life and enhancement of anti-tumor effect was substantial when the WT-FL complex is administered subcutaneously, a convenient route for clinical application. The trimeric nature of this complex is the key to its superior biologic properties. This is novel, especially given the previous concern that such constructs could be inhibitory instead of stimulatory on immune cells. These results support further evaluation of this complex for clinical development.

## **P226 A novel multimeric IL15/IL15R $\alpha$ -Fc complex to enhance neuroblastoma immunotherapy**

*Xu, H.*<sup>2</sup>, *Buhtoiarov, I.*<sup>3</sup>, *Guo, H.F.*<sup>4</sup>, *Cheung, N.K.*<sup>5</sup>

Memorial Sloan Kettering Cancer Center, Pediatrics, New York, USA <sup>2</sup>, Memorial Sloan Kettering Cancer Center, Pediatrics, New York, USA<sup>3</sup>, Memorial Sloan Kettering Cancer Center, Pediatrics, New York, USA<sup>4</sup>, Memorial Sloan Kettering Cancer Center, Pediatrics, New York, Memorial Sloan Kettering Cancer Center<sup>5</sup>

### **Background/Introduction**

The role of T cells and NK cells in controlling a wide spectrum of human cancers is proven. The success requires their continual *in vivo* persistence and their ability to traffic to tumor sites. Yet, there is no clinically approved cytokine available that will expand, maintain, and activate effector T cells or NK cells, other than IL-2 which has many undesirable toxicities. IL-15 has diverse immunologic effects, and plays an important role in the development, homeostasis and function of memory CD8<sup>+</sup> T cells, NK cells, and other immune cells. A soluble IL15/IL15R $\alpha$  complex, using the sushi domain of the IL15R $\alpha$  and the N72D mutation in IL15 (IL15-N72D/IL15R $\alpha$ -Sushi-Fc, ALT-803), has been proven effective in preclinical and clinical trials. GD2 is one of the proven neuroblastoma antigens for immunotherapy, and 3F8 mAbs and hu3F8-BsAb have shown robust anti-tumor activities against neuroblastoma and other GD2<sup>+</sup> tumors both *in vitro* and *in vivo*.

### **Aims**

To develop a novel IL15/IL15R $\alpha$  complex in order to achieve better *in vivo* efficacy than ALT-803 without the disadvantage of introducing mutations in IL15 which could potentially induce neutralizing antibodies.

### **Methods/Materials**

Instead of using Sushi domain (SU) of IL15R $\alpha$  (aa1-66) in the complex, we used full length ectodomain (FL) of IL15R $\alpha$  (aa1-175) fused to the CH2-CH3 of human IgG1 Fc region. Both WT and N72D mutant IL15 (MUT, to enhance affinity for IL15 $\beta\gamma$ c), in complex with either FL or SU domain of IL15R $\alpha$  were made for comparison. All four complexes (WT-FL, MUT-FL, WT-SU and MUT-SU) showed >90% purity and stability with storage.

### **Results**

We successfully engineered a full length IL15R $\alpha$  construct (WT-FL) that allowed the IL15/IL15R $\alpha$  complex to form spontaneous trimers of dimers (6 IL15 + 6 IL15R $\alpha$ ). The complex was able to stimulate PBMC-derived T cells and NK cells with augmented *in vitro* tumor cytotoxicity, increased expression of surface markers such as stimulatory receptors, cytotoxic proteins both intracytoplasmically and on the cell surface. Furthermore, when compared to MUT-SU (ALT-803), the WT-FL complex showed substantially longer serum half-life, with considerable enhancement of anti-neuroblastoma potency in the presence of NK + 3F8 mAbs, or T cells + hu3F8-BsAb, in multiple xenograft models.

### **Summary/Conclusions**

The increase in serum half-life and enhancement of anti-tumor effect was substantial when the WT-FL complex is administered subcutaneously, a convenient route for clinical application. The trimeric nature of this complex is the key to its superior biologic properties. This is novel, especially given the previous concern that such constructs could be inhibitory instead of stimulatory on immune cells. These results support further evaluation of this complex for clinical development.

## **P227 Late disappearance of bone marrow minimal residual disease after KIR-ligand-mismatched allogeneic CBT suggests KIR-mismatched NK cell activity against neuroblastoma**

*Nishio, N.*<sup>2</sup>, *Ichikawa, D.*<sup>3</sup>, *Imaya, M.*<sup>4</sup>, *Yoshida, T.*<sup>5</sup>, *Yamamori, A.*<sup>6</sup>, *Wakamatsu, M.*<sup>7</sup>, *Miwata, S.*<sup>8</sup>, *Narita, K.*<sup>9</sup>, *Kitazawa, H.*<sup>10</sup>, *Hamada, M.*<sup>11</sup>, *Kawashima, N.*<sup>12</sup>, *Nishikawa, E.*<sup>13</sup>, *Okuno, Y.*<sup>14</sup>, *Narita, A.*<sup>15</sup>, *Muramatsu, H.*<sup>16</sup>, *Kojima, S.*<sup>17</sup>, *Takahashi, Y.*<sup>18</sup>

Nagoya University Graduate School of Medicine, Department of Pediatrics, Nagoya, Japan <sup>2</sup>, Nagoya University Graduate School of Medicine, Department of Pediatrics, Nagoya, Japan<sup>3</sup>, Nagoya University Graduate School of Medicine, Department of Pediatrics, Nagoya, Japan<sup>4</sup>, Nagoya University Graduate School of Medicine, Department of Pediatrics, Nagoya, Nagoya University Graduate School of Medicine<sup>5</sup>, Nagoya University Graduate School of Medicine, Department of Pediatrics, Nagoya, Japan<sup>6</sup>, Nagoya University Graduate School of Medicine, Department of Pediatrics, Nagoya, Japan<sup>7</sup>, Nagoya University Graduate School of Medicine, Department of Pediatrics, Nagoya, Japan<sup>8</sup>, Nagoya University Graduate School of Medicine, Department of Pediatrics, Nagoya, Japan<sup>9</sup>, Nagoya University Graduate School of Medicine, Department of Pediatrics, Nagoya, Japan<sup>10</sup>, Nagoya University Graduate School of Medicine, Department of Pediatrics, Nagoya, Japan<sup>11</sup>, Nagoya University Graduate School of Medicine, Department of Pediatrics, Nagoya, Japan <sup>12</sup>, Nagoya University Graduate School of Medicine, Department of Pediatrics, Nagoya, Japan <sup>13</sup>, Nagoya University Graduate School of Medicine, Department of Pediatrics, Nagoya, Japan <sup>14</sup>, Nagoya University Graduate School of Medicine, Department of Pediatrics, Nagoya, Japan <sup>15</sup>, Nagoya University Graduate School of Medicine, Department of Pediatrics, Nagoya, Japan <sup>16</sup>, Nagoya University Graduate School of Medicine, Department of Pediatrics, Nagoya, Japan <sup>17</sup>, Nagoya University Graduate School of Medicine, Department of Pediatrics, Nagoya, Japan <sup>18</sup>

### Background/Introduction:

Killer cell immunoglobulin-like receptor (KIR)/HLA genotypes predictive of missing KIR ligands are reported to be associated with a better outcome after autologous hematopoietic stem cell transplantation in patients with high risk neuroblastoma (NB). We previously reported that KIR-ligand-mismatched allogeneic cord blood transplantation (CBT) as a consolidation therapy reduced relapse of bone marrow and improved survival in children with primary high-risk metastatic neuroblastoma (Nishio et al. ANR 2018). We believe that the detection of bone marrow minimal residual disease (BM-MRD) in patients with NB can be used to monitor their response to therapy and predict relapse.

### Aims:

We evaluated BM-MRD to monitor the response to KIR-ligand-mismatched CBT.

### Methods/Materials:

Seven patients with refractory/relapsed metastatic NB whose BM-MRD remained positive before CBT were included in this study. To detect BM-MRD, PHOX2B and tyrosine hydroxylase (TH) levels were measured by real-time quantitative PCR in BM aspirates before and after CBT, as previously reported. The BM samples were collected from both sides of the posterior iliac crest. BM-MRD was determined to be positive when the level of either PHOX2B or TH was detectable.

### Results:

The median age at diagnosis was 4.1 years (range: 2.8-7.7 years). All patients were heavily treated with a median of 16 courses (range: 13-24 courses) of chemotherapy, and 5 patients previously received high-dose chemotherapy with peripheral blood stem cell rescue before CBT. The conditioning regimen for CBT was 180 mg/m<sup>2</sup> of melphalan and 12 Gy of TBI in 4 patients and 125 mg/m<sup>2</sup> of fludarabine, 140 mg/m<sup>2</sup> of melphalan, and 2 Gy of TBI in 3 patients. The degree of HLA compatibility by serological methods was 5/6 for 3 patients and 4/6 for 4 patients. Five patients had C1/C1 of HLA-Cw and received C1/C2 cord blood (KIR-mismatched donor). One patient received HLA-A11+ cord blood, and another patient received HLA-A11+ and Bw4+ cord blood (KIR-

mismatched donor). The median follow-up period from CBT was 2.0 years (range: 1.2-3.7 years). Engraftment was achieved in all patients, and none of the patients developed either grade II-IV acute graft-versus-host disease (GVHD) or chronic GVHD. Surprisingly, BM-MRD became negative after CBT in 5 patients (71%) at 2, 3, 8, 10, and 12 months of CBT. Three of these patients were alive without relapse until the last follow-up at 18, 31, and 50 months. In the remaining 2 patients, BM-MRD did not disappear. One patient developed brain metastasis at 11 months after CBT, whereas another patient was still alive without disease progression, although BM-MRD was positive at the last follow-up 18 months after CBT.

#### Summary/Conclusions:

Late disappearance of BM-MRD and prolonged relapse-free survival after KIR-ligand-mismatched CBT suggests KIR-mismatched NK cell activity against neuroblastoma.

## **P228 Combined targeting of GD2 ganglioside and BET proteins in *MYCN*-amplified neuroblastoma cell lines - studies involving 3D models**

*Horwacik, I.*<sup>2</sup>, *Durbas, M.*<sup>3</sup>, *Rokita, H.*<sup>4</sup>

Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Laboratory of Molecular Genetics and Virology, Kraków, Poland <sup>2</sup>, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Laboratory of Molecular Genetics and Virology, Kraków, Poland<sup>3</sup>, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Laboratory of Molecular Genetics and Virology, Kraków, Poland<sup>4</sup>

### Background/Introduction:

Treatment of high-risk neuroblastoma patients still remains challenging. This stimulates a quest for new combined treatment modalities. GD2 ganglioside (GD2) is one of the pivotal targets for treatment of neuroblastoma patients. Our previous research has shown that some neuroblastoma cell lines treated with the anti-GD2 14G2a monoclonal antibody (mAb) alter their morphology, exhibit decreased cell viability accompanied by changes in transcriptome and in signaling networks (Horwacik and Rokita. *Apoptosis*. 2015; 20(5):679-688). *MYCN*-amplified neuroblastoma cell lines are sensitive to BET (bromodomain and extra-terminal) protein inhibitors (Puissant et al. *Cancer Discov*. 2013; 3:309-323). The agents are currently tested in clinical trials against various cancers.

### Aims:

We aimed to further characterize effects of the 14G2a mAb on selected signaling pathways and to verify outcome of combined targeting of *MYCN*-amplified neuroblastoma cells with the mAb and BET protein inhibitor CPI203.

### Methods/Materials:

In addition to standard cultures (2D), our research involved cultures of *MYCN*-amplified neuroblastoma cells (IMR-32, CHP-134, CHP-126, KELLY) on low binding surfaces (3D models). This can better approximate conditions in tumors, as cells interact both with each other and extra cellular matrix in 3 dimensions, which is important for testing compounds that are potential drugs. It should be stressed that the recently crystallized 14G2a mAb has the same antigen binding region as the ch14.18 mAb (Horwacik et al. *Mol Cell Proteomics*. 2015; 14(10):2577-2590). Effects of CPI203, OTX015, I-BET762 inhibitors (Sigma-Aldrich) were tested using cell viability assays. Molecular techniques (e.g., RT-qPCR, protein arrays from RayBiotech, Inc.) were applied to investigate proteome and transcriptome changes.

### Results:

Using intra-cellular ATP level measurements in our 2D and 3D models, we confirmed, for IMR-32 cells, that the cytotoxic effect of the 14G2a mAb is observed for both types of cultures. Based on microarrays data, we already reported transcriptome changes after the 14G2a mAb treatment of IMR-32 cells (Horwacik et al. *Acta Biochim Pol*. 2015; 62(3):423-433). Here, RT-qPCR was applied to check levels of transcripts of selected genes involved in RNA metabolism in 14G2a-treated IMR-32 cells in 3D cultures. We measured increased levels of *MSX1* and *JUN* transcripts and decreased levels of *ID1* and *TLX2*.

GD2 accumulates in the outer cell membrane and affects the cancer cell phenotype. We applied RTK phosphorylation antibody arrays to evaluate phosphorylation levels of 71 tyrosine kinases in the 14G2a-treated IMR-32 cells in 3D cultures. Interestingly, we showed a decrease in the tyrosine phosphorylation level of an oncogenic non-receptor kinase ACK1 and as a result the protein was selected for further research. Finally, we showed that IMR-32 cells are sensitive to selected inhibitors of BET proteins and further focused on testing efficacy of CPI203 alone or in combination with the 14G2a mAb in 3D models.

### Summary/Conclusions:

Treatment of high-risk neuroblastoma children requires multi-modal approach. We focused our experiments on combined targeting of GD2 and BET proteins in *MYCN*-amplified neuroblastoma cells, which extends knowledge on neuroblastoma biology and cell fate. Our preclinical data, gathered in 3D models, add to efforts to improve survival of the neuroblastoma patients.

## **P229 Analyzing the influence of KDM1A on neuroblastoma-specific CAR T cell therapy**

*Sulejmani, O.<sup>2</sup>, Grunewald, L.<sup>3</sup>, Andersch, L.<sup>4</sup>, Schwiebert, S.<sup>5</sup>, Klaus, A.<sup>6</sup>, Winkler, A.<sup>7</sup>, Eggert, A.<sup>8</sup>, Schulte, J.H.<sup>9</sup>, Henssen, A.<sup>10</sup>, Anders, K.<sup>11</sup>, Künkele, A.<sup>12</sup>*

Charité–Universitätsmedizin, Department of Pediatric Oncology and Hematology, Berlin, Germany <sup>2</sup>, Charité–Universitätsmedizin, Department of Pediatric Oncology and Hematology, Berlin, Germany<sup>3</sup>, Charité–Universitätsmedizin, Department of Pediatric Oncology and Hematology, Berlin, Germany<sup>4</sup>, Charité–Universitätsmedizin, Department of Pediatric Oncology and Hematology, Berlin, Charité–Universitätsmedizin<sup>5</sup>, Charité–Universitätsmedizin, Department of Pediatric Oncology and Hematology, Berlin, Germany<sup>6</sup>, Charité–Universitätsmedizin, Department of Pediatric Oncology and Hematology, Berlin, Germany<sup>7</sup>, Charité–Universitätsmedizin, Department of Pediatric Oncology and Hematology, Berlin, Germany<sup>8</sup>, Charité–Universitätsmedizin, Department of Pediatric Oncology and Hematology, Berlin, Germany<sup>9</sup>, Charité–Universitätsmedizin, Department of Pediatric Oncology and Hematology, Berlin, Germany<sup>10</sup>, German Cancer Consortium (DKTK), 10117, Department of Pediatric Oncology and Hematology, Berlin, Germany<sup>11</sup>, Charité–Universitätsmedizin, Department of Pediatric Oncology and Hematology, Berlin, Germany, <sup>12</sup>

### **Background/Introduction:**

Neuroblastoma is the most common malignant extracranial solid tumor in children, arising from the sympathoadrenal lineage of the neural crest. Treating refractory or relapsed neuroblastoma remains a great challenge in pediatric oncology. An innovative immunotherapeutic approach uses chimeric antigen receptor (CAR) T cells to target antigens specifically and strongly expressed on neuroblastoma cells, such as L1CAM. However, a major challenge is the escape of antigen-negative tumor cells that cause tumor recurrence. Enzymes involved in epigenetic gene regulation, such as the histone demethylating enzyme, lysine-specific demethylase 1 (KDM1A, previously known as LSD1) are known to suppress the expression of different anti-tumor gene networks. The re-expression of these genes via KDM1A inhibition, might boost the efficacy of a CAR T cell therapy. Therefore, combining KDM1A inhibition with CAR T cell therapy may provide a new strategy to better fight this malignancy.

### **Aims:**

In this study, we aimed to identify changes in gene expression in established neuroblastoma cell lines caused by KDM1A suppression that could be exploited to improve CAR T cell therapy against neuroblastoma.

### **Methods/Materials:**

First, we determined the level of KDM1A expression in 8 neuroblastoma cell lines as well as primary T cells by western blot and RT-PCR. Neuroblastoma cell lines with different levels of KDM1A expression were subjected to titrated amounts of KDM1A inhibitors (SP-2509 and SP-2577) and IC50 values were determined by analyzing cell survival. Next, we determined expression changes of cell surface molecules by flow cytometry of neuroblastoma cells treated for 1, 3 or 6 days with their respective IC50 KDM1A inhibitor concentrations.

### **Results:**

We could show that KDM1A was highly expressed in all 8 neuroblastoma cell lines analyzed. In contrast, KDM1A expression was significantly lower expressed in primary T cells, but still detectable. We chose 3 neuroblastoma cell lines for further experiments, which were then treated with two different KDM1A inhibitors (SP-2509, SP-2577). The different neuroblastoma cell lines showed a differential response to the KDM1A inhibitors. While 50% of neuroblastoma cell line IMR5/75 died upon treatment with 3 µM of both inhibitors, cell line SK-N-BE(2) required higher concentration of both inhibitors. Importantly, our CAR T cells are more sensitive to the KDM1A inhibitor treatment compared to the tumor cells, indicating the need for a sequential rather than simultaneous treatment strategy for experiments in which CAR T cells and inhibitors will be combined. When we treated our 3 selected

neuroblastoma cell lines with SP-2509 or SP-2577 at their respective IC50 concentrations, we found an upregulation of FAS (CD95), a death receptor critical for T cell-mediated killing.

Summary/Conclusions:

KDM1A is highly expressed in neuroblastoma cell lines and we could demonstrate that all analyzed cell lines were susceptible to KDM1A inhibition therapy. KDM1A inhibition resulted in upregulation of Fas on the cell surface of neuroblastoma cells and might be exploited to enable antigen-independent lysis of the tumor cells via CAR T cells.



## **P230 G-CSF as a suitable alternative cytokine to boost neutrophil-mediated antibody immunotherapy of neuroblastoma**

Martinez Sanz, P.<sup>2</sup>

Sanquin Research, Blood Cell Research, Amsterdam, Netherlands <sup>2</sup>

### Background/Introduction:

The current immunotherapy for high-risk neuroblastoma patients targets GD2 ganglioside with the therapeutic antibody dinutuximab. Opsonized tumor cells are killed by antibody-dependent cellular cytotoxicity (ADCC), a process mediated by various immune cells, including neutrophils. The killing capacity of neutrophils to kill dinutuximab-opsonized tumor cells is further enhanced by granulocyte-macrophage colony stimulating factor (GM-CSF), which has been shown in the past to improve clinical response to anti-GD2 antibody therapy. However, GM-CSF is not available for clinical use in Europe, creating a high clinical need for an alternative method to stimulate dinutuximab responsiveness. In this study, we propose to use clinically well-established granulocyte-colony stimulating factor (G-CSF) as a potential replacement for unavailable GM-CSF in the dinutuximab immunotherapy regimen of European neuroblastoma patients.

### Aims:

To show that granulocyte-colony stimulating factor (G-CSF) is an efficacious and safe alternative to GM-CSF to boost neutrophil-mediated antibody therapy.

### Methods/Materials:

We tested the capacity of either fresh (unstimulated) or *in vitro* G-CSF or GM-CSF stimulated neutrophils isolated from healthy donors to kill dinutuximab-opsonized GD2 positive neuroblastoma cell lines. Blocking experiments with F(ab')<sub>2</sub> fragments of antibodies inhibiting either respective FcγR or a major neutrophil integrin CD11b/CD18 were used to decipher a mechanism by which the cytokines affect ability of neutrophils to perform ADCC. Live cell imaging microscopy was used to visualize neutrophil mediated tumor killing. To assess the effect of G-CSF on tumor cells, we cultured neuroblastoma cell lines in the presence or absence of the cytokine in the culture medium for various time points. We analyzed tumor cell proliferation rate, susceptibility for neutrophil-mediated ADCC, and markers of epithelial-to-mesenchymal transition (EMT) by qRT-PCR.

### Results:

We observed that G-CSF was as powerful as GM-CSF in enhancing destruction of dinutuximab-opsonized tumor cells in all studied cell lines. This effect was consistent regardless of dinutuximab concentration, amount of cytokine used, or tumor:neutrophil ratio. Blocking experiments of the three main Fc receptors that could be expressed by neutrophils (FcγRI, FcγRIIa, and FcγRIIIb) revealed that it was FcγRIIa that mediated ADCC in neuroblastoma cell lines, and this was consistent for both stimulation conditions. Neutrophils stimulated either with G-CSF or GM-CSF showed an activated CD11b/CD18 integrin that turned out to be indispensable for efficient ADCC. Live cell imaging experiments showed trogocytosis to be the main killing mechanism by which differentially stimulated neutrophils destroyed antibody-opsonized tumor cells. Importantly, although neuroblastoma cells express the receptor for G-CSF, in our hands their proliferation rate, as well as their susceptibility to neutrophil-mediated ADCC was unaffected upon exposure to the cytokine. Also, no signs of EMT were observed at any analyzed time point.

### Summary/Conclusions:

We demonstrated that G-CSF potentiates neutrophils to kill dinutuximab-opsonized neuroblastoma cells equally well as GM-CSF and exposure to the proposed cytokine did not change the phenotype of tumor cells. Altogether, our data supports the use of G-CSF as an alternative to GM-CSF and warrants further testing of G-CSF in a clinical setting for the improvement of immunotherapy of neuroblastoma.



## **P231 The antibody-drug conjugate D3-GPC2-PBD induces immunogenic cell death and triggers antitumor immune responses in preclinical neuroblastoma models**

*Pascual-Pasto, G.<sup>2</sup>, Buongervino, S.<sup>3</sup>, Zhelev, D.<sup>4</sup>, Erbe, A.<sup>5</sup>, Sondel, P.<sup>6</sup>, Dimitrov, D.<sup>7</sup>, Bosse, K.<sup>8</sup>*

The Children's Hospital of Philadelphia, Division of Oncology, Philadelphia, USA <sup>2</sup>, The Children's Hospital of Philadelphia, Division of Oncology, Philadelphia, USA<sup>3</sup>, University of Pittsburgh, Department of Medicine, Pittsburgh, USA<sup>4</sup>, University of Wisconsin, Department of Pediatrics, Madison, University of Wisconsin<sup>5</sup>, University of Wisconsin, Department of Pediatrics, Madison, USA<sup>6</sup>, University of Pittsburgh, Department of Medicine, Pittsburgh, USA<sup>7</sup>, The Children's Hospital of Philadelphia, Division of Oncology, Philadelphia, USA<sup>8</sup>

### Background/Introduction:

GPC2 is a cell surface *MYCN*-driven immunotherapeutic target in high-risk neuroblastoma (NB). We have previously demonstrated potent antitumor effects of a GPC2-directed antibody drug conjugate (ADC) bearing PBD dimers (D3-GPC2-PBD) in neuroblastoma patient-derived xenografts; however, their tumor immunomodulatory properties remain undefined.

### Aims:

We aim to study whether the D3-GPC2-PBD ADC induces immunogenic cell death (ICD) in NB cells and triggers an antitumor immune response in immunocompetent NB murine models.

### Methods/Materials:

Induction of ICD was evaluated by measuring calreticulin (CALR) membrane translocation by flow cytometry and high mobility group box 1 (HMGB1) secretion by ELISA in 2 human NB cell lines (SMS-SAN and NB-EBC1), 2 *Gpc2* isogenic murine NB cell lines (NXS2-*Gpc2* and 9464D-*Gpc2*) and a *Gpc2* isogenic model melanoma cell line (B78-*Gpc2*) treated with different concentrations of D3-GPC2-PBD (1.8-200 pM). Tumor cell phagocytosis was evaluated by flow cytometry after co-culturing human macrophages with GFP-labeled, ADC-treated NB-EBC1 cells with and without dinutuximab (1 µg/mL). *in vivo* tumor growth and antitumor immune responses were studied after vaccination of A/J mice with ADC-pretreated NXS2-*Gpc2* cells followed by tumor re-challenge with naïve NXS2-*Gpc2* cells.

### Results:

D3-GPC2-PBD ADC treatment of murine *Gpc2* isogenic cell lines induced potent cytotoxicity comparable to that observed in human neuroblastoma cell lines ( $n=3$ ; mean  $IC_{50} = 115$  pM). D3-GPC2-PBD treatment induced CALR membrane translocation in a concentration-dependent manner (maximum CRT fold-increase of 2.2-20 at 96 hours after treatment with 200 pM of D3-GPC2-PBD ADC). HMGB1 secretion also increased after D3-GPC2-PBD treatment in a dose-dependent manner, similarly achieving maximum secretion levels of 37-65 ng/mL at 96 h post 200 pM D3-GPC2-PBD ADC treatment. Across the murine NB *Gpc2* isogenic cell lines, NXS2-*Gpc2* cells showed the most robust biomarkers of ICD, which correlated with their prior reported tumor mutational burden (13.9 vs. 1.5 mutations per MB in the 9464D cell line). The percentage of macrophage engulfed NB cells was higher in ADC pretreated NB-EBC1 cells, a phenomenon that was further enhanced upon combination with dinutuximab treatment. Prophylactic subcutaneous vaccination with  $2 \times 10^6$  ADC pretreated NXS2-*Gpc2* cells prevented subcutaneous tumor re-challenge with  $2 \times 10^6$  viable NXS2-*Gpc2* cells in 72% of animals, and significantly increased murine survival compared to control animals vaccinated with freeze-thawed cells ( $p=0.01$ ). The NXS2-*Gpc2* tumors that grew after vaccination showed increased PD1<sup>+</sup> T cell tumor infiltration compared to controls ( $n=3$ ,  $p=0.04$ ), postulating T cell exhaustion as a potential mechanism of tumor recurrence. Systemic isolated CD8<sup>+</sup> T cells from vaccinated animals underwent potent activation upon *ex-vivo* co-culture with native NXS2-*Gpc2* cells. Percentage of CD137<sup>+</sup> and CD69<sup>+</sup> cytotoxic T cells increased 1.3 and 2.4-fold in ADC-vaccinated animals compared to controls, further supporting a vaccination-induced T-cell mediated antitumor

immune response. Ongoing experiments are further exploring whether ADC-mediated vaccination also prevents disseminated tumor re-challenge in a NXS2-Gpc2 metastatic model.

Summary/Conclusions:

The GPC2-directed antibody drug-conjugate D3-GPC2-PBD induces immunogenic cell death, enhances tumor cell phagocytosis and stimulates antitumor immune responses *in vivo* mediated by cytotoxic T cell activation. Ongoing studies are focused on elucidating the synergistic antitumor activity of the D3-GPC2-PBD ADC combined with anti-GD2 antibodies and/or immune checkpoint inhibitors.

## **P232 89Zr-DFO-Dinutuximab-Beta to assess differential expression of surface GD2 in neuroblastoma.**

*Turnock, S.M.*<sup>2</sup>, *Da Pieve, C.*<sup>3</sup>, *Smith, G.*<sup>4</sup>, *Kramer-Marek, G.*<sup>5</sup>

The Institute of Cancer Research, Radiotherapy and Imaging, Sutton, United Kingdom <sup>2</sup>, The Institute of Cancer Research, Radiotherapy and Imaging, Sutton, United Kingdom<sup>3</sup>, The Institute of Cancer Research, Radiotherapy and Imaging, Sutton, United Kingdom<sup>4</sup>, The Institute of Cancer Research, Radiotherapy and Imaging, Sutton, The Institute of Cancer Research<sup>5</sup>

### Background/Introduction:

The GD2 ganglioside is highly expressed on the surface of neuroblastoma (NB) cells with minimal expression in healthy tissues; as a result it is a key target for immunotherapy in high-risk NB patients. Over 95% of primary NBs express GD2, nevertheless target expression level is heterogeneous both inter- and intra-tumourally. In addition, relapse is associated with a positive, yet low GD2 expression in tumour biopsies. Currently, immunotherapy is given regardless of the degree of positivity in tumour GD2 expression and prospective studies are still needed to accurately identify biomarkers of anti-GD2 response. Non-invasive GD2 immuno-PET (positron emission tomography) would enable real time evaluation of GD2 expression for response assessment; and function as a predictive biomarker for accurate patient selection into clinical trials.

### Aims:

In our studies, we investigated whether a zirconium-89 labelled anti-GD2 antibody could successfully delineate tumour volumes in NB xenograft mouse models and differentiate between tumours with low and high expression of GD2.

### Methods/Materials:

A commercial GD2-PE antibody and an in-house prepared Dinutuximab-Beta-IR700 conjugate (GD2-IR700) were used to characterise the GD2 expression by flow cytometry (FC) and confocal microscopy in Kelly, SK-N-BE(2)C and SK-N-AS NB cells. A DFO-Dinutuximab-Beta immunoconjugate was radiolabelled with zirconium-89 (<sup>89</sup>Zr-DFO-GD2) and used to assess the specificity of binding on the panel of cell lines (4 °C, 1 hr). For PET studies, <sup>89</sup>Zr-DFO-GD2 (10 µg, 1.8 MBq) was injected i.v. into mice bearing SK-N-AS (GD2<sup>high</sup>) and SK-N-BE(2)C (GD2<sup>low</sup>) tumours and images were acquired daily up to 96 hr post-injection. Biodistribution studies, autoradiography (AR), FC and immunofluorescence (IF) analyses of the resected tumours were correlated with PET imaging data.

### Results:

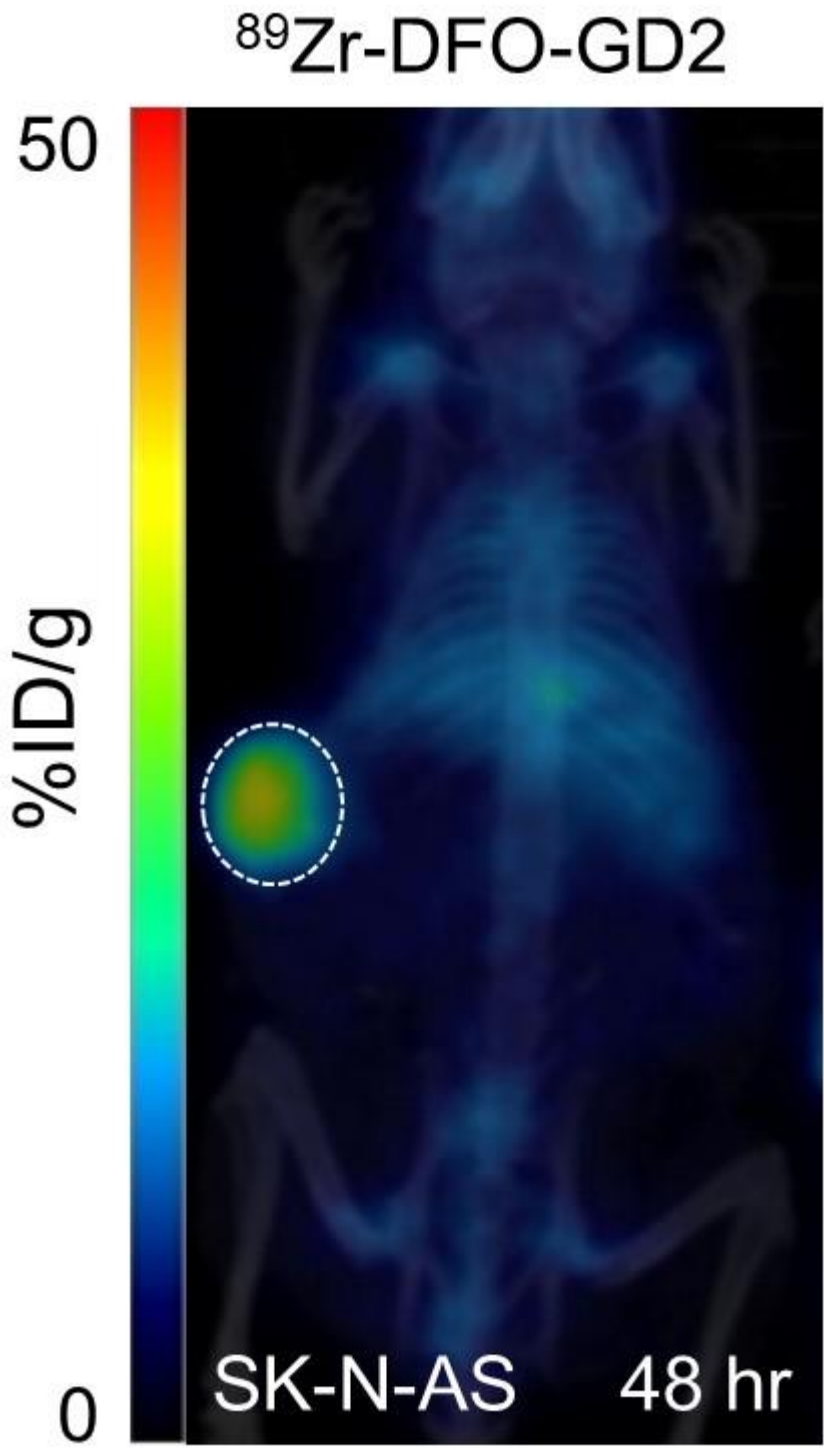
Analysis of GD2 expression by FC and confocal microscopy showed a relatively high target level in SK-N-AS compared to low expression in SK-N-BE(2)C cells. The <sup>89</sup>Zr-DFO-GD2 was prepared and isolated with a 99% radiochemical purity, and a specific activity of 0.19-0.25 MBq/µg. Cell binding of <sup>89</sup>Zr-DFO-GD2 correlated with the GD2 expression level measured via FC, and was successfully blocked with a 50-fold excess of non-labelled GD2 antibody. PET image acquisitions of SK-N-AS and SK-N-BE(2)C xenografts (24-96 hr post-injection) showed specific and receptor dependent tumour uptake of <sup>89</sup>Zr-DFO-GD2. This data was corroborated by *ex vivo* AR and IF of tumour sections. GD2-IR700 FC on fresh tumour samples correlated with <sup>89</sup>Zr-DFO-GD2 binding characteristics. The distribution of GD2 in Kelly cells was distinctly heterogeneous. Therefore our successful isolation of the more homogenous

Kelly-GD2<sup>high</sup> cell population can be used for future investigations.

### Summary/Conclusions:

GD2-IR700 antibody can discriminate expression levels of GD2 *in vitro*, and further supports <sup>89</sup>Zr-DFO-GD2 PET to differentiate between low and high GD2 expression in tumour models of NB, as

early as 48 hours post-injection. Ongoing work in mouse models and patient samples will provide insight in to the potential importance of  $^{89}\text{Zr}$ -DFO-GD2 PET imaging to guide GD2 immunotherapy.



Caption 1: 48 hr scan of mice bearing SK-N-AS GD2 positive tumour



## **P233 Low dinutuximab binding to neuroblastoma patient-derived xenografts is associated with resistance to chemoimmunotherapy and is found in patient marrow samples using multi-color flow cytometry**

*Keyel, M.<sup>2</sup>, Davidson, H.<sup>3</sup>, Reynolds, C.<sup>4</sup>*

Texas Tech University Health Sciences Center, Pediatrics, Lubbock, USA <sup>2</sup>, Texas Tech University Health Sciences Center, Pediatrics, Lubbock, USA<sup>3</sup>, Texas Tech University Health Sciences Center, Pediatrics, Lubbock, USA<sup>4</sup>

### Background/Introduction:

Maintenance therapy with a chimeric anti-GD2 antibody, ch14.18 (dinutuximab) combined with cytokines and isotretinoin improves outcome for high-risk neuroblastoma. A phase II clinical trial combining dinutuximab with temozolomide + irinotecan (TMZ + IRN) showed promising activity in recurrent neuroblastoma (*Lancet Oncol*, 18(7): 946-957, 2017), but a report that ~ 12% of neuroblastoma patients have low or negative GD2 expression (*Pediatr Blood Cancer* 64(1): 46-56, 2017) indicates the need to assess GD2 expression on neuroblastomas and to define expression levels necessary for activity.

### Aims:

### Methods/Materials:

Flow cytometry and directly-labeled dinutuximab quantified % positive and median fluorescence index (MFI = binding intensity) for 26 patient-derived neuroblastoma cell lines and 13 patient-derived xenografts (PDXs) established at diagnosis (n=10) or at progressive disease (PD) (n=29). Gating on neuroblastoma cells with directly-labeled non-GD2 neuroblastoma antibodies (HSAN and 459) that are negative for hematopoietic cells and gating out CD45+ cells we quantified dinutuximab binding in 59 bone marrow samples from neuroblastoma patients (41 pretherapy, 18 at PD). Event-free survival was assessed in nu/nu mice treated with temozolomide (TMZ) + irinotecan (IRN) +/- dinutuximab for neuroblastoma PDXs established from PD patients.

### Results:

We observed three distinct groups of cell lines based on dinutuximab binding. Cell lines with >80% of neuroblastoma cells positive for dinutuximab binding also had significantly (p=0.04) higher density of binding (MFI). Intermediate GD2 (50-80% positive) and low GD2 (< 50% positive and MFI < 1000) was more frequent in cell lines established at PD (56% had intermediate or low GD2) compared to those established at diagnosis (14% had intermediate or low GD2). In a GD2-positive PDX (> 80% GD2-positive cells), dinutuximab + TMZ + IRN significantly (p = 0.001) enhanced mouse event-free survival (EFS) compared to TMZ + IRN. Compared to TMZ+ IRN alone, PDXs with 25-60% GD2-positive cells treated with dinutuximab + TMZ + IRN showed a significant but minimal increase in EFS, while a PDX with < 25% GD2-positive cells + MFI < 1000 showed no increase in EFS with TMZ + IRN + dinutuximab. Neuroblastoma cells were identified by HSAN and 459 staining in 50 of 59 patient marrow samples. Of those, 6/16 (38%) PD samples and 11/34 (32%) pre-therapy samples had neuroblastoma cells with dinutuximab MFI < 2500 and < 10% dinutuximab-positive binding.

### Summary/Conclusions:

Low GD2 expression can occur in neuroblastoma and may be more frequent in PD patients. Neuroblastoma PDXs in nu/nu mice provide a preclinical model to assess dinutuximab activity when combined with chemotherapy. In 34% of patient marrow samples analyzed, dinutuximab binding was comparable to levels seen in PDX's that did not benefit from dinutuximab. Quantifying GD2 expression is a potential biomarker of activity for dinutuximab that warrants evaluation in patients treated with dinutuximab combined with temozolomide + irinotecan. Funded in part by United Therapeutics Corporation.





## **P234 Identification of tumor infiltrating T cells in neuroblastoma by single cell sequencing**

Dong, R.<sup>2</sup>

Children's Hospital of Fudan University, Pediatric surgery, Shanghai, China <sup>2</sup>

### Background/Introduction:

Neuroblastoma (NB) is the most common extracranial solid tumor in children, with highly variable clinical features and outcomes. Anti-GD2 immunotherapy has been proven effective in high-risk neuroblastoma, which highlights the significance of immunotherapy in the treatment of NB. Systematic identification of tumor-infiltrating lymphocytes especially T cells in NB is the footstone of the development and application of immunotherapy.

### Aims:

To identify tumor infiltrating T cells in neuroblastoma at single cell level, and to promote the understanding of the functional state and dynamics of T cells in NB, which might pave the way for new immunotherapy for NB.

### Methods/Materials:

We performed single cell sequencing on 11 NB patients and isolated all together 12,909 T and NK cells, which went through qualification control and unsupervised clustering. The inferred developmental trajectory was performed by Monocle2. Expression scores were calculated by mean expression of specific markers of different cell types. RNA-seq mRNA expression data and clinical parameters were retrieved from cBioportal. CIBERSORT was used to infer T cell compositions in RNA-seq data from TCGA. To correct the effect of T cell levels within each sample, the expression of selected genes in tumor were divided by that of mean of CD3 genes (*CD3D*, *CD3E* and *CD3G*).

### Results:

Ten different large subgroups were identified by distinct expression patterns between clusters, with three subsets CD4+ T cells, six subsets of CD8+ T cells and one subset of NK cells. In general, much more naïve T cells than effector T cells were observed. In terms of effector T cells, a group of CD4+ NKT cells with high expression of *KLRB1* and *GZMK* was identified, while there are three groups of CD8+ cytotoxic T cells, with CD8-APOD group more naïve than CD8-NKG7 cells. We also found that a group of CD4+ exhausted cells, highly expressed *TIGIT*. There was almost no expression of *PD1* and *CTLA4*, which may be why anti-PD1/PDL1 or anti-CTLA4 therapy received no satisfactory results in NB. Cell trajectory was delineated, and moreover with the surge of cytotoxic score in pseudotime, there was a rather slower increase of exhausted score. Several genes such as *APOD*, *CCL4*, *CST7* might be positively correlated with the expression of inhibitory markers. We used CIBERSORT to infer the relative ratio of T cells in Rna-seq, finding that compared with poorly differentiated NB, there was more NKT cells and CD8+ cytotoxic T cells in ganglioblastoma. Furthermore, patients with higher expression of NKT signature gene-*KLRB1* in tumor had a significantly higher survival.

### Summary/Conclusions:

Single-cell RNA-seq reveals distinctive functional composition of T cells in NB. There was almost no expression of *PD1* and *CTLA4*. The majority of T cells are in the un-activated naïve state. Therefore, promoting these cells to more cytotoxic phenotype might be a potentially effective strategy for cancer immunotherapy. A group of CD4+ NKT cells was identified and its signature gene-*KLRB1* was correlated with lower risk stratification and better prognosis.

## **P235 Impact of anti-GD2 antibody at induction on ability to achieve gross total resection of high-risk neuroblastoma**

*Mansfield, S.<sup>2</sup>, McCarville, M.B.<sup>3</sup>, Lucas, J.<sup>4</sup>, Krasin, M.<sup>5</sup>, Federico, S.<sup>6</sup>, Furman, W.<sup>7</sup>, Davidoff, A.<sup>8</sup>*

St. Jude Children's Research Hospital, Department of Surgery, Memphis, USA <sup>2</sup>, St. Jude Children's Research Hospital, Radiology, Memphis, USA<sup>3</sup>, St. Jude Children's Research Hospital, Radiation Oncology, Memphis, USA<sup>4</sup>, St. Jude Children's Research Hospital, Radiation Oncology, Memphis, St. Jude Children's Research Hospital<sup>5</sup>, St. Jude Children's Research Hospital, Oncology, Memphis, USA<sup>6</sup>, St. Jude Children's Research Hospital, Oncology, Memphis, USA<sup>7</sup>, St. Jude Children's Research Hospital, Surgery, Memphis, USA<sup>8</sup>

### Background/Introduction:

High-risk neuroblastoma requires multi-modality therapy including intensive chemotherapy, surgery, high-dose chemotherapy with stem cell rescue, radiation and biologic agents. The addition of anti-disialoganglioside mAb (anti-GD2 mAb) immunotherapy has improved response rates. In previous trials, anti-GD2 antibody therapy was used at the end of therapy to treat minimal residual disease. Two current trials administer the anti-GD2 antibody upfront, during induction, with encouraging results.

### Aims:

The aim of this study was to evaluate the impact of anti-GD2 antibody given during induction on the completeness of surgical resection. Our hypothesis was that an increased percentage of patients would have a gross total resection (>90%) with this induction regimen compared to historical controls. Our control cohort consisted of patients with high risk neuroblastoma treated on regimens that did not include anti-GD-2 antibody administered during induction, operated on by the same surgeon at our institution.

### Methods/Materials:

Following IRB approval, we retrospectively reviewed charts of patients treated on our, institutional phase II trial (NB2012) using a humanized anti-GD2 mAb (hu14.18K322A) in combination with granulocyte-macrophage colony-stimulating factor (GM-CSF) and low-dose IL2 during induction together with induction chemotherapy agents cyclophosphamide, topotecan, cisplatin, etoposide, doxorubicin, and vincristine. We compared these patients to those treated on our previous institutional high-risk neuroblastoma protocols where induction therapy did not include anti-GD-2 mAb immunotherapy. The number of image-defined risk factors (IDRF) at diagnosis, the number of pre-operative cycles of chemotherapy, and the time to resumption of chemotherapy post-operatively were determined. Tumor volume on immediate pre-operative computed tomography (CT) and end of induction CT (after surgical resection of primary tumor) was measured by a single pediatric radiologist and the percent decrease of tumor volume was calculated. Patients who did not have surgery or who had upfront surgery prior to chemotherapy were excluded.

### Results:

There were 36 patients eligible to be reviewed on historic trials from 1999-2009 and 47 on NB2012, after excluding 21 and 18 patients, respectively. The number of patients with 3 or more IDRFs at diagnosis was significantly higher in NB2012 patients (84.8%) compared to those on prior trials (56.7%,  $p=0.009$ ). In the older trials, the mean percent reduction of tumor volume after surgery compared to pre-operative imaging was 88.0% ( $\pm 1.2\%$ ) with 66.7% achieving a >90% resection. For patients on NB2012, the mean percent reduction was 90.7% ( $\pm 17.6\%$ ) with 70.2% achieving a >90% resection. The percent reduction ( $p=0.52$ ) and ability to achieve >90% resection ( $p=0.813$ ) were not significantly different. There was no difference between the two groups in the number of cycles of chemotherapy administered pre-operatively (mean  $3.7 \pm 1.6$  historic vs  $3.2 \pm 1.2$  NB2012,  $p=0.10$ ). Patients in both groups resumed chemotherapy within similar timeframes post-operatively (12.3 days historic versus 11.9 days on NB2012,  $p=0.742$ ).

Summary/Conclusions:

Despite a patient population/referral pattern with significantly more IDRFs at diagnosis, the use of anti-GD2 antibody during induction allowed for similar gross total resection rates without requiring an increased number of pre-operative treatment cycles or resulting in delays in resumption of chemotherapy post-operatively.

## **P236 Survival Impact of Anti-GD2 Antibody Response – A Phase II Ganglioside Vaccine Trial in Relapsed Neuroblastoma**

*Cheung, L.<sup>2</sup>, Cheung, N.K.<sup>3</sup>, Modak, S.<sup>4</sup>, Mauguen, A.<sup>5</sup>, Basu, E.<sup>6</sup>, Roberts, S.<sup>7</sup>, Ragupathi, G.<sup>8</sup>, Kushner, B.<sup>9</sup>*

Memorial Sloan Kettering Cancer Center, Pediatrics, New York, USA <sup>2</sup>, Memorial Sloan Kettering Cancer Center, Pediatrics, New York, USA<sup>3</sup>, Memorial Sloan Kettering Cancer Center, Pediatrics, New York, USA<sup>4</sup>, Memorial Sloan Kettering Cancer Center, Epidemiology-Biostatistics, New York, Memorial Sloan Kettering Cancer Center<sup>5</sup>, Memorial Sloan Kettering Cancer Center, Pediatrics, New York, USA<sup>6</sup>, Memorial Sloan Kettering Cancer Center, Pediatrics, New York, USA<sup>7</sup>, Memorial Sloan Kettering Cancer Center, Medicine, New York, USA<sup>8</sup>, Memorial Sloan Kettering Cancer Center, Pediatrics, New York, USA<sup>9</sup>, ,

### **Background/Introduction:**

High-risk neuroblastoma (HR-NB) relapse carries a dismal prognosis. Recent experience, however, suggests that cure is possible when remission is achieved with salvage therapy that includes anti-GD2 monoclonal antibody (mAb) (OncoImmunol 2015). The favorable survival outcome and safety profile after vaccination with gangliosides GD2 and GD3 in a Phase I trial (n=15, Clinical Cancer Res 2014), and the importance of seroconversion (mounting anti-vaccine antibody response) need to be confirmed.

### **Aims:**

To analyze the kinetics of seroconversion and its prognostic impact on survival in a Phase II trial (Clinicaltrials.gov NCT00911560) amongst HR-NB patients with prior disease progressions.

### **Methods/Materials:**

A cohort of 102 HR-NB patients who achieved complete remission after salvage therapy were enrolled in this trial. They received 7 subcutaneous vaccine injections (week 1-2-3-8-20-32-52) plus oral beta-glucan (starting week 6 at 40 mg/kg/day, 14 days on/14 days off). Each vaccine injection consisted of 30 µg each of GD2 and GD3, lactonized and conjugated to the immunogenic carrier protein keyhole limpet hemocyanin and mixed with the saponin OPT-821 adjuvant (150 µg/m<sup>2</sup>). Serum anti-vaccine antibody titers were quantified by ELISA. Kaplan-Meier analyses and multivariable prognostic models were used for survival estimates.

### **Results:**

Among 102 patients, 63% had one, 21% had 2, and 16% had 3-6 prior disease progressions. 83/100 patients had failed prior anti-GD2 mAb (m3F8, dinutuximab, or naxitamab) therapy before vaccine: one mAb (n=62), two mAb (n=15), or all three (n=6). Common toxicities were self-limited injection-related local reaction and fever. No pain, neuropathy, or grade 3/4 toxicities occurred during or post treatment. Progression-free survival (PFS) was 44%±5% and overall survival (OS) was 88%±4% at 2 years, and 36%±7% and 70%±8% at 5 years, respectively. Serum anti-GD2 (IgG1 and IgM) and anti-GD3 (IgG1) titers had marked increases following the initiation of betaglugan at week 6. In univariate analyses, favorable prognostic factors included: one versus ≥2 prior disease progressions (PFS p=0.005, OS p=0.04); none versus any prior anti-GD2 mAb failures (PFS p=0.003, OS p=0.01); and the induction of ≥150 ng/ml anti-GD2-IgG1 titers by week 8 (PFS p=0.02, OS p=0.06). Factors not prognostic included: time to first NB progression, MYCN amplification status, anti-GD2-IgM, anti-GD3-IgG1 or anti-KLH-IgG1 titers, and treatment with anti-GD2 mAb right before vaccine. In multivariate analyses, week 8 anti-GD2-IgG1 titer ≥150 ng/ml yielded a hazard ratio (HR) of 0.41 [0.20, 0.83], p=0.01 for PFS, and HR=0.15 [0.02, 1.12], p=0.06 for OS. The second independent prognostic variable was the number of prior disease progressions (≥2 vs 1), yielding HR of 2.12 [1.26, 3.58], p=0.005 for PFS, and HR =2.77 (1.03, 7.47), p=0.04 for OS.

### **Summary/Conclusions:**

The absence of toxicities despite seroconversion contrasted sharply with the side effects of conventional anti-GD2 mAb therapy. Even with prior disease progressions and prior failures from anti-GD2 mAb therapy, anti-GD2 (though not anti-GD3) seroconversion was associated with notable long-term survival among HR-NB patients previously thought to be unsalvageable. A randomized trial to assess the role of beta-glucan in seroconversion is actively accruing patients.

## **P237 Adjusted administration of a T-cell utilizing bispecific anti CD3/GD2 construct and CH14.18mAb in relation to immune recovery after alloSCT in patients with relapsed neuroblastoma**

*Lang, A.<sup>2</sup>, Jung, G.<sup>3</sup>, Heubach, F.<sup>4</sup>, Rabsteyn, A.<sup>5</sup>, Schlegel, P.<sup>6</sup>, Seitz, C.<sup>7</sup>, Handgretinger, R.<sup>8</sup>, Lang, P.<sup>9</sup>*

University Children's Hospital Tuebingen, Hematology and oncology, Tuebingen, Germany <sup>2</sup>, University of Tuebingen Interfaculty Institute for Cell Biology, Department of Immunology, Tuebingen, Germany<sup>3</sup>, University Children's Hospital Tuebingen, Hematology and oncology, Tuebingen, Germany<sup>4</sup>, University Children's Hospital Tuebingen, Hematology and oncology, Tuebingen, University Children's Hospital Tuebingen<sup>5</sup>, University Children's Hospital Tuebingen, Hematology and oncology, Tuebingen, Germany<sup>6</sup>, University Children's Hospital Tuebingen, Hematology and oncology, Tuebingen, Germany<sup>7</sup>, University Children's Hospital Tuebingen, Hematology and oncology, Tuebingen, Germany<sup>8</sup>, University Children's Hospital Tuebingen, Hematology and oncology, Tuebingen, Germany<sup>9</sup>

### **Background/Introduction:**

Whereas immunotherapy in high-risk/relapsed neuroblastoma with standard antibody formats targeting the tumor associated antigen GD2 is currently used after autologous/allogeneic stem cell transplantation (SCT), T-cells have rarely been addressed as effectors. Bispecific antibodies can bring T-cells into close proximity to tumor cells and activate them through the CD3 region, which ultimately leads to target cell elimination. They lack the Fc-domain and cannot trigger ADCC or CDC as NK-cells. An example for bispecific antibodies is NG-CU (developed at the Department of Immunology, University of Tuebingen), which recognizes CD3 and GD2 as target antigens. The tumor antigen specific antibody domain, derived from a fab fragment of GD2 14.18 antibody and the T-cell recruiting antibody domain, derived from single chain variable fragment (scFv fragment) of UCHT-1 (CD3) are linked by modified CH2 domain.

### **Aims:**

We investigated whether the bispecific antibody NG-CU might be an alternative to the therapeutic monoclonal antibody CH14.18, which mediates CDC and ADCC through NK-cells.

### **Methods/Materials:**

Different antibody concentrations and effector to target ratios (E:T) were evaluated using the xCELLigence RTCA system, PBMCs (healthy donors and patients after allogeneic/autologous SCT) and the neuroblastoma cell lines LS and LAN-1. CDC was evaluated using autologous serum.

### **Results:**

NG-CU showed enhanced cytotoxicity compared to CH14.18. Median specific lysis (n=3) after 12/24/48 hrs (effectors: healthy PBMCs, E:T=5:1) with LS cells was: 55/73/77% (CH14.18, 1 µg/ml) vs. 70/100/100% (NG-CU, 100ng/ml). P values with Mann-Whitney test: p=0,0244; p<0,0001; p<0,0001. With LAN-1 cells: 56/69/88% (CH14.18, 1 µg/ml) vs. 9/46/99% (NG-CU, 100 ng/ml). P values with Mann-Whitney test: p<0,0001; p=0,1139; p=0,0464.

Taking CDC into account by adding autologous serum, cytotoxicity of CH14.18 was drastically increased. ADCC and CDC eradicated targets with 64% after 4 hrs and 93% after 48 hrs (E:T=5:1). However, using a lower E:T ratio (1:1) lysis of CH14.18 remained static, but not for NG-CU. Median lysis (n=3) after 12/24/48 hrs: 71/74/77% (CH14.18) vs. 25/42/67% (NG-CU). After >48 hrs targets were completely eradicated by NG-CU, while targets treated with CH14.18 still proliferated.

Using patient PBMCs, significant lysis with both constructs was detected dependent on percentages and total numbers of T- and NK-cells.

In the first wave of immune regeneration after SCT, NK-cells represented the majority of effectors. Here, CH14.18 produced higher lysis, whereas later on, associated with increasing T-cell counts, NG-CU was more effective. With both antibody constructs complete eradication of neuroblastoma cells (LS+LAN-1) was detectable.

Summary/Conclusions:

Using healthy PBMCs, NG-CU showed enhanced cytotoxicity compared to CH14.18 even in lower concentrations and E:T ratios. Dependent on the differential recovery of effector cells posttransplant either NK-cell based or T-cell based antibody constructs might result in optimal anti-tumor activity. An adjusted administration of both antibody constructs in relation to the state of immune recovery after SCT could be an alternative to classical neuroblastoma approaches.



## **P238 High-level *MYCN* expression in neuroblastoma impairs L1CAM-directed CAR T cell therapy *in vitro***

Grunewald, L.<sup>2</sup>, Schwiebert, S.<sup>3</sup>, Klaus, A.<sup>4</sup>, Hertwig, F.<sup>5</sup>, Jensen, M.C.<sup>6</sup>, Henssen, A.G.<sup>7</sup>, Eggert, A.<sup>8</sup>, Anders, A.<sup>9</sup>, Schulte, J.H.<sup>10</sup>, Künkele, A.<sup>11</sup>

Charité Universitätsmedizin Berlin, Pediatric Oncology and Hematology, Berlin, Germany<sup>2</sup>, Charité Universitätsmedizin Berlin, Pediatric Oncology and Hematology, Berlin, Germany<sup>3</sup>, Charité Universitätsmedizin Berlin, Pediatric Oncology and Hematology, Berlin, Germany<sup>4</sup>, Deutsches Konsortium für Translationale Krebsforschung, Pediatric Oncology and Hematology, Berlin, Deutsches Konsortium für Translationale Krebsforschung<sup>5</sup>, Ben Towne Center for Childhood Cancer Research, Pediatric Oncology and Hematology, Seattle, USA<sup>6</sup>, Charité Universitätsmedizin Berlin, Pediatric Oncology and Hematology, Berlin, Germany<sup>7</sup>, Charité Universitätsmedizin Berlin, Pediatric Oncology and Hematology, Berlin, Germany<sup>8</sup>, Deutsches Konsortium für Translationale Krebsforschung, Pediatric Oncology and Hematology, Berlin, Germany<sup>9</sup>, Charité Universitätsmedizin Berlin, Pediatric Oncology and Hematology, Berlin, Germany<sup>10</sup>, Charité Universitätsmedizin Berlin, Pediatric Oncology and Hematology, Berlin, Germany<sup>11</sup>

### Background/Introduction:

Current treatment protocols have only limited success in patients with neuroblastomas harboring amplifications of the central oncogenic driver, *MYCN*. CAR T cell therapies have recently entered neuroblastoma clinical trials after their success against hematological malignancies. Initial responses have however been disappointing.

### Aims:

We investigated the influence of *MYCN* amplification on tumor cell response to neuroblastoma-specific CAR T cell therapy with the aim to illuminate mechanisms inhibiting CAR T cell success against neuroblastoma.

### Methods/Materials:

As a starting point, we used the SK-N-AS neuroblastoma cell line with the normal diploid *MYCN* complement (*MYCN*<sup>non-amp</sup>) and overexpressed *MYCN* (*MYCN*<sup>amp</sup>) in these cells using a tetracycline-inducible system. Neuroblastoma cells with the different *MYCN* backgrounds were co-cultured with second-generation L1CAM-targeting CAR T cells harboring either the CD28 or 4-1BB costimulatory domain. L1CAM-CAR T cell function was assessed in assays for activation/inhibitory receptor expression, cytokine release and tumor cell cytotoxicity. Combination of CAR T cells with the indirect *MYCN* inhibitor, MLN8237, was assessed via combinatorial cytotoxic effects and synergism was calculated using R.

### Results:

Co-culture with *MYCN*<sup>amp</sup> SK-N-AS cells reduced activation of L1CAM-CAR T cells (decreased CD25 and CD137 expression) compared to parental *MYCN*<sup>non-amp</sup> cell co-culture. Inhibitory Tim3 receptor expression was increased by almost 2-fold on L1CAM-CAR T cells and they released up to 2-fold less IFNG and IL2 in co-culture with *MYCN*<sup>amp</sup> SK-N-AS cells compared with parental *MYCN*<sup>non-amp</sup> cells. The L1CAM-CAR T cells co-cultured with *MYCN*<sup>amp</sup> SK-N-AS cells also demonstrated less killing potential against the *MYCN*<sup>amp</sup> SK-N-AS cells in comparison to the parental cell line. All effects were observed for L1CAM-CAR T cells, regardless of which costimulatory domain was used in the CAR construct, but CD28 costimulation did produce higher efficacy in all functionality assays. Interestingly, co-culture with L1CAM-CAR T cells downregulated the L1CAM target antigen on the *MYCN*<sup>amp</sup> tumor cells. First hints from patient data emphasizes that lower L1CAM expression is associated with *MYCN* amplified high-risk tumors. Indeed, stable L1CAM expression on tumor cells rescued the negative effects exerted on CAR T cells. A combinational therapy approach using an aurora kinase inhibitor, indirectly inhibiting *MYCN* function, and L1CAM-specific CAR T cells restored L1CAM expression on *MYCN*<sup>amp</sup> neuroblastoma cells in our *in vitro* model. Antigen loss on neuroblastoma cells was not

recovered after combination treatment, but L1CAM-CAR T cell-directed *MYCN*<sup>amp</sup> SK-N-AS cell killing was increased by up to 40%. Nevertheless, a synergistic effect was detected exclusively for *MYCN* overexpressing cells when the indirect *MYCN* inhibitor and L1CAM-targeted CAR T cell therapy were combined.

#### Summary/Conclusions:

*MYCN*-driven L1CAM downregulation on neuroblastoma cells may be one cause for impaired efficacy of L1CAM-specific CAR T cell therapy in patients with neuroblastoma. Whether target antigen downregulation on the tumor cells is the only escape mechanism exerted within *MYCN*-amplified tumors remains elusive. We provide preclinical evidence that combining L1CAM-specific CAR T cells with an aurora a kinase inhibitor might be an option to increase CAR T cell efficacy for patients with *MYCN*-amplified neuroblastomas.

## **P239 Analyzing the impact and mechanism of CAR T cell impairment by soluble neuroblastoma antigen**

Andersch, L.<sup>2</sup>, Schwiebert, S.<sup>3</sup>, Winkler, A.<sup>4</sup>, Klaus, A.<sup>5</sup>, Grunewald, L.<sup>6</sup>, Zirngibl, F.<sup>7</sup>, Jensen, M.C.<sup>8</sup>, Eggert, A.<sup>9</sup>, Künkele, A.<sup>10</sup>, Anders, K.<sup>11</sup>

Charité - Universitätsmedizin, Department of Pediatric Oncology and Hematology, Berlin, Germany<sup>2</sup>, Charité – Universitätsmedizin, Department of Pediatric Oncology and Hematology, Berlin, Germany<sup>3</sup>, Charité – Universitätsmedizin, Department of Pediatric Oncology and Hematology, Berlin, Germany<sup>4</sup>, Charité – Universitätsmedizin, Department of Pediatric Oncology and Hematology, Berlin, Charité – Universitätsmedizin<sup>5</sup>, Charité – Universitätsmedizin, Department of Pediatric Oncology and Hematology, Berlin, Germany<sup>6</sup>, Charité – Universitätsmedizin, Department of Pediatric Oncology and Hematology, Berlin, Germany<sup>7</sup>, Seattle Children's Hospital, Ben Towne Center for Childhood Cancer Research, Seattle, USA<sup>8</sup>, Charité – Universitätsmedizin, Department of Pediatric Oncology and Hematology, Berlin, Germany<sup>9</sup>, Charité – Universitätsmedizin, Department of Pediatric Oncology and Hematology, Berlin, Germany<sup>10</sup>, German Cancer Consortium (DKTK), Department of Pediatric Oncology and Hematology, Berlin, Germany<sup>11</sup>

### Background/Introduction:

Despite high remission rates for hematological malignancies following CAR T cell therapy, targeting solid tumors remains challenging. The hostile and immunosuppressive tumor microenvironment contributes to reduced CAR T efficacy against solid tumors, but the nature of the target antigen still plays a crucial role. L1CAM (formerly CD171), an adhesion molecule playing a fundamental role during brain development, is overexpressed by different tumor entities including neuroblastoma. The cancer-specific glycosylation of the CE7 epitope makes it a suitable target antigen, and CAR T cells targeting it are currently in a phase I clinical trial for high-risk neuroblastoma at the Seattle Children's Hospital (USA). Constitutive ADAM metalloprotease-directed cleavage of the L1CAM extracellular domain releases the L1CAM ectodomain containing the CE7 epitope into blood and ascites, which we propose could impair CAR T cell performance either by neutralizing CAR T cells before they reach the tumor site or exhausting CAR T cells more quickly via direct or excessive CE7 CAR domain binding.

### Aims:

The extent and underlying mechanism of how soluble L1CAM impacts L1CAM-directed CAR T cell therapy will be analyzed to improve this therapeutic strategy for patients with neuroblastoma.

### Methods/Materials:

The extent of constitutive L1CAM shedding during *in vitro* culture of SK-N-BE(2) (L1CAM<sup>high</sup>) and SK-N-AS (L1CAM<sup>low</sup>) neuroblastoma cell lines was assessed using western blotting. L1CAM- or CD19-directed CAR T cells were cultured with increasing amounts of soluble recombinant L1CAM or followed by co-culture with CD19-expressing SK-N-BE(2) cells. Expression of the CD137 activation marker and effector cytokine production were subsequently flow cytometrically analyzed.

### Results:

Soluble L1CAM is constitutively shed *in vitro* and shedding remains stable over time, leading to soluble L1CAM accumulation. Exposing L1CAM-targeting CAR T cells to soluble recombinant L1CAM concentrations as low as 333 ng/ml upregulated CD137 on the T cell surface, indicating that soluble L1CAM caused L1CAM-targeting CAR T cell activation. T cell activation was higher with increasing concentrations of L1CAM. Interestingly, L1CAM-targeting CAR T cells exposed to high levels of soluble L1CAM then challenged with SK-N-BE(2) cells in the presence of soluble L1CAM were incapable of producing IFNG, suggesting that if levels of soluble L1CAM reach a critical amount, it has a neutralizing effect on L1CAM-targeting CAR T cells. Soluble L1CAM had no effect on CD19-directed CAR T cells at any concentration used, indicating a CAR-specific effect.

### Summary/Conclusions:

Here we show that L1CAM is constitutively shed by neuroblastoma cells, and that soluble L1CAM specifically impairs L1CAM-directed CAR T cells by neutralization and early activation distant from the tumor site. Knowing how soluble L1CAM affects L1CAM-directed CAR T cell therapy will enable us to implement strategies to avoid CAR T cell impairment and improve CAR T cell therapy for patients with neuroblastoma.

## **P240 The immunomodulatory role of apoptotic neuroblastoma primed mesenchymal stromal cells on tumor microenvironment**

*Chan, G.C.F.<sup>2</sup>, Li, A.K.Y.<sup>3</sup>*

The University of Hong Kong, Paediatrics, Hong Kong, China <sup>2</sup>, The University of Hong Kong, Paediatrics, Hong Kong, China<sup>3</sup>

### Background/Introduction:

One of the contributory factors for treatment refractoriness of neuroblastoma is the unfavorable tumor microenvironment (TME) for immune cells. Within the TME, mesenchymal stromal cells (MSCs) can migrate through blood vessels and exerts immunosuppression and become major hindrance to immunotherapy. However, studies showed MSCs can be manipulated by the environment and change their phenotype.

### Aims:

We would like to study the influence of apoptotic cells (AC) on MSCs' function.

### Methods/Materials:

The AC primed MSCs could induce T cells proliferation and activation. Moreover T cells were induced to differentiate to pro-inflammatory phenotype. It is associated with reduction in regulatory T cells (Treg) differentiation. In contrast to conventional thinking, T cells co-cultured with AC-primed MSC have a greater cytotoxic function. As immunogenic molecules were not found in the supernatant of the dying neuroblastoma cells, we explored the role of extracellular vesicles (EVs). Interestingly apoptotic neuroblastoma derived EVs contained HMGB1 which is associated with immuno-stimulatory function. There was a up-regulation of the HMGB1 receptor, RAGE and TLR2 on the hTMSC. This eventually led to an activation of the NF- $\kappa$ B signalling pathway and increased the secretion of pro-inflammatory cytokines IL-6, IL-8 and IL-33.

### Results:

The AC primed MSCs could induce T cells proliferation and activation. Moreover T cells were induced to differentiate to pro-inflammatory phenotype. It is associated with reduction in regulatory T cells (Treg) differentiation. In contrast to conventional thinking, T cells co-cultured with AC-primed MSC have a greater cytotoxic function. As immunogenic molecules were not found in the supernatant of the dying neuroblastoma cells, we explored the role of extracellular vesicles (EVs). Interestingly apoptotic neuroblastoma derived EVs contained HMGB1 which is associated with immuno-stimulatory function. There was a up-regulation of the HMGB1 receptor, RAGE and TLR2 on the hTMSC. This eventually led to an activation of the NF- $\kappa$ B signalling pathway and increased the secretion of pro-inflammatory cytokines IL-6, IL-8 and IL-33.

### Summary/Conclusions:

After chemotherapy, abundant ACs release and they alter the immune milieu of TME. Apoptotic neuroblastoma can release HMGB1 to convert the MSCs from immunosuppressive to pro-inflammatory phenotype. Our findings suggest a paradigm shift in anti-cancer concept. This can potentially improve the efficacy of immunotherapy by manipulating the TME towards a more favourable condition for immune surveillance.

## **P241 *MYCN* and differentiation state alter inflammatory signaling in neuroblastoma**

Wolpaw, A.J.<sup>2</sup>, Dong, M.<sup>3</sup>, Volgina, D.<sup>4</sup>, Dang, C.V.<sup>5</sup>, , <sup>6</sup>

Children's Hospital of Philadelphia, Pediatric Oncology, Philadelphia, USA <sup>2</sup>, The Wistar Institute, Molecular and Cellular Oncogenesis, Philadelphia, USA<sup>3</sup>, The Wistar Institute, Molecular and Cellular Oncogenesis, Philadelphia, USA<sup>4</sup>, The Wistar Institute, Molecular and Cellular Oncogenesis, Philadelphia, The Wistar Institute<sup>5</sup>, , , <sup>6</sup>

### Background/Introduction:

While immune checkpoint blockade (ICB) therapies have revolutionized the treatment of some adult cancers, these therapies have been largely ineffective in pediatric solid tumors, including neuroblastoma. In adult cancers, tumors with baseline inflammatory signaling and infiltration with cytotoxic T cells are more likely to respond, while immunologically “cold” tumors are more resistant. Response to ICB can be restored by converting tumors from a cold to a hot immunologic state, which may also enhance response to other immune-mediated therapies. Neuroblastoma, particularly *MYCN*-amplified disease, is a classically cold tumor with a lack of T cell infiltration and very low MHC I expression. To identify pathways that can be used to activate inflammatory signaling in neuroblastoma, we tested the functional responses of neuroblastoma cell lines to inflammatory stimuli and found that responses to some stimuli are consistent and those to others are heterogenous. Exploring the mechanisms behind this heterogeneity demonstrates a role for *MYCN* and mesenchymal/adrenergic differentiation state in regulating inflammatory signaling in neuroblastoma.

### Aims:

- Test the ability of exogenous interferon, toll-like receptor agonists, and cytosolic DNA to activate inflammatory signaling in neuroblastoma cell lines
- Determine the role of *MYCN* in modulating TLR-responsiveness in neuroblastoma cell lines
- Determine the role of mesenchymal-adrenergic differentiation in regulation TLR-responsiveness in neuroblastoma cell lines

### Methods/Materials:

We treated 19 neuroblastoma cell lines with a variety of inflammatory stimuli including exogenous interferon and agonists of toll-like receptors and other pattern recognition receptors. Response was determined by measuring phosphorylation of STAT1 and IRF3, change in mRNA expression of NF-κB and interferon response genes, and activation of an NF-κB reporter construct. We tested the effect of overexpression and knockdown of *MYCN* on responses to these inflammatory stimuli. We then used inducible expression of PRRX1a in adrenergic cell types to convert them to a mesenchymal state and test the impact of this state switch on response.

### Results:

We found that all cell lines tested generated an inflammatory response to treatment with exogenous interferon and none responded to cytosolic DNA, but responses to TLR agonists were heterogenous. *MYCN* amplified cell lines were largely unresponsive and acute *MYCN* activation in non-amplified cell lines attenuated response. Differentiation state accurately classified the cell lines into responsiveness categories, with mesenchymal lines able to respond and adrenergic lines unresponsive. Exogenous expression of PRRX1a converted an unresponsive adrenergic line to a fully responsive state.

### Summary/Conclusions:

Neuroblastoma cell lines are universally sensitive to interferon and insensitive to cytosolic DNA but have heterogenous responses to toll-like receptor agonists. This responsiveness is partially suppressed by *MYCN* signaling but more strongly driven by mesenchymal differentiation. This

suggests a potential vulnerability of mesenchymal cells to ICB and other immune-mediated therapies and future experiments will test this hypothesis.

## **P242 Therapy with dinutuximab beta in combination with chemotherapy in patients with refractory relapsed neuroblastoma. Initial results.**

*Wieczorek, A.<sup>2</sup>, Zaniewska-Tekieli, A.<sup>3</sup>, Gizycka, A.<sup>4</sup>, Balwierz, W.<sup>5</sup>*

Institute of Pediatrics, Jagiellonian University Medical College, Pediatric Oncology and Hematology, Kraków, Poland<sup>2</sup>, Univesity Children's Hospital of Krakow, Pediatric Oncology and Hematology, Kraków, Poland<sup>3</sup>, Univesity Children's Hospital of Krakow, Hospital Pharmacy, Kraków, Poland<sup>4</sup>, Jagiellonian University Medical College, Pediatric Oncology and Hematology, Kraków, Jagiellonian University Medical College<sup>5</sup>

### **Background/Introduction:**

In spite of combined intensive therapy, prognosis in high risk neuroblastoma (NBL) patients with disease relapse or progression remains poor. There are data showing that immunotherapy combined with chemotherapy may be safe and effective treatment option.

### **Aims:**

The aim of the study was evaluation of feasibility, safety and effectiveness of combination of immunotherapy with dinutuximab beta (DB) and chemotherapy with irinotecan and temozolomide in patients with NBL relapse or progression.

### **Methods/Materials:**

Dinutuximab beta (10 mg/m<sup>2</sup>/5 days) with chemotherapy (irinotecan 50 mg/m<sup>2</sup>/5 days, temozolomide 100 mg/m<sup>2</sup>/5 days) was given as compassionate use in patients with therapy resistant NBL relapse or progression. Patients received 1-7 cycles of therapy. For every patient the acceptance of the Ethical Committee was obtained. Therapy was given in 6 patients, age range 2.3-14.8 years. At diagnosis 5 patients were in stage 4 and 1 in stage 3 with *MYCN* amplification. *MYCN* amplification was found in 4/6 patients. There were 3 distant, 2 combined and 1 local relapses. Two patients received immunochemotherapy at the 2nd relapse and 4 patients at the 1st relapse. DB was used in previous therapy in 3 patients (1 with interleukin-2). Before immunochemotherapy all patients received treatment with the second line standard chemotherapy with or without 131-MIBG treatment with autologous stem cell rescue, surgery and radiotherapy.

### **Results:**

Objective response was observed in 4 patients, including complete remission (CR) in 3 patients (2 with disseminated bone relapse and 1 with bone marrow relapse) and 1 very good partial response (VGPR, relapse in the localization of primary tumor). Three of them had previous immunotherapy. In 2 patients, in whom relapse occurred during the 1st line DB therapy (during cycle 2 and after cycle 1), only mild temporary improvement was observed after the first cycle (decrease of NSE and LDH, slight regression of tumor size, reduction of pain), with rapid progression few days after the cycle. Both patients died of disease. The patient with VGPR also died because of disease progression diagnosed during the fifth scheduled course of immunochemotherapy. Three patients are alive in CR (one during therapy, two - 5 and 6 months after end of therapy). Immunochemotherapy was quite well tolerated, with acceptable side effects. Hematological toxicities (stage 3 and 4 CTC WHO) and diarrhea (stage 2 and 3 CTC WHO) were common, but they did not influence therapy. Patients treated previously with DB had good tolerance of immunochemotherapy.

### **Summary/Conclusions:**

Therapy with DB in combination with chemotherapy is feasible, also in patients treated previously with DB. Objective response was observed in 4/6 pts (67%), including 3/6 pts (50%) with CR. It may be an option in therapy resistant patients, however it seems to have no benefit in patients who relapsed during immunotherapy. Clinical trials are needed to confirm its safety and efficacy.





## **P243 Evaluation of the role played by chemokines in shaping the immune microenvironment in neuroblastoma associated with opsoclonus-myoclonus**

*Sartelet, H.*<sup>2</sup>, *Aspard, C.*<sup>3</sup>, *Manches, O.*<sup>4</sup>, *Kacher, J.*<sup>5</sup>, *Plantaz, D.*<sup>6</sup>, *Chaperot, L.*<sup>7</sup>

CHU de grenoble, Department of pathology, Grenoble, France <sup>2</sup>, Etablissement Français du Sang Auvergne-Rhône-Alpes, Inserm U 1209, CNRS UMR 5309,, Grenoble, France<sup>3</sup>, Etablissement Français du Sang Auvergne-Rhône-Alpes, Inserm U 1209, CNRS UMR 5309,, Grenoble, France<sup>4</sup>, Etablissement Français du Sang Auvergne-Rhône-Alpes, Inserm U 1209, CNRS UMR 5309,, Grenoble, Etablissement Français du Sang Auvergne-Rhône-Alpes<sup>5</sup>, CHU de grenoble, Department of pediatrics, Grenoble, France<sup>6</sup>, Etablissement Français du Sang Auvergne-Rhône-Alpes, Inserm U 1209, CNRS UMR 5309,, Grenoble, France<sup>7</sup>

### Background/Introduction:

Neuroblastoma (NB) is a highly heterogeneous disease, and *MYCN* amplification has been associated with a T cell-poor immune microenvironment. Among *MYCN* non-amplified tumors, some tumors are associated with Opsoclonus Myoclonus Syndrome (OMS), a rare neurological disorder linked with autoimmune targeting of the nervous system. Chemokines are a family of soluble cytokines that act as chemoattractants to guide the migration of cells, especially immune cells.

### Aims:

We designed a study to describe and understand the role played by chemokines in shaping the immune microenvironment in neuroblastoma associated with opsoclonus-myoclonus.

### Methods/Materials:

We analyzed chemokine secretion *in vitro* by 4 NB cell lines displaying distinct *MYCN* status and ex vivo with fresh tumor samples obtained from 2 patients with or without OMS, and assessed the ability of tumor supernatants to recruit immune cells from peripheral blood mononuclear cells (PBMC) from healthy volunteers. Chemokine arrays were used to characterize the secretion of 38 chemokines in supernatants from NB cell lines and primary tumors. Transwell migration experiments were performed to analyze immune cell migration driven by these supernatants. The nature of migrating cells in transwell experiments together with the composition of tumor-infiltrating cells from the patients were characterized by multiparametric flow cytometry, using a panel allowing identification of antigen presenting cells (myeloid and plasmacytoid dendritic cells, monocytes and B lymphocytes), T lymphocytes and their subsets (CD4+, CD8+, Treg, CD56+ T cells), and innate effector cells (NK cells - both CD56 bright and dim, gd T lymphocytes and iNKT).

### Results:

NB cell lines without *MYCN* amplification secrete higher level of IL-8, CCL2 and CXCL10 compared to *MYCN*-amplified NB cell lines. When assessing the ability of NB cell line supernatants (SN) to recruit immune cells, we observed that SN from NB cell lines without *NMYC* amplification induced active preferential migration of antigen presenting cells (APC=monocytes, myeloid (mDCs) and plasmacytoid dendritic cells (pDCs)) and of gd T lymphocytes. Blocking experiments clearly demonstrated that this migration was dependent on CCR2/CCL2 axis, CCR2 being expressed at high levels by mDCs, pDCs and monocytes.

Notably, the direct ex vivo analysis of immune cell infiltration by flow cytometry of tumor samples from 2 patients with NB showed an enrichment of mDCs in tumors compared to blood samples, associated with CCL2 secretion observed in tumor supernatants. Interestingly, OMS patient's tumor had higher numbers of effector cells (CD3+CD8+, CD3+CD56+ and  $\gamma\delta$ T lymphocytes), compared to patient's blood and the non-OMS patient's tumor. The chemokines responsible for this preferential recruitment are under investigation, and migration experiments are being performed to decipher the mechanisms involved.

### Summary/Conclusions:

We provide here for the first time an exhaustive characterization of chemokine expression and immune cell infiltration in NB, highlighting the major role of CCR2/CCL2 in the preferential recruitment of monocytes, mDCs and pDCs by *MYCN* non-amplified NB tumors. We also demonstrate that tumor from patient with OMS is infiltrated by higher numbers of effector cells of both adaptive and innate immunity compared to non-OMS patient, which might be directly related to the autoimmune activation leading to OMS symptoms.

## **P244 Immunotherapy Initiated Before Surgical Resection Improves Survival and Reduces Metastatic Burden in Neuroblastoma**

Zobel, M.J.<sup>2</sup>, Abigail, A.K.<sup>3</sup>, Wu, H.W.<sup>4</sup>, Sun, J.<sup>5</sup>, Ourshalimian, S.<sup>6</sup>, Sheard, M.<sup>7</sup>, Seeger, R.C.<sup>8</sup>, Kim, E.S.<sup>9</sup>

Children's Hospital Los Angeles, Pediatric Surgery, Los Angeles, USA <sup>2</sup>, Children's Hospital Los Angeles, Pediatric Surgery, Los Angeles, USA<sup>3</sup>, Children's Hospital Los Angeles, Division of Hematology/Oncology, Los Angeles, USA<sup>4</sup>, Children's Hospital Los Angeles, Division of Hematology/Oncology, Los Angeles, USA<sup>5</sup>, Children's Hospital Los Angeles, Pediatric Surgery, Los Angeles, USA<sup>6</sup>, Children's Hospital Los Angeles, Division of Hematology/Oncology, Los Angeles, USA<sup>7</sup>, Children's Hospital Los Angeles, Division of Hematology/Oncology, Los Angeles, USA<sup>8</sup>, Children's Hospital Los Angeles, Pediatric Surgery, Los Angeles, USA<sup>9</sup>

### Background/Introduction:

High-risk neuroblastoma portends a poor prognosis. While immunotherapy with anti-disialoganglioside (GD2) monoclonal antibody (dinutuximab) is an important therapeutic adjunct to chemotherapy and surgery, a majority of patients with high-risk disease still succumb to recurrent, metastatic disease. Our previous work demonstrated that the addition of adoptively transferred *ex vivo*-activated human natural killer cells (aNK) to dinutuximab results in improved survival and decreased metastatic burden in a neuroblastoma mouse model of post-resection minimal residual disease. While dinutuximab is currently administered after primary disease resection and post-consolidation, the impact of the timing of immunotherapy treatment relative to surgery has yet to be examined.

### Aims:

To demonstrate that immunotherapy with dinutuximab combined with adoptively transferred aNK cells initiated before surgical resection improves survival and reduces metastatic burden relative to therapy initiated after resection in a mouse model of metastatic neuroblastoma.

### Methods/Materials:

One million human neuroblastoma cells (CHLA-255 or KCNR), tagged with luciferase, were implanted beneath the left renal capsule of immunodeficient NOD-scid gamma (NSG) mice. Mice were randomized to three treatment groups: (i) surgical resection alone ("surgery only"), (ii) dinutuximab plus aNK cells initiated two weeks before resection ("early tx + surgery"), or (iii) dinutuximab plus aNK cells initiated after resection ("late tx + surgery"). Gross total resection of the xenograft tumors was performed 17 days after implantation. Immunotherapy and aNK cells were administered with IL2 and IL15 via tail vein injection twice weekly for a total of four weeks. Weekly bioluminescent imaging was used to measure tumor cell flux (photons/sec) in each mouse as a means of quantifying total metastatic burden. Survival data was collected. Mixed-effects logistic regression was used to model flux data; paired t-test was used to calculate significance. Log-rank test was used to calculate significance for survival data. P value < 0.05 was considered significant.

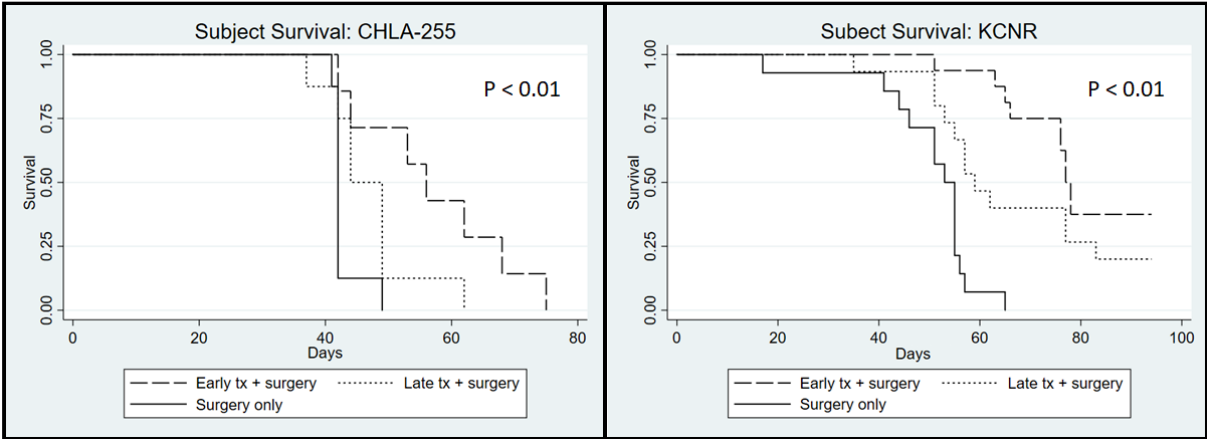
### Results:

In mice implanted with the neuroblastoma cell lines CHLA-255 and KCNR, the total metastatic disease burden of the mice was decreased when immunotherapy was given before tumor resection compared to given after resection (p=0.06) or compared to surgery alone without immunotherapy (p=0.01). Immunotherapy plus aNK cells initiated prior to surgical resection resulted in significantly longer overall survival following implantation with either cell line (p<0.01) (Fig 1).

### Summary/Conclusions:

Our data shows that immunotherapy with an anti-GD2 monoclonal antibody plus adoptively transferred aNK cells administered before surgical resection prolongs survival and reduces the metastatic burden

over time. These findings support the clinical investigation of immunotherapy in high-risk neuroblastoma during the pre-surgical induction phase of chemotherapy.



Caption 1: Figure 1. Survival Curves: CHLA-255, KCNR

## **P245 $\beta$ 3-adrenoreceptor modulates the immune-checkpoint PD-L1 expression and impairs tumor growth in a murine syngeneic neuroblastoma model**

*Bruno, G.<sup>2</sup>, Subbiani, A.<sup>3</sup>, Dabraio, A.<sup>4</sup>, Ciullini Mannurita, S.<sup>5</sup>, Cencetti, F.<sup>6</sup>, Bianchini, F.<sup>7</sup>, Calvani, M.<sup>8</sup>, Favre, C.<sup>9</sup>*

A. Meyer University Children's Hospital, Department of Paediatric Haematology-Oncology, Florence, Italy<sup>2</sup>, A. Meyer University Children's Hospital, Department of Paediatric Haematology-Oncology, A. Meyer University Children's Ho, Florence, Italy<sup>3</sup>, A. Meyer University Children's Hospital, Department of Paediatric Haematology-Oncology, A. Meyer University Children's Ho, Florence, Italy<sup>4</sup>, A. Meyer University Children's Hospital, Department of Paediatric Haematology-Oncology, A. Meyer University Children's Ho, Florence, A. Meyer University Children's Hospital<sup>5</sup>, University of Florence, Department of Experimental and Clinical Biomedical Sciences "Mario Serio", Florence, Italy<sup>6</sup>, University of Florence, Department of Experimental and Clinical Biomedical Sciences "Mario Serio", Unive, Florence, Italy<sup>7</sup>, A. Meyer University Children's Hospital, Department of Paediatric Haematology-Oncology, Florence, Italy<sup>8</sup>, A. Meyer University Children's Hospital, Department of Paediatric Haematology-Oncology, Florence, Italy<sup>9</sup>, ,

### Background/Introduction

Neuroblastoma (NB) is the most frequently observed among extracranial pediatric solid tumors. Despite the availability of numerous therapies, high risk NB is still difficult to cure, therefore, new therapeutic strategies are desperately needed. Immune therapies are the most promising antitumor strategy; moreover, several literature data support the concept that the PD1/PD-L1 immune checkpoint axis, among others, is crucial for allowing NB immune-evasion.  $\beta$ 3-adrenoreceptor ( $\beta$ 3-AR) has been recently identified as a crucial regulator of different tumor-related processes, including immune reactivity. Moreover, we recently demonstrated that  $\beta$ 3-AR-blockade, via sphingosine 1-phosphate (S1P) signaling modulation, is able to affect NB tumor progression using in vivo and in vitro models.

### Aims

The present project aims to investigate the relationship between the  $\beta$ 3-AR pathway, the S1P signaling and the expression of the PD1/PD-L1 immune-checkpoint in NB. Given that, recently, our group demonstrated that  $\beta$ 3-AR is involved in regulating the immune reactivity of melanoma, and that its blockade is also able to impair NB tumor growth, the goal of our study was to investigate whether  $\beta$ 3-AR modulation affects the immune reactivity in NB through S1P signaling. Specifically, we evaluated the expression of PD1/PD-L1 immune-checkpoint in a murine syngeneic model of NB following  $\beta$ 3-AR antagonism.

### Methods/Materials

Female NCI A/J mice 4-weeks-old were bought from Charles River Laboratories (Frederick). Murine NB Neuro-2A cells were subcutaneously implanted in A/J recipient mice by injecting  $1 \times 10^6$  cells in 100  $\mu$ l of PBS in the right flank. Tumor growth rate was evaluated by measuring tumor mass with a caliber, and tumor mass volume was calculated as  $\text{Volume} = [(\text{length} \times \text{width})^2 / 2]$ . Treatments with  $\beta$ 3-AR antagonist (SR59230A) and S1P2 receptor selective agonist (CYM5520) started when Neuro-2A cells formed a palpable tumor. Mice were sacrificed after 8 days of treatment. Tumor masses were then excised and divided to perform western blot, immunofluorescence, and citofluorimetric analyses, in order to evaluate immune-checkpoint expression in tumor cells and tumor infiltrating lymphocytes (TILs). TILs were isolated by tumor mass using anti-CD45 micro-beads and the AutoMACS Separator Pro (Miltenyi Biotec).

### Results

$\beta$ 3-AR pharmacological blockade, induced with SR59230A, strongly reduced NB tumor growth in A/J mice through the involvement of the sphingosine-1-phosphate receptor 2 (S1P2). The expression of the immune-checkpoint PD-L1 decreased in tumour masses excised from SR59230A-treated mice compared to control group. Moreover, this decrease was partially reverted by using a selective S1P2 receptor agonist (CYM5520). These results suggest that  $\beta$ 3-AR is able to regulate the PD1/PD-L1 expression in NB tumor, through the S1P signalling involvement.

### Summary/Conclusions

Even though cancer immune therapy has given great chances of good prognosis to several oncological patients, some of them are still not responsive to these therapies. Thus, the understanding of the molecular mechanisms that regulate the expression of the PD1/PD-L1 and other immune checkpoints during tumor progression could open new therapeutic strategies to overcome tumor resistance of this inauspicious pediatric tumor.

## **P246 Immunology of neuroblastoma. Beyond the immunotherapy**

*Druy, A.E.<sup>2</sup>, Andreeva, N.A.<sup>3</sup>, Koldasheva, M.M<sup>4</sup>, Shamanskaya, T.V.<sup>5</sup>, Kachanov, D.Y.<sup>6</sup>*

D. Rogachev National Medical Research Center of Pediatric Hematology, Oncology, Laboratory of Molecular Oncology, Moscow, Russia <sup>2</sup>, D. Rogachev National Medical Research Center of Pediatric Hematology, Oncology, Laboratory of Molecular Oncology, Moscow, Russia<sup>3</sup>, D. Rogachev National Medical Research Center of Pediatric Hematology, Oncology, Department of Clinical Oncology, Moscow, Russia<sup>4</sup>, D. Rogachev National Medical Research Center of Pediatric Hematology, Oncology, Department of Clinical Oncology, Moscow, D. Rogachev National Medical Research Center of Pediatric Hematology, Oncology<sup>5</sup>, D. Rogachev National Medical Research Center of Pediatric Hematology, Oncology, Department of Clinical Oncology, Moscow, Russia<sup>6</sup>

### **Background/Introduction:**

Nowadays immunotherapy is becoming the standard-of-care in treatment of high-risk neuroblastoma, while molecular and cellular mechanisms underlying tumor-host interaction are still mostly unraveled.

### **Aims:**

Investigation of mechanisms of anti-tumor immune response against neuroblastoma and immune-mediated paraneoplastic opsoclonus-myoclonus syndrome (OMS).

### **Methods/Materials:**

39 tumor specimens from primary chemotherapy and immunotherapy naïve patients with neuroblastoma were subjected to mRNA analysis with Nanostring PanCancer Immune Profiling Panel. This type of the assay implies bulk investigation of mRNA both from tumor cells and cells of microenvironment. All patients were treated or observed according to NB2004 protocol. 9 patients had neuroblastic tumor accompanying by paraneoplastic OMS. All these patients belonged to the observation group. 15 patients with matching clinical characteristics were used as a control. 13 patients belonged to high-risk group (5 harbored *MYCN* amplification) and 2 - to intermediate-risk group.

### **Results:**

Group of patients enriched with OMS cases (6/9) was characterized by significant prevalence of T (CD3D,E,G) and B cells in the tumor microenvironment. Cytotoxic cell population was present mostly by CD56dim NK cells. The major pathway of cellular cytotoxicity was perforin-granzyme mediated, comparing to FAS-dependent. Also population of tumor-infiltrating cells was enriched in MHCII-positive antigen-presenting cells demonstrating marks of activation (CD80/86 expression) and FOXP3-positive T-reg cells (median Log2 fold change is 2.14SE0.50, adjusted p=0.020). However, 3 patients with clinically confirmed OMS did not fell into this group and vice versa 6 patients without OMS demonstrated similar molecular profile.

Tumor cells were characterized by retained expression of MHC1 mRNA, significant gain of expression of integrins (2.93SE0.53, p=0.005), activation of SMAD and inhibition of PIK3CA pathways. Microenvironment cells actively produced mRNA of factors of the innate immunity: complement components (3.00SE0.52, p=0.002), TLRs (2.25SE0.44, p=0.005), pro-inflammatory cytokines and chemokines (mostly CCLs, CXCLs, TNF-superfamily, IL6 with corresponding receptors, 2.45SE0.51, p=0.009).

The analysis of entire patient's cohort let to formation of two independent groups, which were characterized by completely different profile of the expression of genes involved in both innate and adoptive immune response. First group (n=21) contained mostly favorable cases with or without OMS and 8 high-risk patients, while in the second group (n=18) patients from all risk groups were present and prone to have the adverse events. Interestingly, that the expression signature discriminating these groups was identical to the signature depicting OMS positive and negative cases. Similar to the cohort enriched in OMS cases, first group was characterized by upregulation of the mediators of innate and



adoptive immunity. The slight differences were observed in balanced FAS-dependent and perforin-granzyme cytotoxicity, and extensive repertoire of upregulated pro-inflammatory cytokines (2.38SE0.53,  $p=0.003$ ). Among very few genes, which were upregulated in the second group were cancer-testis antigens PBK and TTK (2.28SE0.58,  $p=0.002$ ). But expression of these antigens was not enough to induce anti-tumor immune response.

#### Summary/Conclusions:

Thus, based on mRNA profiling of tumor cells and microenvironment two types of neuroblastoma were recognized - immunological "hot" and "cold" neoplasms. These findings have no direct impact on the prognosis of patients with neuroblastoma but could be useful to predict the efficacy of the immunotherapy and clinical course of immunological paraneoplastic syndromes, such as OMS.

## **P247 Generation and characterization of PDL1 knock NB cell lines**

*Huang, T.*<sup>2</sup>, *Rozen, E.*<sup>3</sup>, *Wigglesworth, K.*<sup>4</sup>, *Shohet, J.*<sup>5</sup>

Umass Medical School, Dept of Pediatrics, Worcester, USA <sup>2</sup>, Umass Medical School, Dept of Pediatrics, Worcester, USA<sup>3</sup>, Umass Medical School, Dept of Pediatrics, Worcester, USA<sup>4</sup>, Umass Medical School, Dept of Pediatrics, Worcester, Umass Medical School<sup>5</sup>

### Background/Introduction:

Immune check points are central to host tumor interactions and blockade of PD-1 / PD-L1 interactions may be an important adjuvant immunotherapeutic approach to neuroblastoma and other solid tumors. PD-L1 expression varies widely across NB cell lines. Multiple tumor and host derived cytokines modulate surface expression of tumor checkpoint ligands to alter the tumor/immune synapse. In addition to its ligand/receptor function, additional cell autonomous functions for PD-L1 modulating tumor cell physiology are under investigation.

PD-L1 is typically upregulated by IFN $\gamma$  although this is highly cell line dependent in part due to epigenetic silencing of the locus.

### Aims:

### Methods/Materials:

In order to better understand the functions of PD-L1 in neuroblastoma we are generating a panel of NB cells with variable PD-L1 expression using CRISPR-Cas12a based ribonucleoprotein approaches to modify expression of endogenous PD-L1 for *in vitro* and *in vivo* phenotypic studies.

### Results:

We examined the expression of PD-L1 by qPCR and flow cytometry in SH-SY5Y, SHEP, CHLA20, GIMEN, BE2, NGP and LAN5 cell lines. Surface expression ranges from almost undetectable (NGP) to 97% positive (GIMEN). In addition, response to IFN $\gamma$  varied significantly (eg. SH-SY5y 5 % untreated increased to 95%-97% positive treated, CHLA-20 2% untreated to 95%-97% positive treated).

Next, we used electroporation of Cas12a-gRNAs to target the first and second exons of PD-L1 and isolated single cell derived subclones. Clones are evaluated by qPCR, flow cytometry and responsiveness to IFN $\gamma$ .

For CHLA-20 clone 1, flow cytometry demonstrated no surface expression or response to IFN $\gamma$ . DNA sequence confirmed bi-allelic frameshift deletions by DNA sequencing. Interestingly, initial evaluation suggests that prolonged culture induces a senescent phenotype in a small fraction of these cells based on senescence associated  $\beta$ -gal staining and morphology.

Subsequent characterization of this clone and others is ongoing, including evaluation cell proliferation kinetics, differentiation and neurosphere formation relative to control PD-L1 +/+ and +/- cell lines. We are also evaluating the *in vivo* tumorigenicity and cytokine responsiveness of these cells compared to wild type CHLA-20 using a novel "humanized" murine xenograft approach. This permits us to assess engraftment in the presence of human CD4 and CD8 T-cells as well as monocytes, macrophages and other fully human immune components.

### Summary/Conclusions:

**Conclusions:** We have developed an efficient approach to isolating and characterizing PD-L1 knockout cell lines for functional evaluation of PD-L1. Both cell intrinsic and ligand/receptor mediated functions of will be evaluated using *in vitro* and novel humanized *in vivo* murine models. We hope to elucidate novel mechanistic insights and novel alternative therapeutic approaches to modulate PD-L1 functions in the clinical setting.



## **P248 Considering new anti-GD2 immunotherapeutic approaches to high-risk neuroblastoma.**

*Bahri, M.*<sup>2</sup>, *Vermeulen, S.*<sup>3</sup>, *Galopin, N.*<sup>4</sup>, *Gauttier, V.*<sup>5</sup>, *Thébaud, E.*<sup>6</sup>, *Fougeray, S.*<sup>7</sup>, *Birklé, S.*<sup>8</sup>

CRCINA, INSERM, Angers University, Nantes University, ONCO, Nantes, France <sup>2</sup>, CRCINA, INSERM, Angers University, Nantes University, CRCINA, Nantes, France<sup>3</sup>, CRCINA, INSERM, Angers University, Nantes University, CRCINA, Nantes, France<sup>4</sup>, OSE Immunotherapeutics, N/A, Nantes, OSE Immunotherapeutics<sup>5</sup>, CHU of Nantes, Oncology, Nantes, France<sup>6</sup>, CRCINA, INSERM, Angers University, Nantes University, ONCO, Nantes, France<sup>7</sup>, CRCINA, INSERM, Angers University, Nantes University, ONCO, Nantes, France<sup>8</sup>

### **Background/Introduction:**

Neuroblastoma (NB) is the most common extracranial tumor of childhood. Dinutuximab, a therapeutic antibody against ganglioside GD2, was approved for treatment of high-risk neuroblastoma patients. NK cells are indicated as the main effector cells for dinutuximab-mediated antibody dependent cellular cytotoxicity (ADCC). Next to ADCC, complement dependent cytotoxicity (CDC) is another effector mechanism of dinutuximab. Unfortunately, complement activation is also an important cause of neuropathic pain, related to the expression of GD2 on peripheral nerves. In addition to this severe toxicity, a third of patient experiences treatment failure. Additional strategies are thus needed to improve outcomes for this malignancy. We have found recently that mAb 8B6 specific for O-acetyl GD2 (OAcGD2) show a strong anti-tumor effect via ADCC and CDC. In addition, mAb 8B6 does not cross-reacts with peripheral nerves. Importantly, macrophages are often found in high numbers within neuroblastoma tumors, contrary to NK cells. In fact, the contribution of macrophages has been marginalized in the past : their complex relationship with tumor cells underscores the potential that they act as immune effector cells. Nonetheless, macrophages may perform antibody-dependent phagocytosis (ADP) of tumor cells.

### **Aims:**

On these grounds, we hypothesized that macrophages play a significant role in the efficacy of anti-OAcGD2 monoclonal antibodies.

### **Methods/Materials:**

We first evaluated 8B6 ADP endowment and further correlated this faculty to innate immune checkpoint expression *in vitro* . We went on studying the efficacy of 8B6 mAb *in vivo*, alone or in combination with proof of concept innate immune checkpoint inhibitors (ICI). We collected tumor samples from these mice to further characterize the immune infiltrate using flow cytometry and gene expression profiling (nCounter analysis).

### **Results:**

We observed that neuroblastoma cells express the “don’t eat me” CD47 immune checkpoint inhibitor that limits macrophages responses to mAb 8B6. Yet, we were able to redirect macrophages to kill mAb 8B6 bearing neuroblastoma targets using ICI reagents.

### **Summary/Conclusions:**

Collectively, these data highlight that macrophages are important mediators of the efficacy of anti-OAcGD2 mAb combined with ICI therapy. Thus, targeting macrophages represents an attractive option to achieve durable responses in a greater number of patients with NB.

**P249 Outcomes and toxicities associated with ch14.18 (dinutuximab), GM-CSF, IL-2 and isotretinoin in the non-randomized cohort of patients treated on ANBL0032: A Children's Oncology Group (COG) Study**

*Desai, A.<sup>2</sup>, Gilman, A.<sup>3</sup>, Ozkaynak, M.F.<sup>4</sup>, Naranjo, A.<sup>5</sup>, London, W.<sup>6</sup>, Tenney, S.<sup>7</sup>, Smith, M.<sup>8</sup>, Seibel, N.<sup>9</sup>, Smith, L.M.<sup>10</sup>, Shimada, H.<sup>11</sup>, Matthay, K.<sup>12</sup>, Cohn, S.<sup>13</sup>, Maris, J.<sup>14</sup>, Bagatell, R.<sup>15</sup>, Sondel, P.<sup>16</sup>, Park, J.<sup>17</sup>, Yu, A.<sup>18</sup>*

University of Chicago, Pediatrics, Chicago, USA <sup>2</sup>, PRA Health Sciences, N/A, Raleigh, USA<sup>3</sup>, New York Medical College, Pediatrics, Valhalla, USA<sup>4</sup>, Children's Oncology Group Statistics and Data Center, University of Florida, Biostatistics, Gainesville, Children's Oncology Group Statistics and Data Center, University of Florida<sup>5</sup>, Dana-Farber/Boston Children's Hospital and Harvard Medical School, Pediatrics, Boston, USA<sup>6</sup>, Children's Oncology Group Statistics and Data Center, University of Florida, Biostatistics, Gainesville, USA<sup>7</sup>, National Cancer Institute, Clinical Investigations Branch, Bethesda, USA<sup>8</sup>, National Cancer Institute, Clinical Investigations Branch, Bethesda, USA<sup>9</sup>, Spring Works Therapeutics, Clinical Research and Development, Hillsborough, USA<sup>10</sup>, Stanford University Medical Center, Pathology and Pediatrics, Stanford, USA<sup>11</sup>, UCSF Benioff Children's Hospital, University of California, San Francisco, Pediatrics, San Francisco, USA, <sup>12</sup>, University of Chicago, Pediatrics, Chicago, USA <sup>13</sup>, Children's Hospital of Philadelphia, Pediatrics, Philadelphia, USA <sup>14</sup>, Children's Hospital of Philadelphia, Pediatrics, Philadelphia, USA <sup>15</sup>, University of Wisconsin, Pediatrics, Madison, USA <sup>16</sup>, Seattle Children's Hospital, University of Washington School of Medicine, Pediatrics, Seattle, USA <sup>17</sup>, University of California, San Diego, Pediatrics, San Diego, USA <sup>18</sup>

**Background/Introduction:**

COG ANBL0032 demonstrated that immunotherapy with the anti-GD2 antibody dinutuximab in combination with sargramostim (GM-CSF), aldesleukin (IL-2) and isotretinoin in the post-consolidation setting improved event-free survival (EFS) and overall survival (OS) for patients with high-risk neuroblastoma (HRNBL). Randomization was halted in 2009, and the immunotherapy arm remained open to enrollment to ensure dinutuximab availability and to obtain additional outcome and toxicity data in a larger cohort of patients.

**Aims:**

Our aims are to: 1) Determine the EFS and OS of patients treated with immunotherapy in the ANBL0032 non-randomized cohort, and 2) Identify the CTCAEv4.0 Grade 3 or higher toxicities associated with immunotherapy.

**Methods/Materials:**

Patients <31 years of age with HRNBL and a pre-autologous stem cell transplant (ASCT) response of partial response (PR) or better by the 1993 International Neuroblastoma Response Criteria (INRC) were eligible. Patient demographics, International Neuroblastoma Staging System (INSS) stage, tumor biology, pre-ASCT response by INRC and toxicities were summarized using descriptive statistics. Five-year EFS and OS estimates from the time of study enrollment post-consolidation were generated.

**Results:**

From 2009-2015, 1,183 patients were non-randomly assigned to receive immunotherapy. Among these, 96.7% (n=1,144) were ≥18 months of age at enrollment and 83.1% (n=765/921) had INSS stage 4 disease. Among those tumors with known biology, 45.1% (n=363/805) harbored MYCN amplification, 94.5% (n=749/793) had unfavorable histology, and 54.9% (n=397/723) had diploid tumors. Pre-ASCT response included complete response (CR) (n=352; 29.8%), very good partial response (VGPR) (n=418; 35.3%), or PR (n=413; 34.9%). Patients underwent a single (n=1,042; 88.1%) or tandem (n=141; 11.9%) ASCT prior to immunotherapy. For the entire cohort, 5-year EFS from the time of enrollment was 61.1±1.9% and 5-year OS was 71.9±1.7%. Among patients ≥18 months of age with INSS stage 4 disease (n=746), 5-year EFS and OS were 58.4±2.3% and 71.0±2.1%, respectively. Five-year EFS and OS were 82.3±4.8% and 86.7±4.2% among patients with INSS stage 3 disease (n=110). EFS but not OS was superior for those with a CR/VGPR pre-

ASCT compared to those with a PR pre-ASCT (5-year EFS: 64.2±2.2% vs. 55.4±3.2%, p=0.0133; OS: 72.7±2.1% vs. 70.5±2.9%, p=0.3811). There was a trend for improved OS for those treated with tandem transplant versus single transplant, although the difference did not reach statistical significance (5-year EFS: 65.9±4.3% vs. 60.4±2.1%, p=0.1282; OS: 76.5±3.8% vs. 71.2±1.9%, p=0.0704). Six cycles of therapy were completed in 83.9% of patients, with IL-2 replaced by GM-CSF in 1.8% of patients. Grade 3 or higher toxicities occurring in >10% of patients during GM-CSF and IL-2-containing cycles, respectively, included pain (15.6/11.4%), fever (15.1/32.7%), anemia (18.9/21.7%), thrombocytopenia (13.9/17.4%), lymphopenia (12.3/16.0%), and hypokalemia (13.3/25.2%). During GM-CSF-containing cycles, hypoxia occurred in 10.1% of patients. During IL-2-containing cycles, additional toxicities included anaphylaxis (12.0%), neutropenia (16.1%), hyponatremia (16.5%), and hypotension (13.8%).

Summary/Conclusions:

Aligned with prior reports, we observed a 5-year EFS of 61.1% among a large cohort of HRNBL patients treated with dinutuximab immunotherapy. EFS was higher for patients with INSS stage 3 disease and pre-ASCT disease response of CR/VGPR. The number of Grade 3 or higher toxicities occurring in >10% patients was greater during IL-2-containing cycles.

## **P250 Immunophenotypic characterization of bone marrow metastases and NK cells aimed at predicting sensitivity / resistance to immunotherapy in patients with high risk neuroblastoma**

Castriconi, R.<sup>2</sup>, Dondero, A.<sup>3</sup>, Morini, M.<sup>4</sup>, Raggi, F.<sup>5</sup>, Eva, A.<sup>6</sup>, Garaventa, A.<sup>7</sup>, Conte, M.<sup>8</sup>, Sementa, A.R.<sup>9</sup>, Bottino, C.<sup>10</sup>

University of Genova, Department of Experimental Medicine and CEBR, Genova, Italy <sup>2</sup>, University of Genova, Department of Experimental Medicine, Genova, Italy<sup>3</sup>, IRCSS, Istituto G. Gaslini, Laboratory of Molecular Biology,, Genova, Italy<sup>4</sup>, IRCSS, Istituto G. Gaslini, Laboratory of Molecular Biology, Genova, IRCSS, Istituto G. Gaslini<sup>5</sup>, IRCSS, Istituto G. Gaslini, Laboratory of Molecular Biology, Genova, Italy<sup>6</sup>, IRCSS, Istituto G. Gaslini, Oncologia Pediatrica, Genova, Italy<sup>7</sup>, IRCSS, Istituto G. Gaslini, Oncologia Pediatrica, Genova, Italy<sup>8</sup>, IRCSS, Istituto G. Gaslini, UOC Anatomia Patologica, Genova, Italy<sup>9</sup>, IRCSS, Istituto G. Gaslini, Laboratory of clinical and experimental immunology, Genova, Italy<sup>10</sup>

### Background/Introduction:

High-risk neuroblastomas (HR-NB) are particularly aggressive childhood tumors characterized by metastatic dissemination, typically affecting the bone marrow. The 5-year survival remains <40% despite the aggressiveness of the therapy which includes autologous stem cell transplantation and immunotherapy with specific antibodies for the GD<sup>2</sup> oncofetal antigen. Patients with similar clinical pathological parameters and receiving the same treatment may have markedly different clinical outcome.

### Aims:

Our study aims to analyze the variables that might reduce/abolish the effectiveness of treatments, in particular the efficacy of anti-GD2 immunotherapy. They could include: i) the presence of GD2<sup>neg</sup> or GD2<sup>low</sup> metastatic variants; ii) the presence of high levels of ligands involved in immune-checkpoints axis (such as B7-H3 or PD-Ls.); iii) a reduced post-transplant number of immune cells, in particular of CD16+ Natural Killer (NK) capable of being effectively engaged by the antibody; iv) surface expression of CD16 allotypes (158V or 158F) with different binding affinity for the Fc portion of the antibody; v) reduced expression of DNAM-1 and NKp30, highly involved in spontaneous NB cell killing; vi) percentage of long-term differentiated NK cells (expressing different markers including CD57 and KIRs).

### Methods/Materials:

We analyzed NB and/or NK cell compartment in more than 20 liquid biopsies (Peripheral blood and bone marrow aspirates) from HR-NB patients at the onset at relapse and post autologous stem cell transplantation by using a polychromatic cytofluorimetric method. Moreover, NK cell degranulation capability against NB cells has been evaluated in combination with different NK cell phenotypes, using an *in vitro* CD107a assay

### Results:

Our study allowed us to set up a cytofluorimetric method showing an almost complete overlapping with results derived from histopathologic analysis in terms of presence/absence of NB infiltration. Rare unmatched results are under investigation. Thus, our method showed a great specificity and sensibility. Moreover, importantly, our analysis allowed quantification of CD56, GD2 and B7-H3 surface expression to be correlated to the efficacy of immunotherapy or other clinical parameters. PD-L1 surface expression has never been detected on the bone marrow metastases analyzed.

About NK cell compartment, at time of immunotherapy a relevant percentage of HR-NB patients showed a significant proportion of CD16 negative cells almost in bone marrow and a heterogeneous surface expression of the other molecules analyzed. A correlation between different NK cell phenotypes and degranulation capability against NB in the presence of dinutuximab beta has been performed

#### Summary/Conclusions:

In conclusion our study set up an unbiased cytofluorimetric method to unequivocally identify NB infiltration in liquid biopsies and to characterize NB in terms of surface expression of GD2, B7-H3 and other molecules possibly impacting on response to therapy and on patients' survival.

Our analysis also provided a wide still lacking characterization of NK cell compartment at onset at relapse and before immunotherapy in HR-NB patients.

Overall our study contributes to optimize diagnosis and to predict response to anti-GD2 immunotherapy in HR-NB patients. Moreover, our results highlight some aspects of the immune landscape in NB patients to be considered for current and future novel therapeutic approaches.



## **P251 Outcome of anti-GD2 immunotherapy in Dutch high-risk neuroblastoma patients**

*Tas, M.L.<sup>2</sup>, Dootjes, E.W.<sup>3</sup>, Fiocco, M.F.<sup>4</sup>, De Krijger, R.R.<sup>5</sup>, Dierselhuis, M.P.<sup>6</sup>, Van Eijkelenburg, N.K.A.<sup>7</sup>, Van Grotel, M.<sup>8</sup>, Kraal, K.C.J.M.<sup>9</sup>, Peek, A.M.L.<sup>10</sup>, Tytgat, G.A.M.<sup>11</sup>, Van Noesel, M.M.<sup>12</sup>*

Princess Máxima Center for Pediatric Oncology, Pediatric Oncology, Utrecht, Netherlands<sup>2</sup>, Princess Máxima Center for Pediatric Oncology, Pediatric Oncology, Utrecht, Netherlands<sup>3</sup>, Princess Máxima Center for Pediatric Oncology, Statistics, Utrecht, Netherlands<sup>4</sup>, Princess Máxima Center for Pediatric Oncology, Pediatric Oncology, Utrecht, Princess Máxima Center for Pediatric Oncology<sup>5</sup>, Princess Máxima Center for Pediatric Oncology, Pediatric Oncology, Utrecht, Netherlands<sup>6</sup>, Princess Máxima Center for Pediatric Oncology, Pediatric Oncology, Utrecht, Netherlands<sup>7</sup>, Princess Máxima Center for Pediatric Oncology, Pediatric Oncology, Utrecht, Netherlands<sup>8</sup>, Princess Máxima Center for Pediatric Oncology, Pediatric Oncology, Utrecht, Netherlands<sup>9</sup>, Princess Máxima Center for Pediatric Oncology, Pediatric Oncology, Utrecht, Netherlands<sup>10</sup>, Princess Máxima Center for Pediatric Oncology, Pediatric Oncology, Utrecht, Netherlands<sup>11</sup>, Princess Máxima Center for Pediatric Oncology, Pediatric Oncology, Utrecht, Netherlands,<sup>12</sup>

### **Background/Introduction:**

In high-risk neuroblastoma (HR-NBL), approximately 50% of patients suffer from recurrent disease. Anti-GD2 immunotherapy has been shown to reduce the number of recurrences in the COG cohort.

### **Aims:**

The aim of this study was to compare the outcome of a Dutch HR-NBL cohort treated with maintenance anti-GD2 immunotherapy to patients treated with single-agent isotretinoin maintenance therapy after GPOH04/DCOG04 or POG9640 induction treatment.

### **Methods/Materials:**

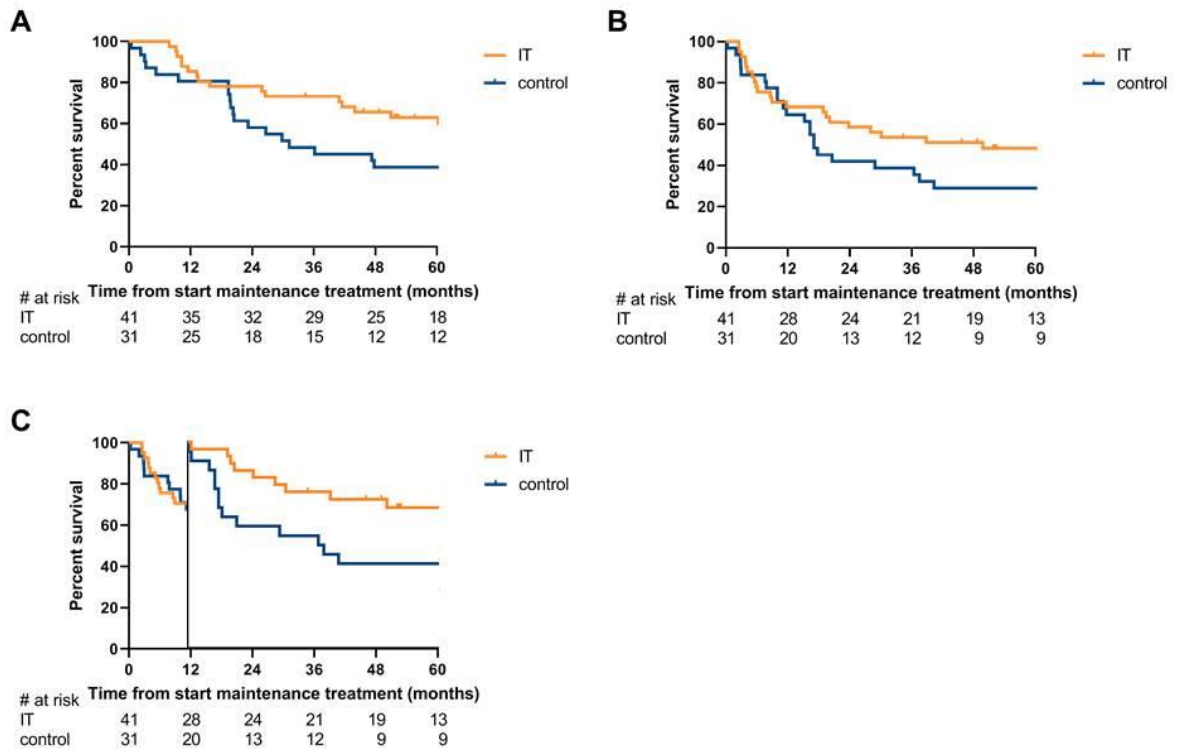
Included in the cohort were Dutch HR-NBL patients treated with immunotherapy between 2009 and 2016 according to the prospective ANBL0032 study (n=47, referred to as the immunotherapy group). They were compared to a historic control group treated with identical induction therapy between 2000 and 2015 with single-agent isotretinoin maintenance therapy (n=37, referred to as the control group). Data were collected of clinical and histological patient characteristics and response to treatment before autologous stem cell transplantation. Survival time was calculated for both groups from start of the maintenance therapy up to an event or the date of last contact. To investigate the effect of immunotherapy on late recurrences in more detail, we performed a landmark analysis at 11 months, when the event-free survival curves started deviating.

### **Results:**

The patients had a median age at diagnosis of 41 months (range 1-196). The study and control group were comparable for baseline characteristics. In this small cohort, a non-significant 14% improved 5-yr overall survival (OS) in favor of the immunotherapy group (immunotherapy 63±7%; control 49±8%; p=0.17) and a 10% improved 5-yr event free survival (EFS) (51±7% vs. 41±8%; p=0.39) were observed. For patients ≥18 months, there was a significant 24% 5-yr OS benefit (63±8% vs. 39±9%; p=0.04; Figure 1A) and a non-significant 19% 5-yr EFS benefit for patients treated with immunotherapy (48±8% vs. 29±8%; p=0.12; Figure 1B). Landmark analysis showed a significantly better 5-yr EFS in the immunotherapy group compared with the control group for patients ≥18 months (68±9% vs. 41±10; p=0.03; Figure 1C), indicating the prevention of late relapses.

### **Summary/Conclusions:**

This study confirms the results of the COG study ANBL0032 that anti-GD2 immunotherapy improves the OS and EFS in patients with HR-NBL. Our small cohort, with a different induction treatment compared to the COG, had a 14% 5-yr OS benefit for patients treated with immunotherapy. We further demonstrated an immunotherapy-induced reduction of late recurrences in patients ≥18 months.



Caption 1: Five-year OS (A), EFS (B) and EFS with landmark at 11 months (C) for patients  $\geq 18$  months, according to treatment group. Abbreviation: IT=immunotherapy

## **P252 Evaluating combination therapy of a bispecific trifunctional antibody and checkpoint inhibition in neuroblastoma models**

*Ivasko, S.<sup>2</sup>, Grunewald, L.<sup>3</sup>, Klaus, A.<sup>4</sup>, Ruf, P.<sup>5</sup>, Lindhofer, H.<sup>6</sup>, Anders, K.<sup>7</sup>, Andersch, L.<sup>8</sup>, Schulte, J.H.<sup>9</sup>, Eggert, A.<sup>10</sup>, Hundsdörfer, P.<sup>11</sup>, Künkele, A.<sup>12</sup>, Zirngibl, F.<sup>13</sup>*

Charité–Universitätsmedizin Berlin, Department of Pediatric Oncology and Hematology, Berlin, Germany <sup>2</sup>, Charité–Universitätsmedizin Berlin, Department of Pediatric Oncology and Hematology, Berlin, Germany<sup>3</sup>, Charité–Universitätsmedizin Berlin, Department of Pediatric Oncology and Hematology, Berlin, Germany<sup>4</sup>, Trion Research GmbH, Trion Research GmbH, Martinsried, Trion Research GmbH<sup>5</sup>, Trion Research GmbH, Trion Research GmbH, Martinsried, Germany<sup>6</sup>, German Cancer Consortium (DKTK), Department of Pediatric Oncology and Hematology, Berlin, Germany<sup>7</sup>, Charité–Universitätsmedizin Berlin, Department of Pediatric Oncology and Hematology, Berlin, Germany<sup>8</sup>, Charité–Universitätsmedizin Berlin, Department of Pediatric Oncology and Hematology, Berlin, Germany<sup>9</sup>, Charité–Universitätsmedizin Berlin, Department of Pediatric Oncology and Hematology, Berlin, Germany<sup>10</sup>, HELIOS Klinikum Berlin Buch, Department of Pediatrics, Berlin, Germany<sup>11</sup>, Charité–Universitätsmedizin Berlin, Department of Pediatric Oncology and Hematology, Berlin, Germany, <sup>12</sup>, Charité–Universitätsmedizin Berlin, Department of Pediatric Oncology and Hematology, Berlin, Germany <sup>13</sup>

### **Background/Introduction:**

Despite advances in treating high-risk neuroblastoma that include combining the monoclonal anti-GD2 antibody ch14.18 with colony stimulating factor 2 (CSF2), interleukin 2 (IL2) or isotretinoin, 50-60% of patients still suffer relapse. Bispecific trifunctional antibodies (trAbs) are a promising innovation in the field of immunotherapies. They are heterodimeric IgG-like molecules that bind to T cells via CD3 and a tumor-associated antigen (TAA) as well as being able to activate antigen-presenting cells via their functional Fc region. A “Tri-cell complex” is created with the tumor cell, T cell and antigen-presenting cell, which induces a tumor-specific immune response. Combination therapy with checkpoint inhibitors, to prevent tumor cell immune escape, has been demonstrated to further improve trAb efficacy in a preclinical mouse model of melanoma.

### **Aims:**

We aimed to evaluate whether immune checkpoint signaling was activated by treatment with the GD2-directed trAb SUREK in neuroblastoma preclinical models, and whether a vaccination effect can be induced in a syngeneic immunocompetent neuroblastoma mouse model by simultaneous administration of irradiated murine neuroblastoma cells and SUREK.

### **Methods/Materials:**

The bispecific trifunctional antibody SUREK binds to the GD2 disialoganglioside on neuroblastoma cells and murine Cd3. The anti-GD2 monoclonal antibody ch14.18 and the tumor-unspecific trifunctional antibody TRBs012 (alphaviurs E1 x anti-mouse-Cd3) served as controls. The murine neuroblastoma cell line NXS2 (stably transduced with a firefly luciferase) was co-cultured with bone marrow-derived dendritic cells and T cells before *in vitro* exposure to SUREK or control antibodies and/or checkpoint inhibitors. Cytotoxicity was assessed using a luciferase-based cytotoxicity assay. Cytokine release by T cells was detected via ELISA. T cell activation and expression of inhibitory receptors were assessed flow cytometrically. The immunocompetent syngeneic neuroblastoma mouse model of NXS2 cells in A/J mice was also used. We vaccinated mice intraperitoneally with  $1 \times 10^5$  irradiated NXS2 cells and treated the animals with an intraperitoneal injection of 5 µg SUREK or control antibodies 21 and 7 days before animals were challenged with  $1 \times 10^6$  viable NXS2 cells (subcutaneous injection). Tumor growth and survival were used to evaluate treatment efficacy.

### **Results:**

The trAb SUREK killed significantly more NXS2 cells than ch14.18 or TRBs012 *in vitro* and this cytotoxic efficacy was dependent on the presence of dendritic cells. Treatment with SUREK

upregulated proteins involved in immune checkpoint signaling, including the immune checkpoint receptors programmed cell death protein 1 (Pdc1) and lymphocyte activation gene 3 (Lag3) on T cells in co-culture with dendritic and NXS2 cells. Administration of irradiated tumor cells and treatment with SUREK prevented engraftment of subcutaneously injected tumor cells and significantly lengthened survival compared to the control groups, creating a vaccination effect.

#### Summary/Conclusions:

Here we provide preclinical evidence that immune checkpoint signaling is induced by treatment with the trAb SUREK and for a specific vaccination effect induced by SUREK in an immunocompetent syngeneic neuroblastoma mouse model. Our results support a rationale to test combination of the trAb SUREK with checkpoint inhibitors to further improve efficacy.

## **P253 Vaccination of high-risk neuroblastoma patients with anti-id antibody mimicking GD2 to maintain protective immunity after passive immunotherapy with anti-GD2 antibody ch14.18/CHO**

*Lode, H.N.<sup>2</sup>, Loibner, H.<sup>3</sup>, Siebert, N.<sup>4</sup>, Troschke-Meurer, S.<sup>5</sup>, Mutschlechner, O.<sup>6</sup>*

University Medicine Greifswald, Pediatric Hematology and Oncology, Greifswald, Germany <sup>2</sup>, AnYxis, Immuno-Oncology, Vienna, Austria<sup>3</sup>, University Medicine Greifswald, Pediatric Hematology and Oncology, Greifswald, Germany<sup>4</sup>, University Medicine Greifswald, Pediatric Hematology and Oncology, Greifswald, University Medicine Greifswald<sup>5</sup>, AnYxis, Immuno-Oncology, Vienna, Austria<sup>6</sup>

### Background/Introduction:

Disialoganglioside GD<sub>2</sub> is an established tumor associated antigen with abundant expression on neuroblastoma (NB) and restricted expression on normal cells. As it is a suitable target for immunotherapy, human/mouse chimeric anti-GD<sub>2</sub> antibodies ch14.18 (dinutuximab) and ch14.18/CHO (dinutuximab beta) were developed and are marketed in US and Europe, respectively, for passive immunotherapy of high-risk NB.

Active immunization based on GD<sub>2</sub> following antibody therapy may induce a long-lasting protective immunity to extend the therapeutic benefit of NB antibody immunotherapy. It has been shown recently that overall survival is improved in NB patients who generated an immune response to ch14.18/CHO, which indicates the induction of immunity via an internal idiotypic network cascade.

Anti-idiotypic antibody ganglidiomab to ch14.18 was raised in mice and shown to carry an internal image of GD<sub>2</sub> *in vitro* and in murine models. Here we describe results of a compassionate need program in NB patients vaccinated by intramuscular injection of alum adsorbed ganglidiomab, following ch14.18/CHO immunotherapy.

### Aims:

To evaluate tolerability, immune response and outcome of vaccination with ganglidiomab in a compassionate use program.

### Methods/Materials:

Between 2012 and 2014, six high-risk NB patients (2-8 y); 4 in CR, 2 with disease following multimodal therapies including stem cell transplant and ch14.18/CHO immunotherapy were repeatedly treated under compassionate need with 0,5mg alum adsorbed ganglidiomab by subcutaneous vaccination (5-10 vaccinations every 2 weeks). Blood for serum measurements was withdrawn at various timepoints. Antibody responses to ganglidiomab, to its idio type and to GD<sub>2</sub> by ELISA as well as complement lysis to a NB cell line were investigated. Last patient follow-up was in 2019.

### Results:

Vaccinations were well tolerated with mild transient local reactions only. In immune sera of all patients a marked induction of antibodies to ganglidiomab and to its variable regions was detected. In 3 of 6 patients also an induction of antibodies to GD<sub>2</sub> was found. Induction of cytolytic activity via complement of GD<sub>2</sub>+ LAN1 NB cells was seen in 2 patients. All six patients were alive without evidence of disease at their last follow up in 2019, i.e. approx. 5 years after start of vaccinations.

### Summary/Conclusions:

Repeated s.c. injections of alum-adsorbed ganglidiomab may evolve as additional well tolerated active immunotherapy modality after ch14.18 antibody treatment to maintain a long-lasting immunity for prevention of progression or re-occurrence of disease, thereby improving overall survival. A clinical trial program is warranted to explore this hypothesis.

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## **P254 Differential Expression of Macrophage-Associated Oncology Markers in Drug-Resistant Neuroblastoma Model.**

*Murphy, C.*<sup>2</sup>, *Soriano, A.*<sup>3</sup>, *Segura, M.F.*<sup>4</sup>, *Piskareva, O.*<sup>5</sup>

Royal College of Surgeons in Ireland, Department of Anatomy and Regenerative Medicine, Dublin, Ireland <sup>2</sup>, Vall d'Hebron Institut de Recerca, Laboratory of Translational Research in Childhood Cancer, Barcelona, Spain<sup>3</sup>, Vall d'Hebron Institut de Recerca, Laboratory of Translational Research in Childhood Cancer, Barcelona, Spain<sup>4</sup>, Royal College of Surgeons in Ireland, Department of Anatomy and Regenerative Medicine, Dublin, Royal College of Surgeons in Ireland<sup>5</sup>

### Background/Introduction:

Aggressive neuroblastoma often displays an immunosuppressive tumour microenvironment (TME) which promotes the polarisation of tumour-associated macrophages (TAMs) to the M2 functional phenotype. These M2 cells display pro-tumour effects by stimulation of tumour cell migration, invasion, intravasation and angiogenesis, as well as remodelling of the extracellular matrix and suppression of adaptive immune responses. Hence, it is critical to understand tumour interactions with these immune cells in the TME and how these interactions are influenced by the expression of several immunology markers.

### Aims:

This study aims to investigate how common variations in drug sensitivity may contribute to differential expression of immune regulatory markers and therefore immunosuppression in neuroblastoma.

### Methods/Materials:

Cisplatin-sensitive KellyLuc and cisplatin-resistant KellyCis83Luc cell lines were injected via the tail vein of mice to establish a metastatic xenograft disease. Metastases were resected after 40 days and were formalin-fixed, paraffin-embedded. H&E staining identified tumour cell enriched regions which were microdissected for RNA-seq analysis using the HTG EdgeSeq ImmunoOncology panel of 549 markers. Markers with significant expression variation between the cisplatin-sensitive and resistant xenografts were analysed via bioinformatics and literature review to assess involvement in macrophage function. These were validated via RT-qPCR analysis on the xenograft samples and on the 2D-grown neuroblastoma cell lines: Kelly, KellyCis83, SH-SY5Y, NB1691, SKNBE and CHP212.

### Results:

Cisplatin-resistant xenografts had an average of 11.4 metastases per mouse vs 6.3 for cisplatin-sensitive, almost a 2-fold increase. 29 of the immuno-oncology markers were differentially expressed in sensitive vs resistant xenografts. 4 markers from this group: Triggering Receptor Expressed on Myeloid Cells 1 (TREM-1), Chemokine Ligand 15 (CCL15), Macrophage Stimulating 1 Receptor (MST1R) and Suppressor of Cytokine Signalling 3 (SOCS3), were found to influence macrophage function in various cancers. SOCS3 was detected in all cell lines tested, with highest expression in CHP212 cells and lowest in Kelly, NB1691 and SKNBE cells, suggesting that these cell lines may create a more immunosuppressive TME if grafted into the murine model. SOCS3 and CCL15 had significantly lower expression in cisplatin-resistant xenografts when analysed via RT-qPCR analysis. Conversely, CCL15 was significantly upregulated in cisplatin-resistant xenografts when analysed via HTG EdgeSeq. TREM-1 and MST1R were not detected in any of the samples tested through RT-qPCR.

### Summary/Conclusions:

Understanding how tumour cells interact with the immune system in the TME is critical to developing new immunotherapies for the treatment of neuroblastoma. It is well known that traditional 2D cell culture is insufficient for assessing these interactions due to poor presentation of immune antigens. In this study, discrepancies were evident upon comparing samples from the xenograft model vs 2D-grown cell lines, highlighting the need to develop more reproducible 3D models for studying

immunosuppression in the neuroblastoma TME *in vitro*. SOCS3 and CCL15 have demonstrated potential as novel targets for neuroblastoma immunotherapy due to their differential expression and impact on macrophage-mediated immunosuppression.



## **P255 Single cell RNA sequencing reveals a pro-inflammatory tumor microenvironment in human neuroblastoma**

Verhoeven, B.M.<sup>2</sup>, Olsen, T.K.<sup>3</sup>, Mei, S.<sup>4</sup>, Gustafsson, K.U.<sup>5</sup>, Pepich, A.<sup>6</sup>, Otte, J.<sup>7</sup>, Mao, Y.<sup>8</sup>, Lundqvist, A.<sup>9</sup>, Kharchenko, P.V.<sup>10</sup>, Wickström, M.<sup>11</sup>, Kogner, P.<sup>12</sup>, Johnsen, J.I.<sup>13</sup>, Baryawno, N.<sup>14</sup>

Karolinska Institutet, Department of Women's and Children's Health, Stockholm, Sweden <sup>2</sup>, Karolinska Institutet, Department of Women's and Children's Health, Stockholm, Sweden<sup>3</sup>, Harvard Medical School, Department of Biomedical Informatics, Boston, USA<sup>4</sup>, Massachusetts General Hospital, Center for Regenerative Medicine, Boston, Massachusetts General Hospital<sup>5</sup>, Karolinska Institutet, Department of Women's and Children's Health, Stockholm, Sweden<sup>6</sup>, Karolinska Institutet, Department of Women's and Children's Health, Stockholm, Sweden<sup>7</sup>, Uppsala University, Immunology, Genetics and Pathology, Uppsala, Sweden<sup>8</sup>, Karolinska Institutet, Department of Oncology-Pathology, Stockholm, Sweden<sup>9</sup>, Harvard Medical School, Department of Biomedical Informatics, Boston, USA<sup>10</sup>, Karolinska Institutet, Department of Women's and Children's Health, Stockholm, Sweden<sup>11</sup>, Karolinska Institutet, Department of Women's and Children's Health, Stockholm, Sweden, <sup>12</sup>, Karolinska Institutet, Department of Women's and Children's Health, Stockholm, Sweden <sup>13</sup>, Karolinska Institutet, Department of Women's and Children's Health, Stockholm, Sweden <sup>14</sup>

### Background/Introduction:

Immunotherapy has been emerging as an important modality for metastatic melanoma and other cancers. Pediatric malignancies, however, are often not treatable with immunotherapy because they are known as 'cold tumors'. Cold tumors lack tumor antigens and infiltration of immune cells, meaning they are not targetable. For neuroblastoma, anti-GD2 immunotherapy has been approved and has increased the survival rate when combined with cytokines. However, still half of high-risk neuroblastoma patients are currently not cured. Certain neuroblastomas respond to anti-inflammatory treatment and recently subsets of high-risk neuroblastomas were reported to show an inflammatory phenotype with infiltrating inflammatory cells of prognostic significance. However, these cells remain to be further classified to establish novel therapeutic options. Therefore, we aimed to characterize the molecular landscape of immune cells in human neuroblastoma by single cell RNA sequencing analysis.

### Aims:

### Methods/Materials:

We used droplet based single-cell RNA-sequencing (10x Chromium Single-Cell 3' v2 protocol) and profiled 17 samples from 15 children. The cohort consisted of primary and recurrent neuroblastomas, untreated and treated, core needle biopsies and surgical specimen, low, intermediate and high-risk patients of different ages and genetic subsets. We used *in silico* analysis and various bioinformatic tools for cell annotations and cell functionality scoring curated from published literature.

### Results:

We profiled ~70,000 single cells from human neuroblastomas, used unbiased clustering and we identified 13 main cell types spanning tumor cells, stromal cells and immune cells. Our data suggest that neuroblastomas consist of high proportions of immune cells including T cells, myeloid cells, NK cells, B cells and mast cells. The highest proportion of immune cells were T cells followed by cells of myeloid origin. Through subclustering *in silico* analysis, we show that the myeloid cell cluster consisted of six subpopulations, from which two subclusters showed high monocyte and inflammatory scores and expressed inflammatory genes such as IL1B and TNF. Further, three subclusters showed high macrophage score with a distinct M2 signature when compared to monocyte subclusters.

The T and NK cells consisted of twelve distinct subpopulations, deconvoluting into two NK cell populations, a population of NKT cells, a population of innate lymphoid cells (ILCs), a population of regulatory T cells and three CD4+ populations including a naïve, an activated and a T helper 17

population of cells. We detected three distinct populations of CD8+ cytotoxic T lymphocytes (CTLs) showing a high cytotoxicity and exhaustion signature score.

Finally, we observed expression of the antigen presenting genes B2M and MHC class I in the *Mesenchymal* tumor cell subpopulation while the *Adrenergic* tumor subpopulation expressed very low levels. Strikingly, the Adrenergic tumor cells expressed NK cell inhibitory ligands suggesting a possible mechanism of NK cell killing evasion.

#### Summary/Conclusions:

In this study, we further analyzed a complex immune cell landscape in human neuroblastomas using single-cell RNA-sequencing. Our results show that the neuroblastoma tumor microenvironment consists of pro-inflammatory cells many of which appear to be fully functional, however, without exerting efficient tumor cell killing. Further studies are required to test functionality of the immune cells and implications for novel immunotherapy.

## **P256 Treatment-related toxicities during anti-GD2 immunotherapy in high-risk neuroblastoma patients in the Netherlands**

*Blom, A.J.<sup>2</sup>, Lurvink, R.C.M.<sup>3</sup>, Aleven, L.H.G.<sup>4</sup>, Mensink, M.O.<sup>5</sup>, Dierselhuis, M.P.<sup>6</sup>, Van Eijkelenburg, N.K.A.<sup>7</sup>, Kraal, K.C.J.M.<sup>8</sup>, Van Noesel, M.M.<sup>9</sup>, Van Grotel, M.<sup>10</sup>, Tytgat, G.A.M.<sup>11</sup>*

Princess Máxima Center for Pediatric Oncology, Research, Utrecht, Netherlands<sup>2</sup>, Princess Máxima Center for Pediatric Oncology, Research, Utrecht, Netherlands<sup>3</sup>, Princess Máxima Center for Pediatric Oncology, Clinical care, Utrecht, Netherlands<sup>4</sup>, Princess Máxima Center for Pediatric Oncology, Clinical care, Utrecht, Princess Máxima Center for Pediatric Oncology<sup>5</sup>, Princess Máxima Center for Pediatric Oncology, Clinical care, Utrecht, Netherlands<sup>6</sup>, Princess Máxima Center for Pediatric Oncology, Clinical care, Utrecht, Netherlands<sup>7</sup>, Princess Máxima Center for Pediatric Oncology, Clinical care, Utrecht, Netherlands<sup>8</sup>, Princess Máxima Center for Pediatric Oncology, Clinical care, Utrecht, Netherlands<sup>9</sup>, Princess Máxima Center for Pediatric Oncology, Clinical care, Utrecht, Netherlands<sup>10</sup>, Princess Máxima Center for Pediatric Oncology, Research, Utrecht, Netherlands<sup>11</sup>

### **Background/Introduction:**

The introduction of immunotherapy using a chimeric anti-GD2 monoclonal antibody (ch14.18, dinutuximab) together with immunostimulatory cytokines [interleukin-2 (IL2) and granulocyte-macrophage colony-stimulating factor (GM-CSF)] has significantly improved survival rates for high-risk neuroblastoma patients. However, this improvement in survival is accompanied by a substantial immunotherapy-related toxicity burden.

Since the approval of ch14.18 for the treatment of neuroblastoma in 2016, high-risk neuroblastoma patients received immunotherapy in accordance with the COG ANBL0032 protocol as post-consolidation therapy in the Netherlands.

### **Aims:**

The primary objective was to describe treatment-related toxicities during immunotherapy with ch14.18 antibody, cytokines IL2 and GM-CSF, and isotretinoin in high-risk neuroblastoma patients after completing standard induction and consolidation according to the DCOG (Dutch Childhood Oncology Group) NBL 2009 treatment protocol.

### **Methods/Materials:**

Descriptive retrospective review of medical records of patients with high-risk neuroblastoma treated with immunotherapy as post-consolidation therapy between August 2016 and October 2019 in a single-center in the Netherlands. Toxicities were recorded and graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE; version 3.0). Although a patient may have reported a given toxicity multiple times per course, only the highest grade of toxicity per patient per type per course is given. Besides toxicities, results of blood cultures and dose modifications of dinutuximab and IL2 were collected.

### **Results:**

Twenty-six patients (11 girls and 15 boys), with a median age at diagnosis of 3.5 years (range 4 months - 18 years) received immunotherapy. In two patients immunotherapy was discontinued after two courses; in one patient due to disease progression and in another patient due to unacceptable toxicities (catheter-related infection with ICU admission in combination with severe sensory and motor neuropathy).

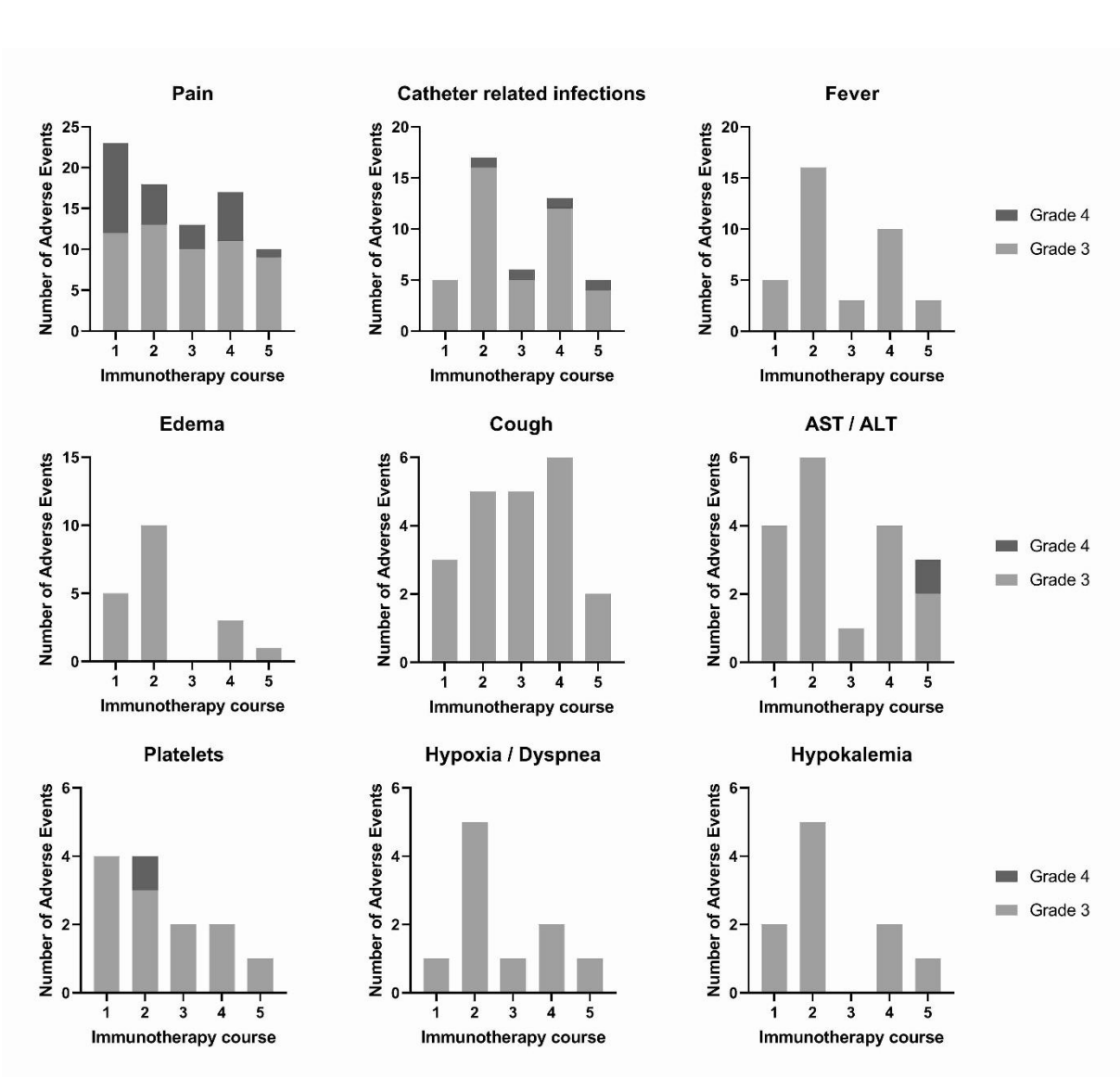
The most common grade  $\geq 3$  toxicities for courses 1-5, respectively, were pain (88, 69, 54, 70, 41%), catheter-related infections (19, 65, 25, 54, 21%), and fever (19, 62, 13, 42, 13%) (Figure 1). In total, 310 grade  $\geq 3$  toxicities were recorded in 124 courses of immunotherapy. Thirty-three grade 4 (11%) and no grade 5 toxicities (death) were seen. Grade  $\geq 3$  toxicities were not evenly distributed among the five courses, with most toxicities described in courses with IL2 [course 2 (36%) and course 4 (23%)].

The total dose of dinutuximab was not administered due to toxicity in 0, 27, 0, 21, 13% of patients in course 1-5, respectively.

All 26 patients experienced fever during immunotherapy; 81% suffered from fever >40.0°C (grade 3). Most fever episodes were recorded in the immunotherapy courses with IL2 (course 2 and 4). Catheter-related bloodstream infections were identified in 81% of patients. Four of these episodes were severe (grade 4) and led to ICU admission followed by full recovery.

Summary/Conclusions:

Immunotherapy-related toxicity after induction and consolidation according to the DCOG NBL 2009 treatment protocol is considerable but manageable.



Caption 1: The most common grade ≥ 3 toxicities of immunotherapy for courses 1-5.

## **P257 Comprehensive immune monitoring in patients with high-risk neuroblastoma during chemo- and immune therapy**

*Szanto, C.L.<sup>2</sup>, Tamminga, S.<sup>3</sup>, De koning, C.C.H.<sup>4</sup>, Van den Beemt, D.<sup>5</sup>, Tas, M.<sup>6</sup>, Kraal, K.<sup>7</sup>, Dierselhuis, M.<sup>8</sup>, Tytgat, L.<sup>9</sup>, Van Noesel, M.<sup>10</sup>, Boelens, J.J.<sup>11</sup>, Nierkens, S.<sup>12</sup>*

University Medical Center Utrecht, Center for Translational Immunology, Utrecht, Netherlands <sup>2</sup>, University Medical Center Utrecht, Center for Translational Immunology, Utrecht, Netherlands<sup>3</sup>, University Medical Center Utrecht, Center for Translational Immunology, Utrecht, Netherlands<sup>4</sup>, University Medical Center Utrecht, Center for Translational Immunology, Utrecht, University Medical Center Utrecht<sup>5</sup>, Prinses Máxima Centrum, Research, Utrecht, Netherlands<sup>6</sup>, Prinses Máxima Centrum, PMC Specialisten SO, Utrecht, Netherlands<sup>7</sup>, Prinses Máxima Centrum, PMC Specialisten SO, Utrecht, Netherlands<sup>8</sup>, Prinses Máxima Centrum, PMC Specialisten SO, Utrecht, Netherlands<sup>9</sup>, Prinses Máxima Centrum, PMC Specialisten SO, Utrecht, Netherlands<sup>10</sup>, Memorial Sloan Kettering Cancer Center, Department of Pediatrics, New York, USA<sup>11</sup>, University Medical Center Utrecht, Center for Translational Immunology, Utrecht, Netherlands, <sup>12</sup>

### Background/Introduction:

Neuroblastoma (NBL) is the most common extracranial solid tumor in childhood. Despite intense treatment, children with high-risk (HR) disease have a poor prognosis. Immunotherapy showed a significant improvement in event-free survival in high-risk neuroblastoma patients receiving chimeric anti-GD2 in combination with cytokines and isotretinoin after myeloablative consolidation therapy. However, response to immunotherapy varies widely, and often therapy is stopped due to severe toxicities. Nassin et al. (*bmt 2018*) showed that most patients with HR NBL do not have full immune reconstitution at the start of immunotherapy and that immune recovery may correlate with disease-related outcomes in patients with HR disease. Data on the presence and function of immune cells during therapeutic interventions after autologous hematopoietic cell transplantation (aHCT) is largely lacking.

### Aims:

The aim of the present study is to describe the dynamics of immune cell subsets and their function at diagnosis and during therapy in HR NBL patients.

### Methods/Materials:

We performed in-depth immune monitoring in peripheral blood of 25 HR NBL patients at diagnosis, during induction chemotherapy, before high dose conditioning and during immunotherapy. Immune subsets (leukocytes, lymphocyte, monocyte, neutrophil, eosinophil and lymphocyte, including subsets within B-cells, NK-cells and T-cells) were measured in fresh peripheral blood samples using multi-color flow cytometry. To assess proliferation of T-cells, PBMCs were labelled with CTV and cultured for 3 days in presence of different stimuli (anti-CD3, PHA) and analyzed using flow cytometry. Suppression of Tregs, was measured similarly in co-cultures of isolated CD4+CD25highCD127low Tregs and PBMC from patients and healthy controls. Supernatants were measured using multiplex immunoassays (IL-2, sIL-2, IL-10, IL-13, IL-17, INF-g, TNFa).

### Results:

The dynamics of immune cells during multi-agent chemotherapy varied largely between patients after chemotherapy. Large variation between cell counts, between patients and between treatment cycles was observed. During immune therapy, we found that IL-2 and GM-CSF induced expansion of suppressive Treg and increased circulating eosinophils. In addition, GM-CSF increased neutrophil, lymphocyte and monocyte counts, but had no effect on CD3+ T cell, NK cell or CD19+ B cell populations. IL-2 administration resulted in increase of both CD16+ and CD56+ NK cells, and had no effect on CD3+ T cell numbers, CD19+ B cell and monocyte counts. Patient Treg were functional albeit a slightly reduced ability to suppress T cell proliferation when compared to Treg from healthy

controls. In contrast, we found that patient T-cell proliferation was significantly impaired at diagnosis and after aHCT. This was confirmed by impaired cytokine profiles measured *in vitro*.

#### Summary/Conclusions:

Our findings show that the status of the immune system is highly variable between patients post-aHCT. Nevertheless, immune therapy protocols do not take the immune status into account for dose calculation or timing of treatment. Moreover, we show that treating patients with immune therapy does not restore the corrupted effector T cell function, but instead induces an expansion of functional Treg. These new insights are valuable for the design of combination strategies and new therapeutic approaches.

## **P258 Combining immunomodulating chemotherapy with checkpoint blockade antibody provides effective therapy in the spontaneous TH-MYC-N transgenic neuroblastoma model**

*Gray, J.C.<sup>2</sup>, Webb, E.<sup>3</sup>, Moreno-Vincente, J.<sup>4</sup>, Cragg, M.S.<sup>5</sup>, Beers, S.A.<sup>6</sup>*

University of Southampton, Paediatric Oncology, Southampton, United Kingdom <sup>2</sup>, University of Southampton, Centre for Cancer Immunology, Southampton, United Kingdom<sup>3</sup>, University of Southampton, Centre for Cancer Immunology, Southampton, United Kingdom<sup>4</sup>, University of Southampton, Centre for Cancer Immunology, Southampton, University of Southampton<sup>5</sup>, University of Southampton, Centre for Cancer Immunology, Southampton, United Kingdom<sup>6</sup>

### Background/Introduction:

Neuroblastoma typically has a low mutational burden, with few neoantigens and an immunosuppressive tumour microenvironment. Not surprisingly, immune checkpoint blockade antibodies (e.g. anti-PD-1), which have achieved remarkable success in adult malignancies, have not shown clinical activity as single agents in neuroblastoma, or indeed many paediatric tumours. However, tumour specific T cells are found in patients with neuroblastoma and, where present, these correlate with favourable outcome. Although chemotherapy is generally considered immunosuppressive, there is increasing recognition that many chemotherapy agents may have favourable effects on the anti-tumour immune response, such as deletion of regulatory T cell and induction of immunogenic cell death.

### Aims:

Here we aimed to investigate the immunomodulating effects of cyclophosphamide and doxorubicin, and explore if these chemotherapy agents can be combined with checkpoint blockade antibodies to provide effective therapy in preclinical models of neuroblastoma .

### Methods/Materials:

The ability of cyclophosphamide and doxorubicin to induce expression of recognised markers of immunogenic cell death in a range of murine neuroblastoma cell lines was assessed by flow cytometry and immunohistochemistry. Syngeneic and spontaneous murine neuroblastoma models were used to examine the effects of different doses of the chemotherapy agents on tumour infiltrating lymphocyte populations, and also to investigate the therapeutic effects of combining chemotherapy with the immune checkpoint blockade (anti-PD-1 monoclonal antibody). An apoptosis resistant neuroblastoma cell line and mouse strain (vav-BCL-2) were used to investigate the mechanism of action of therapeutic synergy between these agents.

### Results:

*In vitro* exposure of neuroblastoma cell lines to either cyclophosphamide or doxorubicin was found to result in induction of ecto-CRT, Hsp-70 and HMGB1, suggesting induction of immunogenic cell death. *In vivo*, low dose cyclophosphamide was found to selectively deplete and reduce the suppressive activity of tumour infiltrating regulatory T cell (Treg) in both NXS2 and 9464D neuroblastoma models, whilst maintaining and activating CD8+ and CD4+ T cells. The combination of low dose cyclophosphamide with anti-PD-1 was demonstrated to improve survival, as compared to either agent alone, in 3 different immunocompetent neuroblastoma models, including the TH-MYC-N transgenic spontaneous tumour model. A metronomic, weekly dosing strategy was found to achieve long term survival in the spontaneous model. The therapeutic effects were lost when wild type neuroblastoma cell lines were inoculated into apoptosis resistant vav-BCL-2 mice, suggesting that host mechanisms (e.g. Treg depletion) are essential in addition to the direct cytotoxic effects of the chemotherapy on tumour cells.

### Summary/Conclusions:

Low dose cyclophosphamide results in depletion of intratumoral Treg cells and synergises with anti-PD-1 antibody to achieve effective long term therapy in the TH-MYC-N spontaneous tumour model.

This combinational therapy warrants further investigation and is potentially easily translatable into clinical trial.



## **P259 IL-15-expanded V $\delta$ 1+ T cells demonstrate prolonged proliferation and anti-tumour activity as a potential platform for immune therapy against neuroblastoma.**

*Ferry, G.<sup>2</sup>, Barisa, M.<sup>3</sup>, Anderson, J.<sup>4</sup>*

UCL Institute of Child Health, Developmental Biology and Cancer - Cancer Section, London, United Kingdom <sup>2</sup>, TC Biopharm, Product Development, Glasgow, United Kingdom<sup>3</sup>, UCL Institute of Child Health, Developmental Biology and Cancer, London, United Kingdom<sup>4</sup>

### Background/Introduction

Chimeric antigen receptor (CAR)-based immunotherapies developed in  $\alpha\beta$  T cells have shown wide success against a number of haematological malignancies. However, their efficacy against neuroblastoma (NB) has been limited; specifically, they have been hindered by on-target, off-tumour toxicity as well as CAR T cell exhaustion and persistence within the NB tumour microenvironment. V $\delta$ 1+ cells, a subset  $\gamma\delta$  T lymphocytes, present a promising cellular platform by which to obviate these drawbacks, as they have distinctive cellular properties that suggest they can protect non-tumour cells from lysis and resist exhaustion in a tissue context. Unfortunately, V $\delta$ 1+ cell expansion and differentiation are not well understood.  $\gamma\delta$  T cells only comprise 1 – 5% of circulating T cells, with 10 – 30% of that fraction being V $\delta$ 1+ cells that have previously been difficult to expand.

### Aims

To study these cells as a potential cell for therapeutic use, we aimed to optimise an V $\delta$ 1+ expansion protocol that included TCR stimulation with anti-CD3 [OKT-3] and IL-15 supplementation to produce cytotoxic V $\delta$ 1+ cells.

### Methods/Materials

We conducted studies with flow cytometric analysis to examine fold change in V $\delta$ 1+ cell number and shifts in their phenotypic marker expression, as well as their proliferation in response to tumour targets. We also investigated expanded V $\delta$ 1+ cell cytokine production using immunosorbent assays.

### Results

Here, we show that our expansion technique is sufficient to produce a large number of V $\delta$ 1+ cells that can proliferate for up to 28 days following stimulation. While these cells express phenotypic markers found in naïve and central memory T cells, they also delay expression of exhaustion markers. Finally, these expanded V $\delta$ 1+ cells demonstrate antitumour activity against NB-specific cell lines through surface marker expression and cytokine release when co-cultured in vitro. [data in progress]

### Summary/Conclusions

Thus, we propose that V $\delta$ 1+ cells can be expanded ex vivo for immunotherapeutic development targeting neuroblastoma.

## **P260 Glycoprotein GD2 presentation on the surface of neuroblastoma cells grown on 3D bio-engineered scaffolds**

Gallagher, C.<sup>2</sup>, Murphy, C.<sup>3</sup>, Rashinkar, S.<sup>4</sup>, O'Brien, F.<sup>5</sup>, Piskareva, O.<sup>6</sup>

RCSI, Pharmacy and Biomolecular Sciences, Dublin, Ireland <sup>2</sup>, RCSI, Pharmacy and Biomolecular Sciences, Dublin, Ireland<sup>3</sup>, RCSI, Pharmacy and Biomolecular Sciences, Dublin, Ireland<sup>4</sup>, RCSI, Tissue Engineering Research Group, Dublin, RCSI<sup>5</sup>, RCSI, Department of Anatomy and Regenerative Medicine, Dublin, Ireland<sup>6</sup>

### Background/Introduction:

Neuroblastoma is an aggressive paediatric cancer of the sympathetic nervous system. Current therapies are not effective in the long term for almost 80% of patients with the clinically aggressive disease (Olga Piskareva and Raymond L. Stallings, 2015). GD2 is a glycoprotein that is abundantly expressed on NB cells. Due to its high expression on NB cells and low expression on other cells of the body it is a useful target for immunotherapy in NB patients. Immunotherapy using anti-GD2 combination therapy has been implemented as a treatment for minimal residual of disease. Yet 1/3 of individuals have a poor outcome due to relapse (Yu et al., 2010). A major hurdle in the drug development pipeline for cancers is the discrepancy between *in vitro* and *in vivo* testing results, leading to only 1 in 10 drugs entering clinical trials and approval by the FDA. Recently, we have characterised the growth of numerous NB cell lines on differing collagen scaffolds with the ability proliferate, infiltrate, deposit extracellular matrix and respond to stimuli similar to *in vivo* biological systems (Curtin et al., 2018).

### Aims:

The aim of this study was to characterize the growth pattern of four well characterized neuroblastoma cell lines on collagen-nanohydroxyapatite (coll-nHA) scaffolds and subsequently evaluate GD2 presentation on the surface of four neuroblastoma cell lines grown in scaffolds using immunohistochemistry.

### Methods/Materials:

Multiple NB cells were characterised for DNA content using the pico green dsDNA assay when grown on coll-nHA scaffolds. Cell growth and distribution on the scaffolds was examined with H&E. Immunohistochemical analysis was performed for the surface marker GD2.

### Results:

Upon analysis of cell numbers per scaffold using the Pico green Assay, results showed cell growth is cell line dependant. This may possibly be in relation to cell line doubling time and invasive nature of each cell line in the scaffolds. Cellular growth peaked at 14 or 21 days before decreasing. This may be due to inability of the cells to infiltrate the scaffold correctly and nutrient availability becoming depleted. We have now optimised immunodetection of GD2 both in 2D and 3D *in vitro*. GD2 is uniformly expressed across all NB cell lines grown in 2D. This data is in agreement with previous studies. At the next step, we investigated GD2 expression in 3D *in vitro* scaffolds to assess the cellular distribution. Results showed differing growth patterns for cell lines, ranging from tight colony clusters to dispersed cell growth.

### Summary/Conclusions:

To conclude, 3D bio-engineered models have great potential to better act as a new platform for testing antibody based immunotherapies that more accurately reflect NB and the ECM. Future studies will assess the ability of the GD2 antibody to induce immune cell-mediated killing in a co-culture of immune cells and NB cells on these new emerging 3D scaffolds.



## **P261 Anti-GD2 antibody internalization by neuroblastoma as a mechanism of resistance to immunotherapy**

*Tibbetts, R.*<sup>2</sup>, *Yeo, K.*<sup>3</sup>, *Muthugounder, S.*<sup>4</sup>, *Asgharzadeh, S.*<sup>5</sup>

Children's Hospital Los Angeles / University of Southern California, Pediatric Hematology-Oncology, Los Angeles, USA <sup>2</sup>, Dana-Farber / Boston Children's Hospital, Pediatric Hematology-Oncology, Boston, USA<sup>3</sup>, Children's Hospital Los Angeles, Pediatric Hematology-Oncology, Los Angeles, USA<sup>4</sup>, Children's Hospital Los Angeles / University of Southern California, Pediatric Hematology-Oncology, Los Angeles, Children's Hospital Los Angeles / University of Southern California<sup>5</sup>

### Background/Introduction:

Neuroblastoma, a sympathetic nervous system cancer, accounts for a disproportionate number of deaths among childhood malignancies despite intensive multimodal therapy including the relatively recent introduction of antibody targeting disialoganglioside (GD2), a common neuroblastoma antigen. The mechanisms underlying therapeutic resistance in some patients, specifically to antibody-based therapy, remains poorly understood.

### Aims:

We hypothesized that differential internalization of anti-GD2 antibody by tumor cells upon binding to surface GD2 may act as a mechanism of escape by decreasing tumor sensitivity to antibody-dependent cellular cytotoxicity (ADCC). We aimed to measure antibody internalization differences across 22 human neuroblastoma cell lines and investigate how it relates to GD2 expression and ADCC sensitivity.

### Methods/Materials:

Antibody internalization was measured using IncucyteS3 live cell imaging and anti-GD2 antibody (14G2a) conjugated to a pH-sensitive red fluorescent dye (pHrodo). GD2 surface expression was quantified by flow cytometry and BD QuantiBrite Beads. Neutrophil-mediated ADCC was measured *in vitro* via one of two methods - DIMSCAN and calcein-labeled tumor cells or IncucyteS3 and GFP-expressing tumor cells. Agents targeting endocytosis (chlorpromazine, cytochalasin-D, methyl-beta-cyclodextrin/M  $\beta$ CD, Pitstop2, and EIPA) were then used to assess inhibition of internalization and subsequently sensitization to ADCC in the highest internalizing cell line, LAN1.

### Results:

Internalization of 14G2a-pHrodo over 24 hours varied significantly across cell lines (AUC range: 50-529) with LAN1 demonstrating the highest internalization AUC (529 +/- 40). Internalization rate was not correlated with GD2 surface expression ( $r=0.15$ ). However, a moderate inverse correlation was found between antibody internalization and sensitivity to neutrophil-mediated ADCC in the 10 cell lines tested ( $r=-0.57$ ). Antibody internalization by LAN1 was significantly inhibited by treatment with EIPA, chlorpromazine, and M $\beta$ CD, all without significant change in surface GD2 expression. Treatment with M  $\beta$ CD (2mM), additionally, was able to sensitize LAN1 to neutrophil-mediated ADCC (52% cytotoxicity) compared to untreated LAN1 (17% cytotoxicity).

### Summary/Conclusions:

Anti-GD2 antibody internalization varies widely across human neuroblastoma cell lines, is inversely correlated with ADCC sensitivity, and, in a high internalizing cell line, can be effectively modulated by inhibitors of pinocytosis. The finding that membrane cholesterol depletion with M  $\beta$ CD sensitized LAN1 to neutrophil-ADCC suggests that membrane organization of GD2, specifically in relation to cholesterol-rich domains, may play a more significant role in antibody internalization than specific endocytosis processes. Elucidating further the mechanisms underlying differential internalization across cell lines could lead to the development of agents targeting internalization and ultimately augmenting tumor sensitivity to antibody-based immunotherapy.



## **P262 Harnessing copper as a novel immune checkpoint therapy for neuroblastoma**

*Vittorio, O.*<sup>2</sup>, *Voli, F.*<sup>3</sup>, *Valli, E.*<sup>4</sup>, *Rouaen, J.*<sup>5</sup>, *Lerra, L.*<sup>6</sup>, *Kimpton, K.*<sup>7</sup>, *Saletta, F.*<sup>8</sup>, *Giorgi, F.M.*<sup>9</sup>, *Mercatelli, D.*<sup>10</sup>, *Shen, S.*<sup>11</sup>, *Murray, J.*<sup>12</sup>, *Cirillo, G.*<sup>13</sup>, *Mayoh, C.*<sup>14</sup>, *Beavis, P.A.*<sup>15</sup>, *Haber, M.*<sup>16</sup>, *Trapani, J.A.*<sup>17</sup>, *Kavallaris, M.*<sup>1</sup>

University of NSW / Children's Cancer Institute, School of Women's and Children's Health UNSW, Sydney, Australia <sup>2</sup>, University of NSW / Children's Cancer Institute, School of Women's and Children's Health UNSW, Sydney, Australia<sup>3</sup>, University of NSW / Children's Cancer Institute, School of Women's and Children's Health UNSW, Sydney, Australia<sup>4</sup>, University of NSW / Children's Cancer Institute, School of Women's and Children's Health, UNSW, Sydney, University of NSW / Children's Cancer Institute<sup>5</sup>, University of NSW / Children's Cancer Institute, School of Women's and Children's Health, UNSW, Sydney, Australia<sup>6</sup>, University of NSW / Children's Cancer Institute, School of Women's and Children's Health, UNSW, Sydney, Australia<sup>7</sup>, Children's Cancer Research Unit, Kids Research Institute, The Children's Hospital, Children's Cancer Research Unit, Westmead, Australia<sup>8</sup>, Department of Pharmacy and Biotechnology, University of Bologna, Department of Pharmacy and Biotechnology, Bologna, Italy<sup>9</sup>, Department of Pharmacy and Biotechnology, University of Bologna, Department of Pharmacy and Biotechnology, Bologna, Italy<sup>10</sup>, University of NSW / Children's Cancer Institute, School of Women's and Children's Health, UNSW, Sydney, Australia<sup>11</sup>, University of NSW / Children's Cancer Institute, School of Women's and Children's Health, UNSW, Sydney, Australia, <sup>12</sup>, University of Calabria, Department of Pharmacy, Health and Nutritional Sciences, Rende, Italy <sup>13</sup>, University of NSW / Children's Cancer Institute, School of Women's and Children's Health, UNSW, Sydney, Australia <sup>14</sup>, Peter MacCallum Cancer Centre, Rosie Lew Cancer Immunology Program, Melbourne, Australia <sup>15</sup>, University of NSW / Children's Cancer Institute, School of Women's and Children's Health, UNSW, Sydney, Australia <sup>16</sup>, Peter MacCallum Cancer Centre, Rosie Lew Cancer Immunology Program, Melbourne, Australia <sup>17</sup>, University of NSW / Children's Cancer Institute, School of Women's and Children's Health, UNSW, Sydney, Australia <sup>18</sup>

### **Background/Introduction:**

Clinical evidence shows that the immune system is unable to guarantee long-lasting control of neuroblastoma (NB), suggesting the existence of mechanisms allowing tumor evasion of host immunity. Cancer immune evasion is recognised as a central hallmark of tumor development. One mechanism that cancer cells use to protect themselves from attack by immune cells is the over-expression of the Programmed Death Ligand 1 (PD-L1). The immune checkpoint protein Programmed Death receptor 1 (PD-1) expressed by lymphocytes instructs T-cells not to attack any cell expressing PD-L1, including tumor cells. Immune checkpoint inhibitors acting through the PD-L1/PD-1 axis have shown efficacy in some chemotherapy-resistant adult cancers, generating interest that these agents may also be helpful to treat certain refractory paediatric malignancies such as NB. However, the factors regulating PD-L1 expression in NB cells are poorly understood and acquired resistance to checkpoint inhibitors is also emerging to limit clinical benefit. Understanding the regulation of PD-L1 in NB is of utmost importance to optimize novel strategies and improve cancer immunotherapies.

### **Aims:**

Our recent preliminary research has identified elevated levels of copper (Cu) in NB tumors compared to normal tissues, with Cu levels strongly influencing the expression of PD-L1 in primary NB. The aim of this study is to evaluate whether copper chelation therapy can inhibit PDL-1 expression in NB and enhance immune responses, reducing tumor growth and increasing survival in vivo.

### **Methods/Materials:**

Experiments were performed using primary NB tissue and three NB cell lines: SK-N-FI, SH-SY5Y, and SK-N-BE(2)C. Protein expression of PD-L1 and copper transporter 1 (CTR1) was determined via western blot. Intracellular copper was measured by Inductively coupled plasma mass spectrometry (ICP-MS). Flow cytometry was used to analyse tumor-infiltrating lymphocytes and natural killer (NK) cells. An RNAseq analysis elucidated the mechanism of copper chelating agents on PD-L1

expression. In vivo activity of these agents was assessed in a syngeneic NB (TH-MYNC) mouse model.

#### Results:

Tissue microarrays from neuroblastoma patient tumors revealed a significant correlation between CTR1 and PD-L1 expression ( $p=0.00014$ ). Media supplementation with copper enhanced PD-L1 expression at both the mRNA and protein levels in NB cell lines. mRNA profiling indicated both copper and IFN $\gamma$  treatment produced similar effects on several key pathways involved in PD-L1 expression and tumor immune evasion. Gene set enrichment analysis (GSEA) from the top 1000 dysregulated pathways, revealed 326 upregulated and 350 downregulated pathways through copper or IFN $\gamma$  stimulation in NB cells. Among these, we identified four signalling cascades involved in tumor immunity: response to cytokines; regulation of cytokine production; IL6/JAK/STAT3 signalling; and TNF- $\alpha$  signalling via NF- $\kappa$ B. These cascades are all key regulators of PD-L1 expression. Conversely, copper chelators were observed to inhibit STAT3 and EGFR phosphorylation and promote ubiquitin-mediated PD-L1 degradation. Furthermore, use of copper chelators in vivo significantly increased the number of tumour-infiltrating CD8 $^+$  T and NK cells, slowed tumor growth and improved survival of TH-MYCN mice.

#### Summary/Conclusions:

This study provides the first evidence of the crucial role of copper in modulating PD-L1 expression and its contribution to tumor immune evasion. Moreover, it raises the potential of repurposing clinically available copper chelating agents to enhance anti-tumor immunity. This novel immunotherapy could target NB and a wide spectrum of other copper-dependent cancers.

## **P263 Risk factor evaluation in high-risk neuroblastoma patients receiving dinutuximab beta (DB) based immunotherapy: A report from the HR-NBL-1/SIOPEN Trial**

*Ladenstein, R.<sup>2</sup>, Poetschger, U.<sup>3</sup>, Valteau-Couanet, D.<sup>4</sup>, Gray, J.<sup>5</sup>, Luksch, R.<sup>6</sup>, Balwierz, W.<sup>7</sup>, Castel, V.<sup>8</sup>, Ash, S.<sup>9</sup>, Beck-Popovic, M.<sup>10</sup>, Laureys, G.<sup>11</sup>, Chi Fung Chan, G.<sup>12</sup>, Ruud, E.<sup>13</sup>, Vetterranta, K.<sup>14</sup>, Owens, C.<sup>15</sup>, Schroeder, H.<sup>16</sup>, Ambros, P.<sup>17</sup>, Sarnacki, S.<sup>18</sup>, Boterberg, T.<sup>19</sup>, Lode, H.<sup>20</sup>*

St. Anna Children's Hospital and Children's Cancer Research Institute (CCRI), Department of Paediatrics, Medical University, Vienna, Austria, Vienna, Austria <sup>2</sup>, Children's Cancer Research Institute (CCRI), Department of Paediatrics, Medical University, Vienna, Austria<sup>3</sup>, Gustave Roussy, Children and Adolescent Oncology Department, Villejuif, France<sup>4</sup>, University Hospital Southampton NHS Foundation Trust and University of Southampt, Paediatric Oncology and Haematology, Southampton, University Hospital Southampton NHS Foundation Trust and University of Southampt<sup>5</sup>, Fondazione IRCCS Istituto Nazionale dei Tumori, Paediatric Oncology and Haematology, Milan, Italy<sup>6</sup>, Jagiellonian University Medical College, Paediatric Haematology Oncology, Krakow, Poland<sup>7</sup>, Hospital Universitario y Politecnico La Fe, Paediatric Oncology and Haematology, Valencia, Spain<sup>8</sup>, Schneider Children's Medical Center of Israel, Sackler Faculty of Medicine Tel Aviv University, Petach Tikvah, Israel<sup>9</sup>, University Hospital Lausanne, Paediatric Oncology and Haematology, Lausanne, Switzerland<sup>10</sup>, Ghent University Hospital, Paediatric Oncology and Haematology, Ghent, Belgium<sup>11</sup>, Queen Mary Hospital, The University of Hong Kong, Department of Pediatrics & Adolescent Medicine, Hong Kong, Hong kong, <sup>12</sup>, Rikshospitalet, Paediatric Oncology and Haematology, Oslo, Norway <sup>13</sup>, University of Tampere and Hospital for Children and Adolescents Univ. of Helsinki, Div. of Hematology-Oncology and Stem Cell Transplantation, Helsinki, Finland <sup>14</sup>, Our Lady's Children's Hospital, Pediatric Haematology/Oncology, Crumlin, Dublin, Ireland <sup>15</sup>, University Hospital of Aarhus, Paediatric Oncology and Haematology, Aarhus, Denmark <sup>16</sup>, Children's Cancer Research Institute (CCRI), Department of Paediatrics, Medical University, Vienna, Austria <sup>17</sup>, Necker Enfants – Malades Hospital, Paris Descartes University, Department of Pediatric Surgery, Paris, France <sup>18</sup>, Ghent University Hospital, Paediatric Oncology and Haematology, Ghent, Belgium <sup>19</sup>, University Medicine Greifswald, Pediatric Hematology and Oncology, Greifswald, Germany <sup>20</sup>

### Background/Introduction:

Prognostic factors at diagnosis were previously incorporated into a multi-variable pseudo-value regression model in HR-NBL1/SIOPEN patients treated without DB to develop a simple score including age, serum lactate dehydrogenase (LDH) and metastatic site index (MSI). Points were allocated in proportion to the log-cumulative hazard ratio (cHR): cHR for LDH >1250U/L was 1.33 (score of 1), cHR for age >5 years and metastatic site involvement (MSI) >1 were 1.79 and 1.86 (scores of 2).

### Aims:

We tested if this score (Morgenstern, PBC 2018) would provide meaningful information in DB treated HR-NBL1/SIOPEN patients (validation cohort).

### Methods/Materials:

High-risk patients (stage 4 ≥1yr.; stage 4 <1yr. with MYCN amplification (MNA); stage 2, 3, 0-21y with MNA) received intensive induction (rapid COJEC or N5-MSKC and TVD for insufficient response), surgery, high dose therapy with BUMEL or CEM and local radiotherapy (21Gy). Patients ≤9 months intervall till HDT/SCT with ≥ partial response prior and no progression thereafter received up to 5 cycles of 100mg/m<sup>2</sup> DB: 2006 to 2013 as short-term (STI: d 8-12) or 2014 to 2018 as long-term infusion (LTI: d8-17) ± 6 (STI) or 3 (LTI) x 10<sup>6</sup> IU/m<sup>2</sup> subcutaneous interleukin 2 (sclL2 over 2 x 5 days) followed by 160mg/m<sup>2</sup> oral isotretinoin (d19-32).

### Results:

1018 patients [512 males; 749 Stage 4(89%), 13 Stage 4s(1%), 86 loc. MNA(10%); median



age at diagnosis 2.8 yrs. (1 day – 20 yrs.)) received DB in 18 countries participating. Their 2-yrs. event-free (EFS)/overall survival (OS) was  $0.65\pm 0.02/0.78\pm 0.01$  and the 5-yrs. EFS/OS of  $0.56\pm 0.02/0.63\pm 0.02$ . 891/1018 patients were evaluable for risk factor and score analysis. STI was used in 541(61%) patients, LTI in 350(39%). DB without sclL2 was given to 550(62%) patients and with sclL2 to 341(38%). There was no difference in EFS/OS neither regarding infusion schedule (5-yrs. EFS of  $0.58\pm 0.02$  for STI and  $0.64\pm 0.03$  for LTI) nor for the addition of sclL2 any schedule (5-yrs. pEFS of  $0.57\pm 0.02$  without and  $0.61\pm 0.03$  with sclL2). Patients in complete remission prior DB treatment had better outcomes than those with residual disease (in CR: 5-yrs. EFS/OS of  $0.60\pm 0.03/0.68\pm 0.03$ ; for non-CR: 5-yrs. EFS/OS of  $0.54\pm 0.03/0.57\pm 0.03$ ). Younger patients fared better than older ones (5-yrs. EFS: <1.5-yrs:  $0.74\pm 0.06$ , 1.5-yrs. to 5-yrs:  $0.58\pm 0.02$  and >5-yrs.  $0.48\pm 0.04$ ) as did localised ones or those with only 1 MSI (5-yrs. pEFS:  $0.77\pm 0.05$ ,  $0.78\pm 0.05$ ,  $0.54\pm 0.02$  >1 MSI). EFS multivariate analysis included age, MSI, LDH  $\geq 1250$ U/L, HDT, response prior DB, DB schedule and use of sclL2: only age >1.5yrs.( $p=0.002$ ) and MSI >1 ( $p=0.002$ ) had independent prognostic prediction. If restricted to stage 4 only, LDH  $\geq 1250$ U/L was an additional independent factor ( $p=0.049$ ). Using the score (0 to 5) relevant risk groups can be identified: 5-yrs. EFS/OS for scores 0&1 are  $0.80\pm 0.06$ & $0.77\pm 0.04$ , score 2/3 are  $0.58\pm 0.03$ & $0.57\pm 0.04$  and for scores 4/5 are  $0.50\pm 0.06$ & $0.43\pm 0.08$ . Similar outcome proportions apply for OS as well as for EFS/OS for stage 4 only.

#### Summary/Conclusions:

The score adds value in DB treatment groups as it differentiates prognostic subgroups facilitating decision making for future DB add on treatments.

## **P264 Tackling MHC class I downregulation to develop effective immunotherapy for neuroblastoma**

Cornel, A.M.<sup>2</sup>, Van den Beemt, D.A.M.<sup>3</sup>, Dunnebach, E.D.<sup>4</sup>, Essing, A.H.W.<sup>5</sup>, Til, N.P.<sup>6</sup>, Molenaar, J.J.<sup>7</sup>, Nierkens, S.<sup>8</sup>

UMC Utrecht, Center for Translational Immunology, Utrecht, Netherlands <sup>2</sup>, UMC Utrecht, Center for Translational Immunology, Utrecht, Netherlands<sup>3</sup>, UMC Utrecht, Center for Translational Immunology, Utrecht, Netherlands<sup>4</sup>, Prinses Máxima Centrum, Research, Utrecht, Prinses Máxima Centrum<sup>5</sup>, UMC Utrecht, Center for Translational Immunology, Utrecht, Netherlands<sup>6</sup>, Prinses Máxima Centrum, Research, Utrecht, Netherlands<sup>7</sup>, Prinses Máxima Centrum, Research, Utrecht, Netherlands<sup>8</sup>

### Background/Introduction:

Neuroblastoma is the most common pediatric solid tumor and responsible for about 15% of all pediatric cancer deaths. The majority of high-risk patients suffers from relapse after intense therapy regimens, resulting in a 5-year survival rate of only 40%. Even though the potential of immune interference in neuroblastoma is shown by the additive effect of anti-GD2 monoclonal antibody therapy to the treatment protocol, long-term follow-up studies reveal that the beneficial effect of immunotherapy diminishes over time. We hypothesize that this is a result of inadequate adaptive immune engagement caused by the extensive immunomodulatory capacity of neuroblastoma and its microenvironment. One of the most remarkable immunomodulatory strategies of neuroblastoma tumors is the downregulation of MHC-I surface expression, thereby preventing cytotoxic T cell recognition and killing. MHC-I lacking cells are known to be subjected to NK-cell mediated cytotoxicity, however, we show that neuroblastoma is able to evade this by temporary upregulating surface expression of MHC-I, thereby becoming more prone to T-cell mediated cytotoxicity. Addition of MHC-I upregulatory cytokines to neuroblastoma cells results in improved MHC-I expression and T-cell cytotoxicity, however, the drawback of toxicity diminishes the potential of these cytokines in therapeutic strategies. Therefore, we are aiming to identify FDA-approved compounds with similar effects on MHC-I expression. Pharmaceutical upregulation of MHC-I expression could foster adaptive anti-tumor immunity in neuroblastoma patients.

### Aims:

To screen FDA-approved drug libraries to identify MHC-I upregulatory compounds to increase neuroblastoma-specific T-cell recognition and killing.

### Methods/Materials:

We have developed a high-throughput flow cytometry antibody staining protocol for adherent cells which we have applied to screen FDA-approved drug libraries to identify compounds affecting MHC-I surface expression in neuroblastoma cell lines. Libraries have been screened at a concentration range between 0.1 nM and 20  $\mu$ M. Observed effects were validated, after which neuroblastoma cell lines have been co-cultured with PRAME reactive tumor-specific T-cells in presence/absence of identified compounds to determine the effect of the observed MHC-I upregulation on T-cell cytotoxicity *in vitro*.

### Results:

~1200 FDA-approved compounds have been screened on their effect on MHC-I surface expression in neuroblastoma cell lines, which resulted in the identification of 21 potential MHC-I upregulatory compounds. The upregulatory effect of two classes of drugs, topoisomerase inhibitors and DNA demethylating agents, have been studied and confirmed in more detail. We show that neuroblastoma cells treated to increase MHC-I cell surface expression show increased susceptibility to PRAME-reactive tumor-specific T-cell mediated cytotoxicity as compared to untreated controls.

### Summary/Conclusions:

We show that pharmacological upregulation of MHC-I can be achieved with cellular treatment of topoisomerase inhibitors and/or DNA demethylating agents. MHC-I upregulation in neuroblastoma

leads to improved T-cell mediated cytotoxicity *in vitro* and is a potential strategy to improve adaptive immune engagement and therewith immunogenicity of neuroblastoma.

## **P265 Innovative armed oncolytic adenovirus immunotherapeutic approach for the treatment of refractory or metastatic neuroblastoma patients**

*Antonucci, L.<sup>2</sup>, Carcarino, E.<sup>3</sup>, Weber, G.<sup>4</sup>, Ferrandino, F.<sup>5</sup>, Nazio, F.<sup>6</sup>, Cerullo, V.<sup>7</sup>, Di Giannatale, A.<sup>8</sup>, Carinci, M.<sup>9</sup>, Galardi, A.<sup>10</sup>, Arnone, C.M.<sup>11</sup>, Belardinilli, T.<sup>12</sup>, Polito, V.A.<sup>13</sup>, Cristantielli, R.<sup>14</sup>, Vernocchi, P.<sup>15</sup>, Quintarelli, C.<sup>16</sup>, De Angelis, B.<sup>17</sup>, De Vito, R.<sup>18</sup>, Castellano, A.<sup>19</sup>, Del Bufalo, F.<sup>20</sup>, Locatelli, F.<sup>21</sup>, Caruana, I.<sup>22</sup>*

IRCCS Bambino Gesù Children's Hospital, Department of Paediatric Haematology/Oncology, Cell and Gene Therapy, Rome, Italy <sup>2</sup>, IRCCS Bambino Gesù Children's Hospital, Department of Paediatric Haematology/Oncology, Cell and Gene Therapy, Rome, Italy<sup>3</sup>, IRCCS Bambino Gesù Children's Hospital, Department of Paediatric Haematology/Oncology, Cell and Gene Therapy, Rome, Italy<sup>4</sup>, IRCCS Bambino Gesù Children's Hospital, Department of Paediatric Haematology/Oncology, Cell and Gene Therapy, Rome, Italy<sup>5</sup>, IRCCS Bambino Gesù Children's Hospital, Department of Paediatric Haematology/Oncology, Cell and Gene Therapy, Rome, Italy<sup>6</sup>, University of Helsinki, Laboratory of Immunovirotherapy, Drug Research Program, Faculty of Pharmacy, Helsinki, Finland<sup>7</sup>, IRCCS Bambino Gesù Children's Hospital, Department of Paediatric Haematology/Oncology, Cell and Gene Therapy, Rome, Italy<sup>8</sup>, IRCCS Bambino Gesù Children's Hospital, Department of Paediatric Haematology/Oncology, Cell and Gene Therapy, Rome, Italy<sup>9</sup>, IRCCS Bambino Gesù Children's Hospital, Department of Paediatric Haematology/Oncology, Cell and Gene Therapy, Rome, Italy<sup>10</sup>, IRCCS Bambino Gesù Children's Hospital, Department of Paediatric Haematology/Oncology, Cell and Gene Therapy, Rome, Italy<sup>11</sup>, IRCCS Bambino Gesù Children's Hospital, Department of Paediatric Haematology/Oncology, Cell and Gene Therapy, Rome, Italy, <sup>12</sup>, IRCCS Bambino Gesù Children's Hospital, Department of Paediatric Haematology/Oncology, Cell and Gene Therapy, Rome, Italy <sup>13</sup>, IRCCS Bambino Gesù Children's Hospital, Department of Paediatric Haematology/Oncology, Cell and Gene Therapy, Rome, Italy <sup>14</sup>, IRCCS Bambino Gesù Children's Hospital, Unit of Human Microbiome, Genetic and Rare Diseases Area, Rome, Italy <sup>15</sup>, IRCCS Bambino Gesù Children's Hospital, Department of Paediatric Haematology/Oncology, Cell and Gene Therapy, Rome, Italy <sup>16</sup>, IRCCS Bambino Gesù Children's Hospital, Department of Paediatric Haematology/Oncology, Cell and Gene Therapy, Rome, Italy <sup>17</sup>, IRCCS Bambino Gesù Children's Hospital, Department of Laboratories - Pathology Unit, Rome, Italy <sup>18</sup>, IRCCS Bambino Gesù Children's Hospital, Department of Paediatric Haematology/Oncology, Cell and Gene Therapy, Rome, Italy <sup>19</sup>, IRCCS Bambino Gesù Children's Hospital, Department of Paediatric Haematology/Oncology, Cell and Gene Therapy, Rome, Italy <sup>20</sup>, IRCCS Bambino Gesù Children's Hospital, Department of Paediatric Haematology/Oncology, Cell and Gene Therapy, Rome, Italy <sup>21</sup>, IRCCS Bambino Gesù Children's Hospital, Department of Paediatric Haematology/Oncology, Cell and Gene Therapy, Rome, Italy <sup>22</sup>

### **Background/Introduction:**

Neuroblastoma (NB) is the most common extracranial solid tumour of childhood. Despite advances in multimodal therapy, children with high-risk (HR-NB) have a poor prognosis and new therapeutic approaches are urgently needed. Immunotherapy based on oncolytic viruses (OV) might represent an important and effective immunotherapeutic strategy but so far even though results in animal experiments are promising, the efficacy in first clinical trials has been suboptimal.

### **Aims:**

Improvement of OV immunotherapy by investigating the mechanisms limiting their efficacy in vivo.

Investigating OV immunotherapy as a platform for combinatorial treatment approaches.

### **Methods/Materials:**

We first investigated the efficacy of viral production after infection with OV in different types of NB cell lines by virus titration focusing first on oncolytic adenovirus (OA). Thereby, we identified that the difference depended on the ability to metastasize and we focused our attention on proteins involved in this process. We silenced these proteins and investigated the effects both at the molecular level (gene

and proteomic expression), virus production (titration) and functional efficiency *in vitro* and *in vivo* (anti-tumour effect, activation of autophagy and production of exosomes).

#### Results:

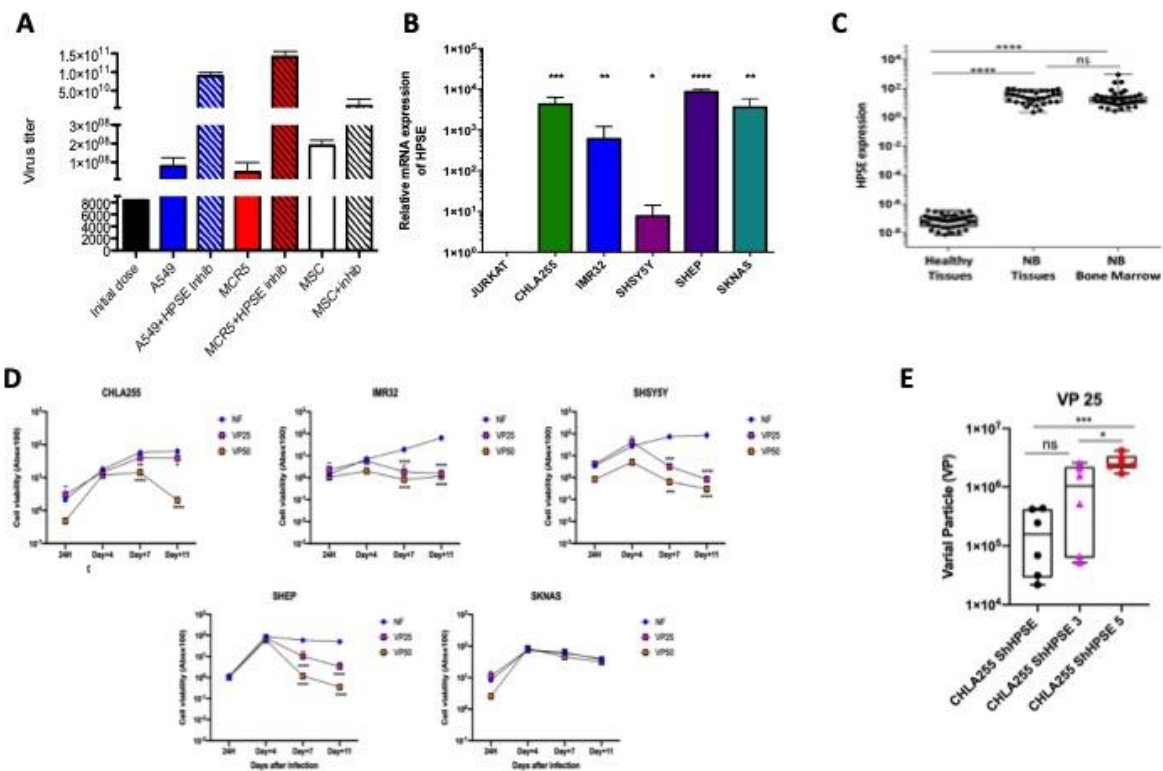
Viral amplification studies revealed a correlation between the reduced number of new viral particles (VP) and the expression of Heparanase (HPSE), an enzyme responsible for modeling the extracellular matrix (ECM) by degradation of heparin-sulfate (HS), a constituent of viral capsids and cellular membranes.

We demonstrated, by immunohistochemistry and qPCR, a significant expression of HPSE in 32 HR-NB ( $p < 0.001$ ) both at diagnosis and relapse. This data was confirmed in a public database (<https://hgserver1.amc.nl/cgi-bin/r2/main.cgi>), showing a negative correlation between HPSE expression and outcome of these patients.

CHLA-255 and SKNAS NB cell lines show high levels of HPSE, metastatic behaviour in an orthotopic xenograft mouse model and a significant resistance to OA ( $p < 0.05$ ). Lentiviral viruses encoding different sh-HPSE have been used to silence the two cell lines. Silencing induced a strong inhibition of HPSE expression and impaired proliferation both *in vitro* and *in vivo*. Notably, reduced expression of HPSE leads to a decreased tumour progression, a significant reduction of metabolic activity and exosome production. Following OA infection, the silenced cells produce a greater amount of VP ( $p = 0.001$ ), show a block of autophagy and a down-regulation of pro-tumoural genes. This phenomenon translates in a different protein expression profile compared to the controls. Furthermore, silenced and infected tumour cells show a strong cytopathic effect of OA as well as a more efficient and robust immune presentation increasing the response against adenovirus antigens and tumour.

#### Summary/Conclusions:

In conclusion, HPSE modulation is a promising strategy to implement viral oncolytic immunotherapies in patients with oncological disease. This strategy might be a useful component for the development of a combined treatment with gene modified cellular products such as Chimeric Antigen Receptor T cells.



Panel A shows the impact of HPSE on virus production after OA infection. Panel B and C show the expression of HPSE in NB cell lines and primary NB samples. Panel D shows the susceptibility of NB cell lines to OA infection. Panel E reveals the improvement of VP production upon silencing of HPSE in CHLA255 NB cell line.

Caption 1: Influence of HPSE expression on new VP production.

## **P266 1RG-CART, a CRUK-sponsored phase I trial to evaluate a novel chimeric antigen receptor-transduced autologous T cells in children with neuroblastoma**

*Jain, N.<sup>2</sup>, Flutter, B.<sup>3</sup>, Wallace, R.<sup>4</sup>, Barone, G.<sup>5</sup>, Jain, N.<sup>6</sup>, Loka, T.<sup>7</sup>, Depani, S.<sup>8</sup>, Thomas, S.<sup>9</sup>, Cheung, G.<sup>10</sup>, Collura, A.<sup>11</sup>, Gileadi, T.<sup>12</sup>, Barton, J.<sup>13</sup>, Wright, G.<sup>14</sup>, Inglott, S.<sup>15</sup>, Biassoni, L.<sup>16</sup>, McHugh, K.<sup>17</sup>, Edwards, D.<sup>18</sup>, Barton, C.<sup>19</sup>, Dyer, K.<sup>20</sup>, Brook, S.<sup>21</sup>, Robson, L.<sup>22</sup>, Westwood, N.<sup>23</sup>, Howe, K.<sup>24</sup>, Pule, M.<sup>25</sup>, Anderson, J.<sup>26</sup>*

Great Ormond Street Hospital, Paediatric Oncology, London, United Kingdom <sup>2</sup>, Institute of Child Health, Experimental Paediatric Oncology, London, United Kingdom<sup>3</sup>, Institute of Child Health, Experimental Paediatric Oncology, London, United Kingdom<sup>4</sup>, Great Ormond Street Hospital, Paediatric Oncology, London, Great Ormond Street Hospital<sup>5</sup>, Great Ormond Street Hospital, Paediatric Oncology, London, United Kingdom<sup>6</sup>, Great Ormond Street Hospital, Paediatric Oncology, London, United Kingdom<sup>7</sup>, Great Ormond Street Hospital, Paediatric Oncology, London, United Kingdom<sup>8</sup>, Institute of Child Health, Experimental Paediatric Oncology, London, United Kingdom<sup>9</sup>, Institute of Child Health, Experimental Paediatric Oncology, London, United Kingdom<sup>10</sup>, Institute of Child Health, Experimental Paediatric Oncology, London, United Kingdom<sup>11</sup>, Institute of Child Health, Experimental Paediatric Oncology, London, United Kingdom, <sup>12</sup>, Great Ormond Street Hospital, Paediatric Haematology, London, United Kingdom <sup>13</sup>, Great Ormond Street Hospital, Clinical reporting, London, United Kingdom <sup>14</sup>, Great Ormond Street Hospital, Clinical Laboratories, London, United Kingdom <sup>15</sup>, Great Ormond Street Hospital, Clinical Radiology, London, United Kingdom <sup>16</sup>, Great Ormond Street Hospital, Clinical Radiology, London, United Kingdom <sup>17</sup>, Cancer Research UK, Centre for Drug Development, London, United Kingdom <sup>18</sup>, Cancer Research UK, Centre for Drug Development, London, United Kingdom <sup>19</sup>, Cancer Research UK, Centre for Drug Development, London, United Kingdom <sup>20</sup>, Cancer Research UK, Centre For Drug Development, London, United Kingdom <sup>21</sup>, Cancer Research UK, Centre for drug development, London, United Kingdom <sup>22</sup>, Cancer Research UK, Centre for Drug Development, London, United Kingdom <sup>23</sup>, Great Ormond Street Hospital, Oncology and Haematology Research Department, London, United Kingdom <sup>24</sup>, Institute of Child Health, Experimental Paediatric Oncology, London, United Kingdom <sup>25</sup>, Institute of Child Health, Experimental Paediatric Oncology, London, United Kingdom <sup>26</sup>

### **Background/Introduction:**

The re-programming of a patient's immune system through genetic modification of the T cell compartment with chimeric antigen receptors (CARs) has led to durable remissions in chemotherapy refractory B cell cancers expressing CD19 antigen. CARs cause redirection of T cell specificity and rely upon identification of a tumour cell surface antigen absent from critical normal tissues. Successful targeting of solid cancers by CAR T cells has been limited by the requirement for infiltration and expansion within the inhibitory tumour microenvironment.

### **Aims:**

Disialoganglioside GD2 is abundant on the surface of most neuroblastomas but absent from most normal cells outside the CNS. To avoid the theoretical inhibitory effects of antibodies against the 14.18 antibody previously administered to many relapsed patients, we devised a second generation (CD28-zeta) anti-GD2 CAR, which incorporates a novel humanised single chain antibody (huk666). We have evaluated safety and feasibility of manufacturing and administering patient-derived CAR-transduced T cells in the setting of relapsed or refractory neuroblastoma

### **Methods/Materials:**

The target cell dose was successfully manufactured in 15 out of 15 heavily pretreated patients undergoing apheresis, and 9 patients have received single infusions of either 10<sup>6</sup> • transduced cells/m<sup>2</sup> (n= 6; 4 without conditioning, 1 x cyclophosphamide (Cy) alone, 1 x Cy and fludarabine (Flu)), or 10<sup>9</sup>/m<sup>2</sup> (n=3 with Flu/Cy) or 10<sup>9</sup>/m<sup>2</sup> (n=3 with Flu/Cy).

### **Results:**

No patients receiving  $10^9/m^2$  showed CAR T cell engraftment or clinical response. Of three patients receiving  $10^8/m^2$  T cells, one patient with high burden disease, experienced grade 2 cytokine release syndrome necessitating therapeutic reversal with IL-6 antagonist tocilizumab, sustained low level CAR-T cell engraftment, improvement in performance status, widespread necrosis of previously heavily infiltrated bone marrow, and partial resolution of bone metastatic disease. New complete clinical outcome data on the final cohort of patients receiving  $10^9/m^2$  will be presented at the meeting. No patients experienced dose limiting toxicity.

Summary/Conclusions:

Manufacture of greater than  $10^8/m^2$  1RG-CART cells is feasible and no dose limiting toxicity is found in any patients. Current research efforts are focussed on understanding and boosting persistence.



## **P267 Trogocytosis: a potential mechanism of resistance in neuroblastoma following anti-GD2 immunotherapy**

Zamora, A.K.<sup>2</sup>, Zobel, M.J.<sup>3</sup>, Sun, J.<sup>4</sup>, Lin, T.Y.<sup>5</sup>, Sheard, M.A.<sup>6</sup>, Seeger, R.C.<sup>7</sup>, Kim, E.S.<sup>8</sup>

Children's Hospital Los Angeles, Pediatric Surgery, Los Angeles, USA <sup>2</sup>, Children's Hospital Los Angeles, Pediatric Surgery, Los Angeles, USA<sup>3</sup>, Children's Hospital Los Angeles, Hematology Oncology, Los Angeles, USA<sup>4</sup>, Children's Hospital Los Angeles, Hematology Oncology, Los Angeles, Children's Hospital Los Angeles<sup>5</sup>, Children's Hospital Los Angeles, Hematology Oncology, Los Angeles, USA<sup>6</sup>, Children's Hospital Los Angeles, Hematology Oncology, Los Angeles, USA<sup>7</sup>, Children's Hospital Los Angeles, Pediatric Surgery, Los Angeles, USA<sup>8</sup>

### Background/Introduction:

Children with high-risk neuroblastoma (NB) have a poor prognosis with an overall survival of less than 50%. The anti-GD2 monoclonal antibody, dinutuximab, has been shown to improve survival, but many children recur with resistant disease. We recently discovered that treating neuroblastoma cells with activated natural killer cells (aNK) and dinutuximab leads to tumor cell death and the acquisition of the neuroblastoma membrane marker GD2 by the natural killer cells, a phenomenon called trogocytosis.

### Aims:

To characterize the process of trogocytosis following immunotherapy in neuroblastoma and to determine the efficacy of GD2+ NK cells in neuroblastoma cytotoxicity.

### Methods/Materials:

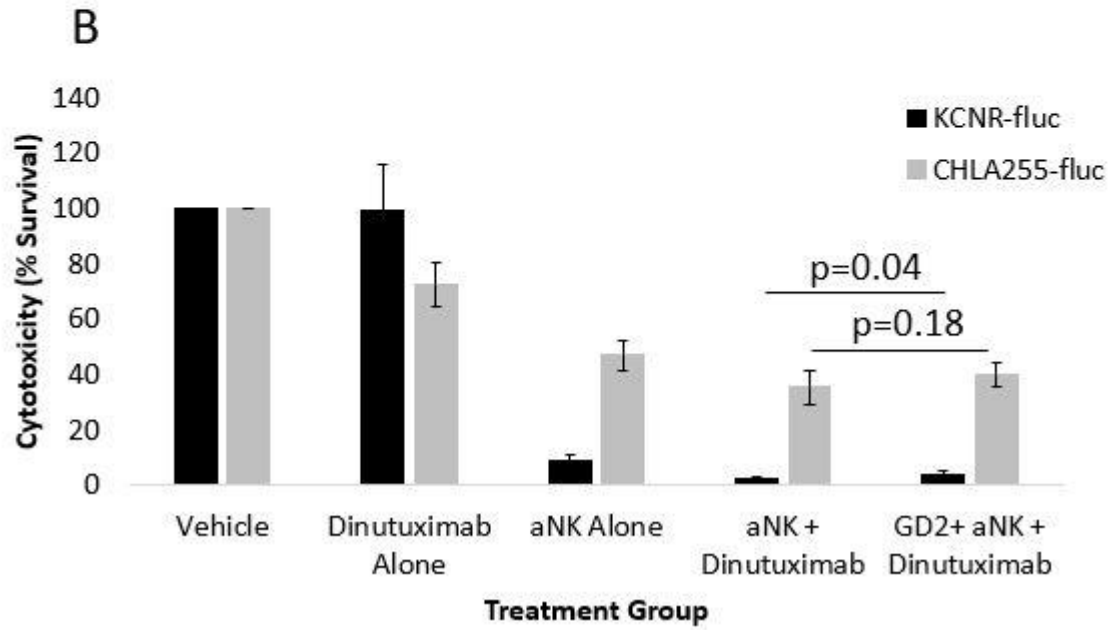
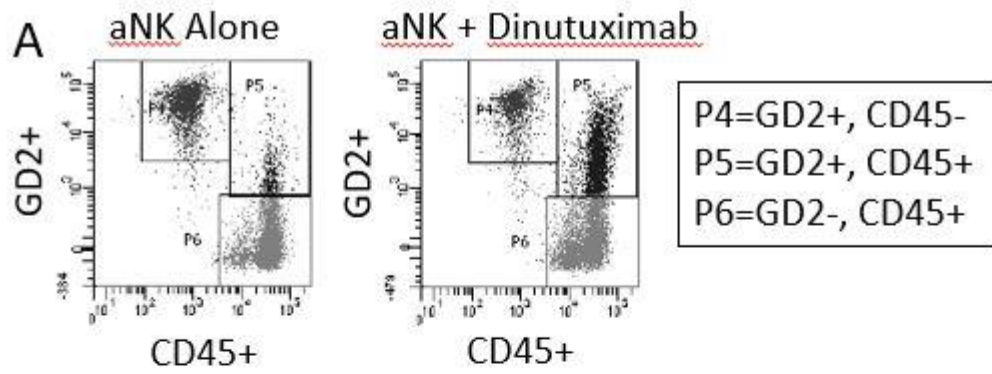
Human NB cells from the CHLA255-fluc and KCNR-fluc cell lines were treated with vehicle control, dinutuximab (100ng/mL) alone, aNK cells alone (E:T 1:1), or aNK cells plus dinutuximab. The treatment groups were then incubated, harvested hourly from 0 minutes to 6 hours, and analyzed by flow cytometry to evaluate expression of GD2 (NB cell marker, APC) and CD45 (NK cell marker, AlexaFluor-488). Live cell microscopy images were obtained at various timepoints. The GD2+ NK cells were then incubated with fresh NB cells for 24 hours to assess cytotoxicity. Luciferase cytotoxicity assays were performed to evaluate the efficacy of the GD2+ NK cells compared to fresh, naïve aNK cells. Student's t-test was performed;  $p < 0.05$  was considered significant.

### Results:

Following treatment with aNK cells and dinutuximab, a novel GD2+/CD45+ population was observed and reached maximal expression at 3 hours (Figure 1A). As tumor cells were killed, there was a notable time-dependent loss of GD2 expression from the NB cell population with a concomitant rise in a GD2+/CD45+ population. Live cell imaging demonstrated the acquisition of NB membrane-bound GD2 by the CD45+ NK cells, resulting in GD2+ NK cells. Lastly, compared to fresh aNK cells, GD2+ NK cells show decreased cytotoxicity to CHLA255 and KCNR neuroblastoma cells alone ( $p=0.11$ ,  $p=0.008$ ) and in combination with dinutuximab ( $p=0.18$ ,  $p=0.04$ ), respectively (Figure 1B).

### Summary/Conclusions:

Our study demonstrates the phenomenon of trogocytosis following aNK cell and dinutuximab immunotherapy *in vitro* which may represent a novel mechanism of resistance in neuroblastoma. Ongoing *in vitro in vivo*, and patient specimen studies are being conducted to further characterize and validate the functional effects of trogocytosis on immunotherapy for neuroblastoma.



## P268 Results of multiantigen DNA-vaccination against neuroblastoma in mouse model

Stegantseva, M.<sup>2</sup>, Shinkevich, V.<sup>3</sup>, Tumar, E.<sup>4</sup>, Mikhaleuskaya, T.<sup>5</sup>, Meleshko, A.<sup>6</sup>

Belarusian Research Center for pediatric Oncology, Hematology and Immunology, Scientific department, Minsk, Belarus <sup>2</sup>, Belarusian Research Center for pediatric Oncology, Hematology and Immunology, Scientific, Minsk, Belarus<sup>3</sup>, Institute of Bioorganic Chemistry of the National Academy of Sciences, Scientific, Minsk, Belarus<sup>4</sup>, Belarusian Research Center for pediatric Oncology, Hematology and Immunology, Patomorphology group, Minsk, Belarusian Research Center for pediatric Oncology, Hematology and Immunology<sup>5</sup>, Belarusian Research Center for pediatric Oncology, Hematology and Immunology, Scientific, Minsk, Belarus<sup>6</sup>

### Background/Introduction:

In high-risk neuroblastoma (NB) patients the percentage of relapse reaches 65%, and the survival rate is 50% despite the use of a wide range of therapeutic approaches. DNA vaccination can be used to prevent relapse in patients with neuroblastoma. A vaccine's efficacy mainly depends on the antigen (AG) chosen as the target, as well as the delivery method.

### Aims:

To compare the immunogenicity of DNA vaccines based on three NB-associated AG (tyrosine hydroxylase (TH), Survivin, Phox2b) and to evaluate the effect of vaccine delivery using polyethyleneimine (PEI) and *Salmonella enterica* on tumor progression

### Methods/Materials:

Sixty-four C57Bl/6 mice were used for immunogenicity study. The mice were injected intramuscularly three times with 10 µg TH/Survivin/Phox2b-PVXCP DNA vaccines (together or separately) in combination with a 20 kDa PEI (DNA-PEI), or an oral gavage of a bacterial suspension of an *Salmonella enterica* attenuated strain (DNA-SE) was performed. A/J mice (n = 58) were used to assess the effect of vaccination on tumor progression when 10<sup>6</sup> NB41A3 cell line were engrafted subcutaneously. The immune response (IR) was evaluated by ELISPOT (production of interferon gamma (IFN $\gamma$ )) and cytotoxicity test (CTT) against NB41A3 murine neuroblastoma cells.

### Results:

According to the CTT results the delivery of the DNA-PEI conjugate caused a significant increase in cellular IR for all AGs and their combination, except TH (p <0.05). Oral delivery with *S. enterica* induced a strong IR, which was at least 2 times higher compared to DNA-PEI for all AGs and significantly higher compared to the control. The median percentage of lysed NB41A3 cells in the group of mice vaccinated with *Salmonella* was significantly increased compared to DNA-PEI: 10.2% versus 4.9% for TH; 23.8% versus 11.5% for Survivin; 21.23% versus 7.9% for Phox2b and 34.21% versus 8.16% for TriMix. Comparison of AGs showed a sequential increase in immunogenicity in the following row: TH → Survivin → Phox2b → TriMix.

IFN $\gamma$  secretion in response to costimulatory protein PVXCP was recorded in 62.5% of vaccinated mice. The highest AG-specific cytokine production was observed after incubation with TH and Phox2b, where the results were 7.2 and 8.4 times higher (respectively) than in the control group. Vaccination with TriMix-SE showed the best results, because the IFN $\gamma$  response was obtained for all tested AGs.

The rate of tumor progression was evaluated only for monovaccinations. PEI-DNA showed better results than DNA-SE in all experimental groups. The median tumor volume after vaccination with TH-PEI on the 25th day of monitoring was 918.2 versus 2328 mm<sup>3</sup> in TH-SE. The final tumor volume after vaccination with Survivin-PEI was 2.7 times smaller than in the control (512 versus 1417 mm<sup>3</sup>), and after vaccination with Survivin-SE it was 2 times smaller (1302 versus 2664 mm<sup>3</sup>). Vaccination with Phox2b-PEI reduced the final tumor volume in 6.3 times (420 mm<sup>3</sup>) compared with the control group (2664 mm<sup>3</sup>), and Phox2b-SE decreased in 4.7 times (634 mm<sup>3</sup>). The low vaccination efficiency with *Salmonella* is probably associated with a toxicity of tumor and bacterial load and potentially can be eliminated by reducing the last one.

### Summary/Conclusions:

The use of a multi-antigen vaccine is more effective and can help prevent the tumor IR escape by suppressing targeted antigen expression. Delivery of the DNA vaccine with *Salmonella enterica* was accompanied by a stronger IR than the DNA-PEI conjugate, but the latter caused more significant tumor regression.

## **P269 Clinical analysis of 458 children with high-risk neuroblastoma in a single center in China**

*Su, Y.<sup>2</sup>, Ma, X.L.<sup>3</sup>, Wang, H.M.<sup>4</sup>, Qin, M.Q.<sup>5</sup>, Zhang, F.Q.<sup>6</sup>, Jin, M.<sup>7</sup>, Zeng, Q.<sup>8</sup>, He, L.J.<sup>9</sup>, Ni, X.<sup>10</sup>*

Beijing Children's Hospital, Capital Medical University, National Center for Chil, Hematology Oncology Center, Beijing, China <sup>2</sup>, Beijing Children's Hospital, Capital Medical University, National Center for Chil, Hematology Oncology Center, Beijing, China<sup>3</sup>, Beijing Children's Hospital, Capital Medical University, National Center for Chil, Department of Surgical Oncology, Beijing, China<sup>4</sup>, Beijing Children's Hospital, Capital Medical University, National Center for Chil, Hematology Oncology Center, Beijing, Beijing Children's Hospital, Capital Medical University, National Center for Chil<sup>5</sup>, Beijing Union Hospital, Chinese Academy of Medical Sciences, Department of Radiation Oncology, Beijing, China<sup>6</sup>, Beijing Children's Hospital, Capital Medical University, National Center for Chil, Hematology Oncology Center, Beijing, China<sup>7</sup>, Beijing Children's Hospital, Capital Medical University, National Center for Chil, Department of Thoracic Surgery, Beijing, China<sup>8</sup>, Beijing Children's Hospital, Capital Medical University, National Center for Chil, Department of Pathology, Beijing, China<sup>9</sup>, Beijing Children's Hospital, Capital Medical University, National Center for Chil, Department of Otorhinolaryngology Head and Neck Surgery, Beijing, China<sup>10</sup>

### **Background/Introduction:**

Despite the multi-disciplinary treatment, the prognosis of high-risk neuroblastoma (HR-NB) is still poor. The treatment of HR-NB is still one of the biggest challenges in pediatric oncology.

### **Aims:**

To analyze clinical characteristics and prognosis of HR-NB children in the single center in China.

### **Methods/Materials:**

The clinical data of children with HR-NB who were diagnosed and treated at the hematology-oncology center of Beijing Children's Hospital (BCH) from February 1st, 2007 to June 30th, 2018 were retrospectively analyzed. The clinical features were summarized and the prognosis was evaluated. The SPSS 19.0 statistical package was used for data analysis.

### **Results:**

A total of 458 children with HR-NB were enrolled in this study, including 265 males (57.9%) and 193 females (42.1%), with a median age of 40 months old (4.5-148 months old). Primary tumors were located on retroperitoneal/adrenal glands in 374 cases (81.7%) and on mediastinum in 64 cases (14%). The bone marrow metastases were seen in 338 cases (73.8%), bone metastases were seen in 316 cases (69%) and the distant lymph node metastasis were seen in 272 cases (59.4%). The median follow-up time was 22 months (0.2-138 months), and the median time of event was 14 months (0.2-72 months). 233 patients survived and 196 died. 172 patients (87.8%) died from tumor progression/recurrence, 9 patients (4.6%) died from treatment-related complications, and 15 patients (7.7%) died in the early. The 3-year event-free survival (EFS) of 458 patients was 42.3%±2.6% and the 5-year EFS was 33.8%±2.8%. The 3-year overall survival (OS) was 53.9%±2.7% and the 5-year OS was 43.1%±3.2%. 166 cases (36.2%) received autologous peripheral blood stem cell transplantation (APBSCT). There was no significant difference in EFS and OS between the patients who received transplantation and those who did not (3-year OS 56.8%±4.2% vs. 52.5%±3.4%, 5-year OS 39.4%±5.2% vs. 46.8%±3.9%, P=0.123; 3-year EFS 44%±4.2% vs 41.5%±3.3%, 5-year EFS 28.9%±4.3% vs 37.9%±3.5%, P=0.237). The median time of tumor recurrence/progression was longer in transplantation group than in nontransplantation group (17.5 months vs 11 months, P=0.000). The children with bone metastasis or bone marrow metastasis had higher OS in transplantation group than in non-transplantation group (P=0.004, P=0.002). There were 86 cases (20.6%) with MYCN gene amplification. The 3-year OS of children with MYCN gene amplification was 28%±5.6%, and the 5-year OS was 28%±5.6%, which was significantly lower than that of children without MYCN amplification (3-year OS 62.6%±3.1%, 5-year OS 47.1%±4.2%, P=0.000). Comparison of transplantation group and nontransplantation

group in children with MYCN gene amplification was 3-year EFS 26.8%±8% vs 20.9%±6.5% (P=0.021), and 3-year OS 31.4%±8.6% vs 26.1%±7.4% (P=0.07). Univariate analysis showed poor prognosis in children with MYCN gene amplification, bone or bone marrow metastasis, and LDH>1500 U/L (P<0.05).

#### Summary/Conclusions

The 3-year OS of HR-NB in the single center was 53.9%±2.7%, and 5-year OS was 43.1%±3.2%. APBSCT can improve the prognosis of patients with bone or bone marrow metastasis and can retard recurrence or progression in patients with MYCN amplification. Seeking more effective treatment method during maintenance period is the key to improve the prognosis of children with HR-NB.

## **P270 Quantitative assessment by liquid biopsy of *MYCN* and chromosome 11q LOH in neuroblastoma**

*Shirai, R.*<sup>2</sup>, *Osumi, T.*<sup>3</sup>, *Ueno-Yokohata, H.*<sup>4</sup>, *Yamada, Y.*<sup>5</sup>, *Yoshida, M.*<sup>6</sup>, *Yoshida, K.*<sup>7</sup>, *Shioda, Y.*<sup>8</sup>, *Kiyotani, C.*<sup>9</sup>, *Terashima, K.*<sup>10</sup>, *Tomizawa, D.*<sup>11</sup>, *Takasugi, N.*<sup>12</sup>, *Miyazaki, O.*<sup>13</sup>, *Kiyokawa, N.*<sup>14</sup>, *Hishiki, T.*<sup>15</sup>, *Matsumoto, K.*<sup>16</sup>, *Yoshioka, T.*<sup>17</sup>, *Kato, M.*<sup>18</sup>

National Center for Child Health and Development, Pediatric Hematology and Oncology Research, Tokyo, Japan<sup>2</sup>, National Center for Child Health and Development, Pediatric Hematology and Oncology Research, Tokyo, Japan<sup>3</sup>, National Center for Child Health and Development, Department of Pediatric Hematology and Oncology Research, Tokyo, Japan<sup>4</sup>, National Center for Child Health and Development, Pediatric Hematology and Oncology Research, Tokyo, National Center for Child Health and Development<sup>5</sup>, National Center for Child Health and Development, Pediatric Hematology and Oncology Research, Tokyo, Japan<sup>6</sup>, National Center for Child Health and Development, Pediatric Hematology and Oncology Research, Tokyo, Japan<sup>7</sup>, National Center for Child Health and Development, Children's Cancer Center, Tokyo, Japan<sup>8</sup>, National Center for Child Health and Development, Children's Cancer Center, Tokyo, Japan<sup>9</sup>, National Center for Child Health and Development, Children's Cancer Center, Tokyo, Japan<sup>10</sup>, National Center for Child Health and Development, Children's Cancer Center, Tokyo, Japan<sup>11</sup>, University of Tokyo, Department of Pediatrics, Tokyo, Japan,<sup>12</sup> National Center for Child Health and Development, Department of Radiology, Tokyo, Japan<sup>13</sup>, National Center for Child Health and Development, Department of Pediatric Hematology and Oncology Research, Tokyo, Japan<sup>14</sup>, National Center for Child Health and Development, Division of Surgical Oncology, Tokyo, Japan<sup>15</sup>, National Center for Child Health and Development, Children's Cancer Center, Tokyo, Japan<sup>16</sup>, National Center for Child Health and Development, Department of Pathology, Tokyo, Japan<sup>17</sup>, National Center for Child Health and Development, Department of Pediatric Hematology and Oncology Research, Tokyo, Japan<sup>18</sup>

### Background/Introduction:

The 'liquid biopsy' detecting genomic alterations of tumors using cell-free DNA (cfDNA) in plasma, has recently attracted attention as a non-invasive alternative of surgical tissue biopsy. However, most of liquid biopsy is qualitative, detecting only presence targeting specific genomic alterations.

### Aims:

We here challenged quantitative liquid biopsy to detect *MYCN* amplification and 11q LOH, important prognostic factors of neuroblastoma.

### Methods/Materials:

Plasma samples at the onset were obtained 17 neuroblastoma patients diagnosed from June 2017 to April 2019. The cfDNA was extracted from 1 ml of plasma and the analyzed results were compared to that of genomic DNA from tumor tissues. The copy number of *MYCN* and *NAGK* genes were quantitatively analyzed by droplet digital PCR (ddPCR). All tumor specimens were routinely examined for *MYCN* amplification using fluorescent in situ hybridization (FISH). LOH at 11q was assessed by detecting allelic imbalances of heterozygous SNPs in the q arm of chromosome 11 using ddPCR. Multiplex ligation probe amplification (MLPA) was also performed to investigate aberrations of 11q in tumor DNA. The proportion of circulating tumor DNA in plasma cfDNA was estimated by examining the variant allele frequency (VAF) of a tumor-specific single nucleotide variant (SNV) identified by whole-exome sequencing for tumor DNA.

### Results:

The median age of the 17 patients was 29 months old (range, 0-80) [INRGSS L1 (n = 1), L2 (n = 4), M (n = 11) and MS (n = 1)]. The presence (n = 6) and absence (n = 10) of *MYCN* amplification in tumor specimens using FISH analysis were correctly detected by liquid biopsy in all cases. Results of *MYCN* copy number analysis using cfDNA were highly concordant to that of tumor specimens, and the correlation coefficient was 0.73 with statistical significance (P = 0.007). 11q LOH was also detected in

five cases using MLPA. Analysis of cfDNA using ddPCR detected 11qLOH in all 4 cases with stage M except a case with stage L2 which could not be detected in cfDNA at the time of recurrence. In terms of VAF of tumor specific SNV, very high correlation of 0.964 ( $P=0.003$ ) was observed in patients with stage M. Interestingly, the VAF of tumor specific SNV in cfDNA was higher than that in tumor DNA in 2 of 5 patients analyzed.

Summary/Conclusions:

*MYCN* copy number and 11q LOH could be accurately analyzed in plasma cfDNA by ddPCR, especially in stage M. Liquid biopsy is a non-invasive and repeatable method which can detect quantitative genomic alterations in NBs.



## **P271 Role of radiotherapy dose-escalation for high-risk neuroblastoma with post-surgical primary site gross residual disease: a report from the COG ANBL0532 study**

*Liu, K.,<sup>1</sup> Park, J.<sup>2</sup>, Naranjo, A.<sup>3</sup>, Zhang, F.<sup>4</sup>, DuBois, S.<sup>5</sup>, Braunstein, S.<sup>6</sup>, Voss, S.<sup>7</sup>, Khanna, G.<sup>8</sup>, London, W.<sup>9</sup>, Doski, J.<sup>10</sup>, Geiger, J.<sup>11</sup>, Kreissman, S.<sup>12</sup>, Grupp, S.<sup>13</sup>, Diller, L.<sup>14</sup>, Park, J.<sup>15</sup>, Haas-Kogan, D.<sup>16</sup>*

Dana-Farber Cancer Institute, Radiation Oncology, Boston, USA<sup>1</sup>, Seattle Children's Hospital, Pediatrics, Seattle, USA<sup>2</sup>, University of Florida, Children's Oncology Group Statistics and Data Center, Gainesville, USA<sup>3</sup>, Children's Oncology Group Statistics and Data Center, Children's Oncology Group Statistics and Data Center, Monrovia, USA<sup>4</sup>, Dana-Farber/Boston Children's Cancer and Blood Disorders Center, Pediatric Oncology, Boston, Dana-Farber/Boston Children's Cancer and Blood Disorders Center<sup>5</sup>, University of California San Francisco, Radiation Oncology, San Francisco, USA<sup>6</sup>, Boston Children's Hospital, Radiology, Boston, USA<sup>7</sup>, St. Louis Children's Hospital, Radiology, St. Louis, USA<sup>8</sup>, Dana-Farber/Boston Children's Cancer and Blood Disorders Center, Pediatric Oncology, Boston, USA<sup>9</sup>, University of Texas Health Science Center, Surgery, San Antonio, USA<sup>10</sup>, C.S. Mott Children's Hospital, Surgery, Ann Arbor, USA<sup>11</sup>, Duke University Medical Center, Pediatrics, Durham, USA<sup>12</sup>, Children's Hospital of Philadelphia, Pediatrics, Philadelphia, USA<sup>13</sup>, Dana-Farber/Boston Children's Cancer and Blood Disorders Center, Pediatric Oncology, Boston, USA<sup>14</sup>, Seattle Children's Hospital, Pediatrics, Seattle, USA<sup>15</sup>, Dana-Farber Cancer Institute, Radiation Oncology, Boston, USA<sup>16</sup>

### **Background/Introduction:**

Despite an intensive multimodal approach, including chemotherapy, multi-agent myeloablative regimens with stem cell transplantation, surgery, radiotherapy, and immunotherapy, outcomes for high-risk neuroblastoma remain inadequate with relapse occurring in approximately 50% of patients. Locoregional relapse continues to be a significant contributor to treatment failure for high-risk neuroblastoma patients even after radiotherapy (RT).

### **Aims:**

We aimed to examine the effects of increasing local dose of radiation to the residual primary tumor for patients with less than a gross total resection on cumulative incidence of local progression (CILP), event-free survival (EFS), and overall survival (OS) in the Children's Oncology Group (COG) ANBL0532 phase 3 study, a question that constituted a Primary Objective of the trial.

### **Methods/Materials:**

Newly diagnosed high-risk neuroblastoma patients were enrolled on COG ANBL0532 from November 2007 to February 2012. Patients were randomized or assigned to receive single vs. tandem autologous stem cell transplantation (SCT). Local control of the primary tumor consisted of surgical resection during induction chemotherapy and RT following last SCT. Patients were prescribed RT (21.6 Gy in 1.8 Gy fractions) to the preoperative primary tumor volume. For patients with incomplete surgical resections of the primary tumor (defined as >1 cm<sup>3</sup> residual soft tissue density as assessed by central review of end-induction scans), an additional boost of 14.4 Gy was delivered to the gross residual tumor for a total dose of 36 Gy. OS, EFS, and CILP were compared to COG A3973 historical cohort, in which all patients received 21.6 Gy without a boost. Kaplan-Meier curves of OS and EFS were compared using a log-rank test. CILP curves, with adjustment for competing risks (previous progression outside the primary, or death), were compared using Gray's test.

### **Results:**

323 patients received RT on ANBL0532. Of these, 133 patients received boost RT. For all patients receiving RT, five-year CILP, EFS, and OS were 11.2±1.8%, 56.2±3.4%, and 68.4±3.2% for the COG ANBL0532 cohort (n=323) compared to 7.1±1.4% (p=0.0590), 47.0±3.5% (p=0.0090), and 57.4±3.5% (p=0.0088) for COG A3973 cohort (n=330), respectively. Five-year CILP, EFS, and OS for COG A3973 patients who had incomplete resection and received RT (n=47) were 10.6±4.6%, 48.9±10.1%, and 56.9±10.0%, respectively. In comparison, five-year CILP, EFS, and

OS for COG ANBL0532 patients receiving boost RT (n=133) were 15.1±3.1% (p=0.4632), 52.1±5.4% (p=0.4053), and 67.0±5.1% (p=0.2863), respectively; and five-year CILP, EFS, and OS for COG ANBL0532 patients randomized or assigned to single SCT and received boost RT (n=74) were 16.3±4.3% (p=0.4126), 50.9±7.0% (p=0.5084), and 68.1±6.7% (p=0.2835), respectively.  
Summary/Conclusions:

CILP was not improved on COG ANBL0532 despite the implementation of boost RT to gross residual tumor present at end-induction. New strategies are needed to decrease the risk of locoregional failure.

## **P272 High-risk neuroblastoma: mIBG Curie scores at the time of diagnosis and the end of induction, their prognostic value in comparison with data of MMD and MRD at the same time.**

*Praliskouskaya, I.<sup>2</sup>, Petrovskaya, A.<sup>3</sup>, Kushnerova, E.<sup>4</sup>, Konoplya, N.<sup>5</sup>*

Belarusian center for pediatric oncology, hematology and immunology, Deputy director of the clinic, Minsk, Belarus <sup>2</sup>, Belarusian center for pediatric oncology, hematology and immunology, X-rays diagnostic, Minsk, Belarus<sup>3</sup>, Belarusian center for pediatric oncology, hematology and immunology, MOLECULAR LABORATORY, Minsk, Belarus<sup>4</sup>, Belarusian center for pediatric oncology, hematology and immunology, Director, Minsk, Belarusian center for pediatric oncology, hematology and immunology<sup>5</sup>,

### Background/Introduction:

Despite a multi-modality approach combining chemotherapy, surgical resection, radiotherapy, autologous stem cell transplant and biotherapy, survival for high risk neuroblastoma remains poor, with 5-year EFS 30-49%. The use of the anti-GD2 chimeric antibody post-transplant time was improvements in EFS. For patients who develop relapsed disease, specifically those who relapse 6-18 months from initial diagnosis, 5-year OS is < 20%. The identification of prognostic markers of response and survival early in a patient's treatment may have significant impact on therapy and outcome.

### Aims:

Assessment of prognostic value for patients with high-risk neuroblastoma of the mIBG data in comparison with data of MMD and MRD at the time of diagnosis and the end of induction.

### Methods/Materials:

44 patients (pts) who underwent I 123 MIBG scintigraphy from April 2011 till December 2017. Sex: 24 - boys, 20- girls. Age median 2.84 years (from 0.76 years to 7.71 years). The distribution according to the stages is the following: stage 2 - 1 pts, stage 3 – 3 pts, stage 4 - 40 pts. All pts received treatment according to NB2004 protocol. Determination of MMD and MRD by PCR, the relative expression level of TH and PHOX2B genes in the bone marrow was evaluated. To assess the significance of differences, the  $\chi^2$  test, the Whitney U-test were used. EFS rate for pts was estimated using the Kaplan-Meier method and survival function was compared using the long-rank test.

### Results

It was revealed that the patient's with the presence of mIBG avid disease at the time of diagnosis according to I123 mIBG scintigraphy of more than 5 CS (Curie scores) significantly worsens the results of therapy (5-year EFS  $74 \pm 13\%$ , vs.  $32 \pm 14\%$ ). In addition, when evaluating the response to therapy after the end of induction treatment, it was found that the preservation of the the presence of mIBG avid disease according to I123 MIBG scintigraphy more than 3 CS significantly worsens the results of the therapy (4-year EFS  $54 \pm 9\%$ , vs.  $13 \pm 12\%$ ).

The data of MIBG scintigraphy are comparable with the data on MMD and MRD in bone marrow. Thus, differences in the medians of relative expression of TH and PHOX2B genes at the time of diagnosis of the disease at 5 points (CS) was 0.003 versus 0.0776 ( $p = 0.012$ ) and 0.0078 against 0.0503 ( $p = 0.059$ ), respectively. At the end of the induction, the differences in the medians of the relative expression of TH and PHOX2B genes in bone marrow of the disease at 3 points (CS) was 0 against 0.0062 ( $p = 0.0056$ ) and 0 against 0.0001 ( $p = 0.0075$ ).

### Summary/Conclusions

The cut-off data for the relative expression level of PHOX2B gene at the end of induction therapy coincides with the median if there are less than 3 (SC) points. For the relative expression level of TH gene in the bone marrow at the time of induction, the cut-off data are within the same order (0.002 and 0.006), respectively. Taking into account the above, we consider it reasonable to use both indicators simultaneously to highlight the ultra-high risk group of patients with neuroblastoma. This group is characterized by an unfavorable prognosis in terms of EFS, the correction of the therapy for this group of patients is definitely necessary.



## **P273 Investigation of a novel approach in the detection of circulating tumor cells in pediatric neuroblastoma patients**

*Yu, U.<sup>2</sup>, Liu, Z.<sup>3</sup>, Chen, S.<sup>4</sup>, Xu, H.<sup>5</sup>, Chen, X.<sup>6</sup>, Liu, S.<sup>7</sup>, Yuan, X.<sup>8</sup>*

Shenzhen Children's Hospital, Department of Hematology and Oncology, Shenzhen, China <sup>2</sup>, Zigzag Biotechnology, -, Shenzhen, China<sup>3</sup>, Shenzhen Children's Hospital, Department of Hematology and Oncology, Shenzhen, China<sup>4</sup>, Shenzhen Children's Hospital, Department of Hematology and Oncology, Shenzhen, Shenzhen Children's Hospital<sup>5</sup>, Shenzhen Children's Hospital, Institute for Medical Research, Shenzhen, China<sup>6</sup>, Shenzhen Children's Hospital, Department of Hematology and Oncology, Shenzhen, China<sup>7</sup>, Shenzhen Children's Hospital, Department of Hematology and Oncology, Shenzhen, China<sup>8</sup>,

### **Background/Introduction:**

Neuroblastoma (NB) is the most common extracranial solid tumor in children. The prognosis of NB varies greatly. Children with high-risk NB often have poor prognosis. Although a variety of aggressive therapies have been taken, this situation is still not optimistic.

### **Aims:**

The aims of this study are 1) to establish a new model to capture circulating tumor cells (CTCs) in peripheral blood of NB patients; 2) to examine the efficacy of the utilization of CTCs in the monitoring of patient outcomes and to identify early relapses.

### **Methods/Materials:**

A newly designed microfluidic chip was developed for the isolation of CTCs in the peripheral blood of NB patients based on the size differences between tumor cells and normal peripheral blood cells. All red blood cells and over 99.99% of white blood cells were successfully excluded from the blood samples. The remaining cells were applied for immunofluorescence staining with markers to identify NB cells. CD45-GD2+PHOX2B<sup>+</sup> cells were classified as NB CTCs.

### **Results:**

The average capture rate of CTCs was evaluated by spiking SH-SY5Y NB cells into healthy blood samples. We examined the capture rate by spiking 6 to 87 cells per 3 ml of blood of each of the 7 donors. The capture rate varied between 83.3% and 90.8%. In sum, 93 patient blood samples were tested and 4 healthy blood samples were used as controls. CTCs were detected in none of the healthy samples. NB patients were divided into low-risk (stage 1 and 2 NB) and high-risk (stage 3 and 4) groups based on INSS classification. The average count of CTCs per 3ml of blood from the 49 low-risk patients was  $0.61 \pm 0.29$  (mean  $\pm$  SEM). In comparison, the average count of CTCs per 3ml of blood from the 46 high-risk patients was  $12.30 \pm 4.55$  (mean  $\pm$  SEM). There was a significant increase of CTC count between the high-risk group and the low-risk group (\*\*P < 0.01, Mann-Whitney U test). We tested 7 samples that were collected from newly diagnosed NB patients before the initiation of any treatment. CTCs were positively detected in all of these 7 samples, with an average count of  $6.29 \pm 2.78$  per 3ml of blood (mean  $\pm$  SEM). Furthermore, we tested 9 patients for CTCs counts both before and after chemotherapy. There were significant reductions of CTCs counts of all of these patients after chemotherapy ( $10.22 \pm 3.27$  vs.  $1.11 \pm 0.66$ , \*\*P < 0.01, Mann-Whitney U test).

### **Summary/Conclusions:**

A newly designed microfluidic chip was presented for enriching, purifying, and counting circulating tumor cells (CTCs) in peripheral blood of NB patients with high sensitivity and specificity. CTCs detection in NB patients showed promising applications in diagnosis, evaluation of treatment, and dynamic monitoring of NB disease.



## P274 Therapeutic targeting of KSP in high-risk neuroblastoma

*Hansson, K.*<sup>2</sup>, *Radke, K.*<sup>3</sup>, *Aaltonen, K.*<sup>4</sup>, *Saarela, J.*<sup>5</sup>, *Mañas, A.*<sup>6</sup>, *Sjölund, J.*<sup>7</sup>, *Smith, E.M.*<sup>8</sup>, *Påhlman, S.*<sup>9</sup>, *Wennerberg, K.*<sup>10</sup>, *Gisselsson, D.*<sup>11</sup>, *Bexell, D.*<sup>12</sup>

Lund University, Translational Cancer Research, Department of Laboratory Medicine, Lund, Sweden <sup>2</sup>, Lund University, Translational Cancer Research, Department of Laboratory Medicine, Lund, Sweden<sup>3</sup>, Lund University, Translational Cancer Research, Department of Laboratory Medicine, Lund, Sweden<sup>4</sup>, University of Helsinki, Institute for Molecular Medicine Finland, Helsinki, University of Helsinki<sup>5</sup>, Lund University, Translational Cancer Research, Department of Laboratory Medicine, Lund, Sweden<sup>6</sup>, Lund University, Translational Cancer Research, Department of Laboratory Medicine, Lund, Sweden<sup>7</sup>, Lund University, Department of Molecular Medicine and Gene Therapy, Lund Strategic Center for Stem Cell Research, Lund, Sweden<sup>8</sup>, Lund University, Translational Cancer Research, Department of Laboratory Medicine, Lund, Sweden<sup>9</sup>, University of Copenhagen, BRIC - Biotech Research & Innovation Centre, Copenhagen, Denmark<sup>10</sup>, Lund University, Division of Clinical Genetics, Department of Laboratory Medicine, Lund, Sweden<sup>11</sup>, Lund University, Translational Cancer Research, Department of Laboratory Medicine, Lund, Sweden, <sup>12</sup>

### Background/Introduction:

High-risk neuroblastoma patients have a very poor prognosis and relapse is common despite heavy treatment. Neuroblastomas exhibit relatively few recurrent and potentially druggable targets. In addition, high-risk neuroblastomas show significant inter- and intratumoral heterogeneity, suggesting that any highly specific targeting may need to be complemented with a broader treatment strategy, for example cell-cycle inhibition, to completely eradicate tumors and minimize the risk of acquired resistance.

### Aims:

To perform a high-throughput drug screen to identify and test novel treatments against high-risk neuroblastoma.

### Methods/Materials:

We used PDX-derived tumor organoids representing multiple high-risk *MYCN*-amplified neuroblastomas and performed a high-throughput drug screen consisting of approved and/or emerging oncology drugs, thereby facilitating rapid translation of candidates to the clinics. The drug screen was performed at different oxygen tensions and drugs were tested against control cells to stringently select drugs with high predicted activity but low potential for off-target effects. Selected drugs were further tested by cell viability and cell death assays, cell cycle analysis, RNA sequencing, and PDX-testing *in vivo*.

### Results:

Several novel anti-neuroblastoma drugs, approved for other conditions, were identified; we selected kinesin spindle protein (KSP) inhibition to overcome potential tumor heterogeneity effects. Bioinformatic analyses of large data sets revealed that high expression of the KSP encoding gene *KIF11* is associated with poor outcome in neuroblastoma and across multiple tumor types, *KIF11* gene dependency is highest in neuroblastoma. KSP inhibition showed efficacy in multiple high-risk neuroblastoma organoid models. Mechanistically, KSP inhibition resulted in formation of abnormal monoastrial spindles, mitotic arrest, upregulation of mitosis-associated genes, and apoptosis. *in vivo*, KSP inhibition caused complete regression of neuroblastoma PDXs and increased survival of orthotopic high-risk neuroblastoma PDXs.

### Summary/Conclusions:

Our results suggest that KSP inhibition could be a promising treatment strategy in children with high-risk neuroblastoma.





## **P275 Depletion of CD11b+ myeloid cells augments anti-neuroblastoma immune response induced by the anti-GD2 antibody dinutuximab beta**

Siebert, N.,<sup>1</sup> Von Lojewski, L.<sup>2</sup>, Von Lojewski, L.<sup>3</sup>, Troschke-Meurer, S.<sup>4</sup>, Zumpe, M.<sup>5</sup>, Marx, M.<sup>6</sup>, Lode, H.<sup>7</sup>

University Medicine Greifswald, Pediatric Hematology and Oncology, Greifswald, Germany<sup>1</sup>,  
University Medicine Greifswald, Pediatric Hematology and Oncology, Greifswald, Germany<sup>2</sup>,  
University Medicine Greifswald, Pediatric Hematology and Oncology, Greifswald, Germany<sup>3</sup>,  
University Medicine Greifswald, Pediatric Hematology and Oncology, Greifswald, Germany<sup>4</sup>,  
University Medicine Greifswald, Pediatric Hematology and Oncology, Greifswald, University Medicine  
Greifswald<sup>5</sup>, University Medicine Greifswald, Pediatric Hematology and Oncology, Greifswald,  
Germany<sup>6</sup>, University Medicine Greifswald, Pediatric Hematology and Oncology, Greifswald,  
Germany<sup>7</sup>

### Background/Introduction:

We recently showed CD11b+ myeloid cell-mediated upregulation of the PD-1/PD-L1 checkpoint on neuroblastoma (NB) cells treated with the chimeric anti-GD2 antibody dinutuximab beta and a blockade of this checkpoint increased anti-tumor efficacy of a GD2-directed treatment.

### Aims:

Here, we addressed whether depletion of CD11b+ immune suppressive cells augments immunotherapeutic effects of dinutuximab beta against NB.

### Methods/Materials:

Expression of CD11b+ myeloid cell-associated and modulating genes (*M-CSF*, *M-CSFR*, *GM-CSF*, *CCL2*, *TGF- $\beta$ 1*, *IL-1 $\beta$* , *IL-4*, *IL-6*, *IL-6R*, *CXCL8*, *IL-10*, *VEGF-A*, *Arg1*, *IDO*, *NOS2* and *IFN- $\gamma$* ) was analyzed using RT-PCR. Flow cytometry and immunohistochemistry were used to assess tumor infiltrating leukocytes. Anti-tumor effects of a GD2-directed treatment with dinutuximab beta (15 mg/kg, days 4-8 after tumor cell inoculation) in combination with depletion of CD11b+ myeloid cells by anti-CD11b Ab (25 mg/kg, days 4, 7, 11, 14 and 18) or 5-Fluorouracil (5-FU; 50 mg/kg, days 4, 11 and 18) were evaluated in a murine syngeneic NB model.

### Results:

53% of all leukocytes found in tumor tissue were CD11b+. Analysis of mRNA expression in NB cells used for tumor induction showed high expression of *M-CSF*, *TGF- $\beta$ 1*, *IL-6*, and *VEGF-A* genes that were further induced in primary tumor tissue. Although analysis of tumors revealed strong mRNA expression of all genes analyzed, the strongest induction was observed for *MCSFR*, *CCL2*, *IL-1 $\beta$* , *IL-4*, *IL-6R*, *CXCL8*, *Arg1* and *NOS2*. Compared to controls, application of anti-CD11b antibodies resulted in reduction of both tumor infiltrating CD11b+ cells and mRNA expression of the genes analyzed. Additionally, CD11b blockade showed delayed tumor growth and prolonged overall survival that could be further improved by either dinutuximab beta or 5-FU treatment. Importantly, the combinatorial immunotherapy with dinutuximab beta and 5-FU showed the strongest anti-tumor effects and superior survival rates.

### Summary/Conclusions:

Depletion of immune suppressive myeloid cells augments anti-neuroblastoma efficacy of a dinutuximab beta-based immunotherapy representing a new effective treatment strategy against GD2-positive cancers.

## **P276 A phase IIa trial of molecular radiotherapy with 177-Lutetium DOTATATE in children with primary refractory or relapsed high-risk neuroblastoma**

*Gaze, M.N.<sup>2</sup>, Gains, J.E.<sup>3</sup>, Moroz, V.<sup>4</sup>, Aldridge, M.D.<sup>5</sup>, Wan, S.<sup>6</sup>, Wheatley, K.<sup>7</sup>, Laidler, J.<sup>8</sup>, Peet, C.<sup>9</sup>, Bomanji, J.B.<sup>10</sup>*

University College London Hospitals, Oncology, London, United Kingdom <sup>2</sup>, University College London Hospitals, Oncology, London, United Kingdom<sup>3</sup>, University of Birmingham, Cancer Research UK Clinical Trials Unit, Birmingham, United Kingdom<sup>4</sup>, University College London Hospitals, Oncology, London, United Kingdom<sup>5</sup>, University College London Hospitals, Nuclear Medicine, London, United Kingdom<sup>6</sup>, University of Birmingham, Cancer Research UK Clinical Trials Unit, Birmingham, United Kingdom<sup>7</sup>, University of Birmingham, Cancer Research UK Clinical Trials Unit, Birmingham, United Kingdom<sup>8</sup>, University College London Hospitals, Oncology, London, United Kingdom<sup>9</sup>, University College London Hospitals, Nuclear Medicine, London, United Kingdom<sup>10</sup>

### **Background/Introduction:**

Current treatments for high-risk neuroblastoma are unsatisfactory, as more than half of patients will either respond poorly to induction chemotherapy, or relapse during or after therapy. Innovative treatments are required. Molecular radiotherapy with 177-Lutetium DOTATATE (LuDO) which targets somatostatin receptors significantly prolongs survival in adults with metastatic neuroendocrine cancers. Many neuroblastomas also express somatostatin receptors, and LuDO has shown promise in small pilot studies.

### **Aims:**

The objective of this Phase IIa, open label, single centre, single arm, two stage clinical trial was to evaluate the safety and activity of LuDO molecular radiotherapy in neuroblastoma.

### **Methods/Materials:**

Children with relapsed or refractory metastatic high-risk neuroblastoma were treated with up to four courses of LuDO. The sample size calculation was based on a Simon Two Stage Minimax design. The administered activity was 75 (first course) to 100 (subsequent courses) MBq kg<sup>-1</sup> per course, spaced at 8 to 12 week intervals, planned not to exceed a cumulative renal radiation dose of 23 Gy. Outcomes were assessed by the International Neuroblastoma Response Criteria (primary outcome), progression-free survival (PFS) and overall survival (OS).

### **Results:**

Forty three patients were screened for the trial, and 21 were recruited between September 2013 and July 2017. Ten were male, and 11 female. The median age was 5.4 years (range 1.7-16.9 years). Only one patient had *MYCN* amplified disease. Eight received the planned four courses, two received two courses and ten received only one course. There was dose limiting hematologic toxicity in one case, but no other significant hematologic or renal toxicity. The median cumulative renal radiation dose in the six patients who received four courses of treatment and for whom full dosimetric data were recorded was 16.5 (range 9.5 - 21.5) Gy. None of 14 evaluable patients had an objective response at one month after completion of treatment (Wilson 95% CI: is 0.0, 0.22). The trial did not therefore proceed to the second stage. Three patients had a minor reduction in the semi-quantitative 123-I mIBG scan score after two courses. Eighteen patients have died from their disease, the median follow up for survivors is 17.3 months (13.1 to 37.4). The median PFS was 2.96 months (95% CI: 1.71, 7.66), and the median OS was 13.0 months (95% CI: 3.0, 21.5).

### **Summary/Conclusions:**

In the absence of any objective responses, the use of LuDO as a single agent at the dose schedule used in this study is not recommended for the treatment of neuroblastoma. There are several reasons why this treatment schedule may not have resulted in objective responses, for example suboptimal dosing and too long between courses. As other studies do show responses, the treatment should not

be regarded as being of no value. Further trials designed to overcome this schedule's limitations are planned with international collaboration.

## **P277 Results of the NB-2015 protocol for treating neuroblastoma in China: experience from multiple centers**

Zhao, Q.<sup>2</sup>, Liu, Y.<sup>3</sup>

Tianjin Medical University Cancer Institute & Hospital, The Pediatric Cancer Department, Tianjin, China<sup>2</sup>, Tianjin Medical University Cancer Institute & Hospital, Tianjin Medical University Cancer Institute & Hospital, Tianjin, China<sup>3</sup>

### Background/Introduction:

Risk-based treatment approaches for neuroblastoma (NB) have been continuously developed for decades. The survival rates of children with NB have been improved through multidisciplinary treatment. However, the diagnosis and treatment strategies are varied across different regions and institutions in China. The collaborative efforts of the Chinese Children's Cancer Group (CCCG) have led to advances in our understanding of NB biology, a standardized classification, and stratified strategies, resulting in improved outcomes in China.

### Aims:

We aimed to assess the NB-2015 protocol formulated by the CCCG in 2015 and evaluate the extent of the impact of autologous peripheral blood stem cell transplantation (APBSCT).

### Methods/Materials:

This study enrolled patients with NB between 2015 and 2018 from seven medical centers in China. The clinical characteristics of the patients were noted, and the therapeutic effect was evaluated as the impact on survival rate.

### Results:

The data of one hundred sixty-one patients with previously untreated NB were reviewed. The 3-year overall survival (OS) and event-free survival (EFS) rates of the low-, intermediate-, and high-risk groups were 100%, 95.5% and 97.2% and 91.6%, 65.6%, and 51.1%, respectively. In total, 3 out of 7 (42.8%) patients with stage 2B disease, none of whom had *MYCN* amplifications, relapsed. A total of 47 of the 78 patients in the high-risk group underwent external-beam radiation to the primary tumor bed (dose ranging from 18 to 36 Gy). The therapeutic effect was significantly different between patients who received radiation therapy and those who did not (3-year OS  $P=0.041$ ). Within the high-risk group, the 3-year OS rates of the patients with and without APBSCT were 79.0% and 58.3%, respectively ( $P=0.014$ ). The 3-year EFS rates of the patients with and without APBSCT were 72.6% and 37.1%, respectively ( $P=0.008$ ).

### Summary/Conclusions:

This study showed that using the NB-2015 protocol achieved satisfactory results without the occurrence of fatal complications. For patients in the stage 2B group, chemotherapy should be intensified. Multidisciplinary treatment was essential; APBSCT can effectively improve the survival rate of patients with NB, especially high-risk patients. A longer follow-up period is needed to survey the survival rate and incidence of complications for Chinese NB patients who received chemotherapy, radiotherapy and APBSCT.

## **P278 Evaluation of the Effect of Trial Participation on Outcomes for High-Risk Neuroblastoma Patients: A Report from the International Neuroblastoma Risk Group Project**

*Balyasny, S.<sup>2</sup>, Lee, S.<sup>3</sup>, Desai, A.<sup>4</sup>, London, W.<sup>5</sup>, Volchenbourn, S.<sup>6</sup>, Naranjo, A.<sup>7</sup>, Bagatell, R.<sup>8</sup>, Pearson, A.<sup>9</sup>, Park, J.<sup>10</sup>, Hogarty, M.<sup>11</sup>, Schleiermacher, G.<sup>12</sup>, Irwin, M.<sup>13</sup>, Cohn, S.<sup>14</sup>, Applebaum, M.<sup>15</sup>*

Pennsylvania State University, College of Liberal Arts, State College, USA <sup>2</sup>, University of Chicago, Department of Public Health Sciences, Chicago, USA<sup>3</sup>, University of Chicago, Department of Pediatrics, Chicago, USA<sup>4</sup>, Harvard Medical School, Boston Children's Hospital and Dana-Farber Cancer Institute, Boston, Harvard Medical School<sup>5</sup>, University of Chicago, Department of Pediatrics, Chicago, USA<sup>6</sup>, University of Florida, Children's Oncology Group Statistics and Data Center, Department of Biostatistic, Gainesville, USA<sup>7</sup>, Children's Hospital of Philadelphia, Division of Oncology and Center for Childhood Cancer Research, Philadelphia, USA<sup>8</sup>, Institute of Cancer Research and Royal Marsden Hospital, Institute of Cancer Research and Royal Marsden Hospital, Surrey, United Kingdom<sup>9</sup>, University of Washington, Seattle Children's Hospital, Seattle, USA<sup>10</sup>, Children's Hospital of Philadelphia, Division of Oncology and Center for Childhood Cancer Research, Philadelphia, USA<sup>11</sup>, Institut Curie, RTOP, U830 INSERM, Paris, France, <sup>12</sup>, Hospital for Sick Children, Department of Pediatrics, Toronto, Canada <sup>13</sup>, University of Chicago, Department of Pediatrics, Chicago, USA <sup>14</sup>, University of Chicago, Department of Pediatrics, Chicago, USA <sup>15</sup>

### **Background/Introduction:**

Therapeutic trials allow for groundbreaking advancements in care, leading to new treatment options and superior survival outcomes. Even for those assigned to standard of care, participants in therapeutic trials may benefit by tight adherence to best practices per study requirements. We hypothesized neuroblastoma patients enrolled on therapeutic trials would have improved outcomes compared to biology trial patients. Historically, African Americans and other minorities have been underrepresented in therapeutic trials. Therefore, we speculated a low proportion of African American patients would enroll on neuroblastoma therapeutic trials.

### **Aims:**

We sought to determine the associations of demographics, clinical phenotype, and tumor biology with therapeutic trial participation and if trial participation impacted event-free survival (EFS).

### **Methods/Materials:**

The INRG database restricted to high-risk patients enrolled on an upfront CCG, POG, or COG therapeutic or biology trial, diagnosed from 1991-2016, was analyzed. Patients were dichotomized as either enrolled on an upfront therapeutic trial or a biology trial only. Differences between clinical and tumor factors between groups were assessed by t-tests and chi-squared tests. As outcomes for patients enrolled on ANBL0532 were not available, patients were classified pre-2006 by treatment Era (1: 1991-1998 and 2: 1999-2006). Five-year EFS was estimated using the Kaplan-Meier method and groups were compared with the log-rank test.

### **Results:**

The analytic cohort included 5,334 high-risk patients. Of these, 2,199 were enrolled on an upfront therapeutic trial (73% on CCG3891, A3973, or ANBL0532) and 3,135 were enrolled on biology trials only (94% on ANBL00B1 or POG9047). The enrollment of African American patients on therapeutic trials was similar to US census data (14.8% and 14.1%, respectively). Compared to patients enrolled only on biology trials, those enrolled on a therapeutic trial were more likely to have INSS stage 4 disease (87.0% vs. 81.6%,  $p<0.01$ ), unfavorable histology (95.7% vs. 93.8%,  $p=0.01$ ), and undifferentiated or poorly differentiated tumors (97.2% vs. 95.0%,  $p<0.01$ ). There were no detectable differences according to age, sex, *MYCN*-amplification, MKI, or ploidy. There was no difference in EFS between patients according to therapeutic trial enrollment or not for either Era 1 ( $p=0.1$ ) or Era 2 ( $p=0.2$ ). A subgroup analyses of patients diagnosed between 1991 and 1996 showed no differences in 5-year EFS for those on the control arm of CCG3891 ( $n=284$ , EFS 25.2%, 95%CI 20.6-30.9%)

compared to those only on biology trials (n=220, EFS 30.7%, 95% CI 25.2-37.6%) (p=0.3). A similar analysis comparing all patients enrolled on A3973 (n=440, EFS 39.0%, 95%CI 34.7-43.9% to biology trial patients diagnosed between 2001 and 2005 (n=547, EFS 41.8%, 95% CI 37.7-46.4%) also failed to detect a difference (p=0.3).

#### Summary/Conclusions:

The clinical characteristics and tumor biology of patients enrolled on therapeutic trials are similar to those who only participate in biology trials. Therapeutic trial participants reflect the demographics of the North American population, with high representation of African American patients. The similarity in outcomes between patients enrolled on therapeutic trials and those only registered on biology trials likely reflects a close adherence to contemporary best clinical practices for all patients regardless of trial participation.

**P279 Relative bioavailability and comparative pharmacokinetics of a novel 13-cis-retinoic acid oral liquid formulation versus drug extracted from capsules: a randomised, open label, multi-dose, cross-over clinical trial in children with neuroblastoma**

*Veal, G.J.<sup>2</sup>, Tweddle, D.A.<sup>3</sup>, Errington, J.<sup>4</sup>, Visser, J.<sup>5</sup>, Buck, H.<sup>6</sup>, Mulla, H.<sup>7</sup>*

Newcastle University, Newcastle University Centre for Cancer, Newcastle Upon Tyne, United Kingdom<sup>2</sup>, Newcastle University and Great North Children's Hospital, Newcastle University Centre for Cancer, Newcastle Upon Tyne, United Kingdom<sup>3</sup>, Newcastle University, Newcastle University Centre for Cancer, Newcastle Upon Tyne, United Kingdom<sup>4</sup>, Cambridge University Hospitals NHS Foundation Trust, Department of Paediatrics, Cambridge, Cambridge University Hospitals NHS Foundation Trust<sup>5</sup>, Nova Laboratories Limited, Clinical Consultant, Leicester, United Kingdom<sup>6</sup>, Nova Laboratories, University Hospitals of Leicester and University of Leicester, Department of Pharmacy, Leicester, United Kingdom<sup>7</sup>, , , , ,

**Background/Introduction:**

In the last decade, the administration of 13-cis-retinoic acid (13-CRA) differentiation therapy in high risk neuroblastoma (HR-NBL) patients with minimal residual disease has been proven to be effective, and reported to prevent tumour relapse after myeloablative therapy. 13-CRA is now established as standard treatment alongside immunotherapy and is administered orally using the licensed capsule form. As few young children are capable of swallowing 13-CRA capsules, carers often have to administer the drug by first extracting liquid out of the capsule. This method of administration is associated with up to 20-fold variability in patient exposure, potentially increasing the risk of treatment failure. Furthermore, the practice of extracting drug from capsules puts mothers of childbearing age at risk of accidental exposure with 13-CRA, which can cause birth defects in unborn babies. Nova Laboratories Limited have developed a convenient, ready-to-use, multi-dose, oral liquid formulation of 13-CRA, which has now been investigated in a clinical trial.

**Aims:**

The aim of the current trial was to investigate the comparative pharmacokinetics, safety, tolerability and palatability of 13-CRA oral liquid (test) and 13-CRA extracted from capsules (reference), in patients aged from 0 months to < 21 years.

**Methods/Materials:**

This was a multi-centre, open label, randomised, multiple dose, cross-over pharmacokinetic study. The decision to initiate oral 13-CRA therapy was independent of the study protocol and was conducted in patients requiring at least two 14-day treatment cycles of 13-CRA. 13-CRA was prescribed at a dose of 200mg/m<sup>2</sup>/day (if bodyweight ≥ 12kg) or 160mg/m<sup>2</sup> (if body weight < 12kg) for both test and reference product. Blood samples for pharmacokinetic analysis were taken 0.5, 1, 1.5, 2, 3, 4, 5, 6 and 24-48 hours post-dose, on Day 1 and Day 14 of each of the two treatment cycles. Primary end-points were population pharmacokinetics (of both 13-CRA and 4-oxo-13-CRA metabolite); secondary end-points were safety, tolerability and palatability of the formulations.

**Results:**

22 patients consented and 20 completed the study, across 12 sites in the UK. 12 patients received the treatment via naso-gastric tubes, 8 orally. The pharmacokinetics of 13-CRA were adequately described by a one-compartment linear model. Drug formulation had an effect on the relative bioavailability and absorption kinetics of 13-CRA. Absorption of the oral liquid was best described by transit compartments, indicating a delay in absorption. No such delay was observed for the capsule. The first order absorption parameter for the oral liquid was 3-fold higher than the capsule, indicating a faster absorption compared to the capsule, once the delay period is over. Relative bioavailability was estimated to be 1.23 (23% increase for oral liquid vs capsule), although the CI was wide (0.81 - 1.65). 22 serious adverse events were reported (2 SUSARS) but there were no safety concerns during the

study and no differences between the two formulations. Palatability feedback was good for the oral liquid and parents preferred the treatment to the capsule formulation.

**Summary/Conclusions:**

The pharmacokinetics, safety, tolerability and palatability of a novel oral liquid formulation of 13-CRA compare favourably to 13-CRA extracted from the licensed capsule formulation.



## **P280 Nivolumab and dinutuximab beta in refractory neuroblastoma**

*Ehlert, K.<sup>2</sup>, Hansjuergens, I.<sup>3</sup>, Zinke, A.<sup>4</sup>, Otto, S.<sup>5</sup>, Siebert, N.<sup>6</sup>, Henze, G.<sup>7</sup>, Lode, H.<sup>8</sup>*

Greifswald University Medicine, Pediatric Hematology and Oncology, Greifswald, Germany <sup>2</sup>, Greifswald University Medicine, Pediatric Hematology and Oncology, Greifswald, Germany<sup>3</sup>, Greifswald University Medicine, Department of Nuclear Medicine, Greifswald, Germany<sup>4</sup>, Greifswald University Medicine, Institute for Diagnostic Radiology, Greifswald, Greifswald University Medicine<sup>5</sup>, Greifswald University Medicine, Pediatric Hematology and Oncology, Greifswald, Germany<sup>6</sup>, Charite University Medicine Berlin, Campus Virchow Klinikum, Pediatric Oncology and Hematology, Berlin, Germany<sup>7</sup>, Greifswald University Medicine, Pediatric Hematology and Oncology, Greifswald, Germany<sup>8</sup>

### **Background/Introduction:**

Already at first diagnosis, more than 50% of patients present with widespread or advanced localized neuroblastoma are at high risk of treatment failure. In these patients, event-free and overall survival after multimodal conventional therapy have been improved by the addition of dinutuximab beta, an anti-GD<sub>2</sub> antibody. The prognosis of children and adolescents with refractory or relapsed neuroblastoma remains poor, however. In the past decade, immunotherapy approaches with checkpoint inhibitors for patients with malignant diseases have gained increasing interest.

### **Aims:**

Evaluate the tolerability and response to treatment in patients with refractory neuroblastoma under the novel immunotherapeutic approach of nivolumab with dinutuximab beta.

### **Methods/Materials:**

Two patients with refractory neuroblastoma, a 9-year old girl and a 26-year old man, were treated with nivolumab (3mg/kg BW every second week) and dinutuximab beta (100mg/m<sup>2</sup> BSA every 6 weeks) for 10 months and 6 months, respectively. MRI and mIBG or PET-CT were checked every 3-5 months.

### **Results:**

Both patients had been treated with any available conventional therapies in the past including treatment with dinutuximab beta + interleukin-2. After the primary diagnosis of neuroblastoma in 2014, the girl had reached a first remission in 2015, but relapsed in January 2017. Combined treatment with chemotherapy and dinutuximab beta from December 2017 to September 2018 had resulted in transient improvement, but then again in progressive disease. The young man's primary diagnosis was in April 2010. He experienced local relapses in 2011, 2012 and 2014. In June 2018, a metastatic relapse and a large abdominal tumor bulk were found. After transient improvement with chemotherapy, tumor surgery and radiotherapy, progressive skeletal metastases were observed in May 2019. As both patients still had a satisfactory quality of life, treatment with dinutuximab beta and nivolumab was initiated. Seven months after starting this therapy, the girl achieved a second complete remission. She is living without disease 3 months after the end of therapy. In the second patient, the soft tissue lesions have disappeared, and his skeletal lesions have regressed after 6 months of therapy.

### **Summary/Conclusions:**

The novel combination of the anti-GD<sub>2</sub>-antibody dinutuximab beta with the checkpoint inhibitor nivolumab was capable of inducing a complete and a very good partial remission in two heavily pre-treated patients with refractory neuroblastoma. Further trials are warranted to clarify the role of this novel approach in a larger number of patients.

## **P281 RESULTS OF HIGH-DOSE TREOSULFAN-CONTAINING REGIMEN IN PATIENTS WITH HIGH-RISK NEUROBLASTOMA**

*Shamanskaya, T.,<sup>1</sup> Kachanov, D.<sup>2</sup>, Kachanov, D.<sup>3</sup>, Hachatryan, L.<sup>4</sup>, Khismatullina, R.<sup>5</sup>, Moiseenko, R.<sup>6</sup>, Teleshova, M.<sup>7</sup>, Skorobogatova, E.<sup>8</sup>, Druy, A.<sup>9</sup>, Zemcova, L.<sup>10</sup>, Kurnikova, E.<sup>11</sup>, Tereschenko, G.<sup>12</sup>, Nechesnyuk, A.<sup>13</sup>, Akhaladze, D.<sup>14</sup>, Fomin, D.<sup>15</sup>, Grachev, N.<sup>16</sup>, Likar, Y.<sup>17</sup>, Maschan, M.<sup>18</sup>, Varfolomeeva, S.<sup>19</sup>, Novichkova, G.<sup>20</sup>*

Dmitry Rogachev National Research Center of Pediatric Hematology, Oncology, Clinical Oncology, Moscow, Russia<sup>1</sup>, Dmitry Rogachev National Research Center of Pediatric Hematology, Oncology, Clinical Oncology, Moscow, Russia<sup>2</sup>, Dmitry Rogachev National Research Center of Pediatric Hematology, Oncology, Clinical Oncology, Moscow, Russia<sup>3</sup>, Dmitry Rogachev National Research Center of Pediatric Hematology, Oncology, Pediatrics, Moscow, Russia<sup>4</sup>, Dmitry Rogachev National Research Center of Pediatric Hematology, Oncology, Bone marrow transplantation 1, Moscow, Dmitry Rogachev National Research Center of Pediatric Hematology, Oncology<sup>5</sup>, Dmitry Rogachev National Research Center of Pediatric Hematology, Oncology, Clinical Oncology, Moscow, Russia<sup>6</sup>, Dmitry Rogachev National Research Center of Pediatric Hematology, Oncology, Clinical Oncology, Moscow, Russia<sup>7</sup>, Russian Children's Clinical Hospital, Bone marrow transplantation, Moscow, Russia<sup>8</sup>, Dmitry Rogachev National Research Center of Pediatric Hematology, Oncology, Molecular Oncology, Moscow, Russia<sup>9</sup>, Dmitry Rogachev National Research Center of Pediatric Hematology, Oncology, Cytogenetics, Moscow, Russia<sup>10</sup>, Dmitry Rogachev National Research Center of Pediatric Hematology, Oncology, Transfusion Medicine, Moscow, Russia<sup>11</sup>, Dmitry Rogachev National Research Center of Pediatric Hematology, Oncology, Radiology, Moscow, Russia<sup>12</sup>, Dmitry Rogachev National Research Center of Pediatric Hematology, Oncology, Radiation Oncology, Moscow, Russia<sup>13</sup>, Dmitry Rogachev National Research Center of Pediatric Hematology, Oncology, Surgery, Moscow, Russia<sup>14</sup>, Russian Scientific Center of Roentgenoradiology, Nuclear Medicine, Moscow, Russia<sup>15</sup>, Dmitry Rogachev National Research Center of Pediatric Hematology, Oncology, Surgery, Moscow, Russia<sup>16</sup>, Dmitry Rogachev National Research Center of Pediatric Hematology, Oncology, Nuclear Medicine, Moscow, Russia<sup>17</sup>, Dmitry Rogachev National Research Center of Pediatric Hematology, Oncology, Bone marrow transplantation, Moscow, Russia<sup>18</sup>, N.N. Blokhin National Medical Research Centre of Oncology, Pediatric Oncology, Moscow, Russia<sup>19</sup>, Dmitry Rogachev National Research Center of Pediatric Hematology, Oncology, Hematology, Moscow, Russia<sup>20</sup>

### **Background/Introduction:**

High-dose chemotherapy is reduced the risk of relapse in high-risk neuroblastoma (NB). Busulfan-containing regimens are considered to be the standard, while the efficacy of treosulfan has not yet been defined.

### **Aims:**

The aim of the study was to analyze the results of therapy of high-risk NB using treosulfan/ melphalan conditioning regimen.

### **Methods/Materials:**

171 patients with high-risk NB were treated for the period 01.2012-12.2018 (84 months). Patients were stratified and treated according to the modified German NB2004 protocol. Patients with *MYCN*-amplified stage 1 were included. 29 (17%) patients didn't receive hematopoietic stem cell transplantation (HSCT). Among 142 transplanted patients high-dose preparative regimens included carboplatin/etoposide/melphalan (CEM) (till June 2013) in 28 (19.7%) cases and treosulfan/melphalan (TreoMel) (since July 2013) in 114 (80.3%). Since July 2014 patients with clear MIBG-positive residual primary tumor and/or metastases prior to HSCT received <sup>131</sup>I-MIBG-therapy. Database was locked on 20.12.2019.

### **Results:**

114 patients who received Treo/Mel were included in the analysis. Male: female ratio was 1.15:1. The median age at the diagnosis was 33.4 months (range 1.3-169.6). Age was older than 12 months in 100 (87.7%) and 18 months in 93 (81.5%) cases. Adrenal gland/retroperitoneum was the primary site in 99 (86.8%) cases. *MYCN* amplification was observed in 53 (46.4%) cases. 95 (83.3%) patients had stage 4 NB. Distribution of patients according to the response prior to HSCT was as follows: complete - 24 (21.0%), very good partial - 34 (29.8%), partial - 46 (40.4%), mixed response - 5 (4.4%), stable disease - 5 (4.4%).

Median follow-up time for all patients was 36.7 months (range 5.6-74.8), for alive patients 43.5 months (range 10.7-74.8). 3-year and 5-year EFS was 42.9% (95%CI 33.0-52.8) and 38.0% (95%CI 27.7-48.3). 3-year and 5-year OS - 71.5% (95%CI 62.4-80.6%) and 61.4% (95%CI 50.3-72.6). One patient died as a result of transplant-related complications. There were no cases of veno-occlusive disease, including patients who underwent MIBG-therapy.

Cumulative incidence of relapse at 36 and 60 months was 56.2% (95%CI 47.1-67.1) and 61.1% (95% CI 51.7-72.4), transplant-related mortality (TRM) 0.9% (95%CI 0.1-6.3).

Univariate analysis showed significantly better EFS in children < 18 months (3-year EFS 75.9% (95%CI 57.5-94.3) vs. 35.0% (95%CI 24.3-45.7) in patients > 18 months,  $p=0.01$ ) and patients with stage 1-3/4S (82.6% (95%CI 64.4-100.0) vs. 34.9% (95%CI 24.4-45.3) in stage 4,  $p=0.004$ ). Topography of the primary tumor and response prior to HSCT didn't impact EFS. Multivariate analysis showed that stage was the independent prognostic factor for EFS (HR 3.4, 95% CI 1.2-9.7).

The only factor that impacted the OS was stage: 3-year OS was 89.5 (95%CI 75.7-100.0) in patients with stage 1-3/4S versus 67.8% (95%CI 57.5-78.2) in stage 4 ( $p=0.02$ ). Age at diagnosis, topography of the tumor and response didn't impact OS.

Presence of *MYCN* amplification significantly decreased EFS ( $p=0.008$ ) and OS ( $p=0.001$ ) in stage 4 patients with know *MYCN* status ( $n=91$ ).

#### Summary/Conclusions:

We presented the results of treatment of a large cohort of high-risk NB patients who received treosulfan-containing conditioning regimen. Treosulfan-melphalan regimen was associated with low TRM and safe use in patients who had received MIBG-therapy. Direct comparison with therapeutic programs based on busulfan-containing conditioning regimen is not possible due to different induction chemotherapy in NB2004 and SIOPEN protocols. Additional studies are needed to assess the long-term prognosis of patients who received treosulfan.

## **P282 THE EVALUATION OF THE TREATMENT RESULTS ACCORDING TO THE RELATIONSHIP OF THE RENAL VESSELS AND TUMOR IN LOW-RISK PATIENTS WITH ABDOMINAL NEUROBLASTOMA – AN ANALYSIS OF THE PATIENTS WHO ENROLLED IN THE LOW-RISK PROTOCOL (JN-L-10) FROM THE JAPAN CHILDREN'S**

*Yoneda, A.<sup>2</sup>, Iehara, T.<sup>3</sup>, Yokota, I.<sup>4</sup>, Takahashi, H.<sup>5</sup>, Teramukai, S.<sup>6</sup>, Kamijyo, T.<sup>7</sup>, Nakazawa, A.<sup>8</sup>, Takimoto, T.<sup>9</sup>, Kikuta, A.<sup>10</sup>, Ikeda, H.<sup>11</sup>, Nakagawara, A.<sup>12</sup>, Tajiri, T.<sup>13</sup>*

Osaka City General Hospital, Pediatric Surgery, Osaka, Japan <sup>2</sup>, Japan Children's Cancer Group (JCCG) Neuroblastoma Committee (JNBSG), Pediatrics, Kyoto, Japan<sup>3</sup>, Japan Children's Cancer Group (JCCG) Neuroblastoma Committee (JNBSG), Biostatistics, Kyoto, Japan<sup>4</sup>, Japan Children's Cancer Group (JCCG) Neuroblastoma Committee (JNBSG), Biostatistics, Kyoto, Japan Children's Cancer Group (JCCG) Neuroblastoma Committee (JNBSG)<sup>5</sup>, Japan Children's Cancer Group (JCCG) Neuroblastoma Committee (JNBSG), Biostatistics, Kyoto, Japan<sup>6</sup>, Japan Children's Cancer Group (JCCG) Neuroblastoma Committee (JNBSG), Research Institute for Clinical Oncology, Kyoto, Japan<sup>7</sup>, Japan Children's Cancer Group (JCCG) Neuroblastoma Committee (JNBSG), Clinical Research, Kyoto, Japan<sup>8</sup>, Japan Children's Cancer Group (JCCG) Neuroblastoma Committee (JNBSG), Clinical Epidemiology, Kyoto, Japan<sup>9</sup>, Japan Children's Cancer Group (JCCG) Neuroblastoma Committee (JNBSG), Pediatric Oncology, Kyoto, Japan<sup>10</sup>, Japan Children's Cancer Group (JCCG) Neuroblastoma Committee (JNBSG), Pediatric Surgery, Kyoto, Japan<sup>11</sup>, Japan Children's Cancer Group (JCCG) Neuroblastoma Committee (JNBSG), Clinical Oncology, Kyoto, Japan, <sup>12</sup>, Japan Children's Cancer Group (JCCG) Neuroblastoma Committee (JNBSG), Pediatric Surgery, Kyoto, Japan <sup>13</sup>

### **Background/Introduction:**

Image-defined risk factors (IDRFs) have been used to evaluate surgical risks before any treatments. An INRG staging system was developed based on the clinical criteria and IDRFs. The Japan Children's Cancer Group (JCCG) Neuroblastoma Committee (JNBSG) conducted the JN-L-10 for low-risk patients using IDRFs as the main factor for determining treatment. According to the new guideline (NG) for assessing IDRFs (Brisse HJ, et al. Radiology, 2011), patients should be considered "IDRF-present", even if the tumor is in contact with the renal vessels only, despite being previously diagnosed as "IDRF-not present." However, this new definition may change the IDRF status in a considerable number of patients.

### **Aims:**

We analyzed the treatment results of the patients who enrolled in the JN-L-10 according to the relationship between the renal vessels and tumor in order to clarify whether or not "contact with renal vessels" should be considered an IDRF.

### **Methods/Materials:**

Of the 60 patients enrolled in JN-L-10, the 37 with an abdominal tumor who had precise information available for IDRFs were selected in this study. Patients were divided into the 3 groups according to the relationship between the renal vessels and tumor: Group S (n=14), separated; Group C (n=16), in contact; Group E (n=7), encased. We analyzed the treatment results, including the surgical results, such as operation time, intra-operative blood loss and surgical complications. Data were shown as the median with the range.

### **Results:**

In Groups S, C and E, 5 (35.7%), 2 (12.5%) and 4 (57.1%) patients did not undergo any surgery other than a biopsy until the end of the treatment, 9 (64.3%), 13 (81.3%) and 0 (0%) patients underwent primary resection, and 0 (0%), 1 (6.3%) and 3 (42.9%) patients underwent resection after neoadjuvant chemotherapy, respectively. Therefore, 26 of 37 patients (9 in Group S, 14 in Group C, 3 in Group E) underwent surgery rather than a biopsy. In Groups S, C and E among these 26 patients, the operation time (minutes) was 161.5 (85-304), 168.5 (103-500) and 235.0 (207-268), and the intra-operative

blood loss (ml) was 10 (0-25), 44 (5-941) and 103 (85-144), respectively. There were some trends, but no significant differences were noted among the three groups in the operation time or intra-operative bleeding. Surgical complications were observed in four patients, all in Group C (ages: 1, 4, 76 and 134 months). Three of these four patients underwent primary resection. The surgical complications were as follows: class IV intra-operative bleeding, partial renal atrophy, vena cava injury and adrenal vein injury.

#### Summary/Conclusions:

Group E patients tended to have a longer operation time and greater intra-operative bleeding than other patients. Surgical complications were observed only in Group C patients. Although there was only one patient who suffered a complication directly related to the renal vessels, 'contact with renal vessels' might be a potential surgical risk for abdominal neuroblastoma. Whether or not neoadjuvant chemotherapy can reduce the surgical risks in Group C patients should be prospectively investigated in a larger cohort study in the future.

## **P283 Long-term outcome and role of biology within risk-adapted treatment strategies: The Austrian neuroblastoma trial A-NB94**

*Fiedler, S.<sup>2</sup>, Ambros, I.M.<sup>3</sup>, Glogova, E.<sup>4</sup>, Benesch, M.<sup>5</sup>, Urban, C.<sup>6</sup>, Mayer, M.<sup>7</sup>, Ebetsberger-Dachs, G.<sup>8</sup>, Bardi, E.<sup>9</sup>, Jones, N.<sup>10</sup>, Gamper, A.<sup>11</sup>, Meister, B.<sup>12</sup>, Crazzolara, R.<sup>13</sup>, Amann, G.<sup>14</sup>, Dieckmann, K.<sup>15</sup>, Horcher, E.<sup>16</sup>, Ziegler, A.<sup>17</sup>, Ambros, P.F.<sup>18</sup>, Ladenstein, R.<sup>19</sup>*

St. Anna Children's Hospital, Department of Pediatric Hematology and Oncology, Vienna, Austria <sup>2</sup>, St. Anna Children's Cancer Research Institute, Tumor Biology, Vienna, Austria<sup>3</sup>, St. Anna Children's Cancer Research Institute, SSIRP, Vienna, Austria<sup>4</sup>, Medical University Graz, Medical University Graz, Graz, Medical University Graz<sup>5</sup>, Medical University Graz, Division of Paediatric Haematology and Oncology, Department of Paediatrics and A, Graz, Austria<sup>6</sup>, Medical University Graz, Medical University of Graz, Graz, Austria<sup>7</sup>, Kepler Medical University Linz, Kepler Medical University Linz, Linz, Austria<sup>8</sup>, St. Anna Children's Hospital, Department of Pediatric Hematology and Oncology, Vienna, Austria<sup>9</sup>, Paracelsus Medical University Salzburg, Paracelsus Medical University Salzburg, Salzburg, Austria<sup>10</sup>, Paracelsus Medical University Salzburg, Paracelsus Medical University Salzburg, Salzburg, Austria<sup>11</sup>, Medical University Innsbruck, Medical University of Innsbruck, Innsbruck, Austria, <sup>12</sup>, Medical University Innsbruck, Medical University Innsbruck, Innsbruck, Austria <sup>13</sup>, Medical University Vienna, Department of Pathology, Vienna, Austria <sup>14</sup>, Medical University Vienna, Department of Radiotherapy, Vienna, Austria <sup>15</sup>, Medical University Vienna, Department of Pediatric Surgery, Vienna, Austria <sup>16</sup>, St. Anna Children's Cancer Research Institute, Tumor Biology, Vienna, Austria <sup>17</sup>, St. Anna Children's Cancer Research Institute, Tumor Biology, Vienna, Austria <sup>18</sup>, St. Anna Children's Cancer Research Institute, SSIRP, Vienna, Austria <sup>19</sup>

### Background/Introduction:

The Austrian neuroblastoma trial A-NB94 was initiated in 1994 to apply risk-adapted treatment intensities based on then recognized factors of tumor stage by the International Neuroblastoma Staging System (INSS), age at diagnosis, and *MYCN* oncogene amplification (MNA) status.

### Aims:

To evaluate the long-term outcomes and association with genomic profiles in the Austrian Neuroblastoma Trial A-NB94 applying a risk-adapted strategy of treatment (RAST).

### Methods/Materials:

RAST depended on INSS stage, age ( $\leq$ / $>$ 12 months), and MNA status and included a surgery only approach in the low to intermediate risk group with up to 6 cycles of CV (cyclophosphamide, vincristine) or CAV (cyclophosphamide, doxorubicin, vincristine) alternated with CBDCA/VP16 (carboplatin, etoposide) depending on age and response. Stage 4  $\leq$ / $>$ 12 months with MNA and stage 4  $>$ 12 months independent of *MYCN* status were considered high-risk patients and received six alternating cycles of HD-CAV and CDDP/VP16 with surgery after 4 cycles. Thereafter, they received a response-depending high-dose chemotherapy (HDT), either a single HDT (etoposide, carboplatin, melphalan) or multiple courses of HDT (1<sup>st</sup> course: thiotepa, carboplatin; 2<sup>nd</sup> course: thiotepa, cyclophosphamide; 3<sup>rd</sup> course: melphalan, etoposide) followed by autologous stem cell rescue (ASCR). Irradiation to the primary tumor site was reserved for MNA tumors (24 to 30 Grey, depending on age). In 1996, anti-GD<sub>2</sub> ch14.18/SP0/2 became available to optimize treatment for HR patients. Isotretinoin was not part of the concept. Segmental chromosomal alterations (SCAs) were investigated retrospectively using multi- and pan-genomic techniques. The median follow-up time was 12 years.

### Results:

The A-NB94 trial enrolled 163 patients. The proportion of patients with localized disease was high (n=109;  $\leq$ 12 months n=41,  $>$ 12 months n=68), likely related to the Austrian screening program. Their ten-year event free survival (10y-EFS) and overall survival (10y-OS) was excellent as apart from one unrelated death all patients became long-term survivors. Stage 4S infants (n=10) had a 10y-EFS and

OS of  $80\pm 13\%$  and  $90\pm 9\%$ , respectively, and infants with non-MNA stage 4 ( $n=6$ )  $83\pm 15\%$  each. High-risk patients (stage 4, either  $>12$  months or MNA,  $n=38$ ), receiving intensified induction (response rate 74%) and, depending on response, one or multiple courses of HDT (response rate 85%), had a 10y-EFS of  $45\pm 8\%$  and 10y-OS of  $47\pm 8\%$ . SCAs were present in increasing frequencies according to stage and age: in 29% of localized tumors but in 92% of stage 4 tumors ( $p<0.001$ ), and in 39% of patients  $\leq 12$  months but in 63% of patients  $>12$  months ( $p<0.001$ ).

#### Summary/Conclusions:

RAST achieved remarkable long-term results, reducing exposure to chemotherapy in low- and intermediate-risk patients successfully. Notably, the intensified HDT approach for high-risk patients showed very good response rates and markedly improved long-term outcomes along with acceptable toxicity profiles and morbidity. SCAs were significantly correlated with stage and age.

## **P284 EGFR inhibitors are effective against chemoresistant neuroblastoma models**

*García, M.<sup>2</sup>, Gómez, S.<sup>3</sup>, Rodríguez-Hernández, C.J.<sup>4</sup>, Mateo-Lozano, S.<sup>5</sup>, Carcaboso, A. M.<sup>6</sup>, Mora, J.<sup>7</sup>, Lavarino, C.<sup>8</sup>*

Fundació Sant Joan de Déu, Developmental Tumor Biology Laboratory, Barcelona, Spain <sup>2</sup>, Fundació Sant Joan de Déu, Developmental Tumor Biology Laboratory, Barcelona, Spain<sup>3</sup>, Fundació Sant Joan de Déu, Developmental Tumor Biology Laboratory, Barcelona, Spain<sup>4</sup>, Fundació Sant Joan de Déu, Developmental Tumor Biology Laboratory, Barcelona, Spain<sup>5</sup>, Fundació Sant Joan de Déu, Developmental Tumor Biology Laboratory, Barcelona, Spain<sup>6</sup>, Fundació Sant Joan de Déu, Developmental Tumor Biology Laboratory, Barcelona, Spain<sup>7</sup>, Fundació Sant Joan de Déu, Developmental Tumor Biology Laboratory, Barcelona, Spain<sup>8</sup>

### **Background/Introduction:**

Patients with high-risk neuroblastoma (HR NB) are treated with multimodal therapy including intensive chemotherapy, radiation, surgery and immunotherapy. Approximately 60% of HR NB patients that initially respond to chemotherapy will eventually suffer disease relapse and/or develop resistance to conventional treatments. Recent studies have shown that molecular profiles of relapsed NB tumors are significantly different from those at diagnosis mainly because of genetic aberrations induced and/or selected by chemoradiotherapy. In most cancers, including HR NB, epidermal growth factor receptor (EGFR) expression increases in therapy-induced chemoresistant cancer cells. Preclinical studies have provided evidence that treatment with EGFR-specific tyrosine kinase (RTKs) inhibitors results in suppression of tumor growth and increases the sensitivity to diverse cytotoxic drugs in chemoresistant cancer cell lines.

### **Aims:**

We established *in vitro* and *in vivo* chemoresistant NB models to explore the activity of EGFR inhibitors and provide insights into the biology of the chemorefractory disease.

### **Methods/Materials:**

We generated five NB cell line models with induced resistance to doxorubicin, cisplatin and vincristine by long-term exposure to increasing drug concentrations. Drug sensitivity was measured overtime using the half maximal inhibitory concentration (IC<sub>50</sub>) value for each cytotoxic drug. Molecular markers associated to drug resistance were evaluated by RTqPCR in chemoresistant cells as well as *in vitro* functional assays. Genome-wide expression profiles were obtained for paired chemoresistant and non-chemoresistant cell lines to explore the effect of drugs. Drug sensitivity and proliferation assays using an EGFR inhibitor were performed in chemoresistant cells. The *in vivo* efficacy of EGFR inhibitor treatment was evaluated by measuring tumor growth in paired chemoresistant and non-chemoresistant HR NB patient-derived xenograft (PDX) models.

### **Results:**

Induced chemoresistant NB cell lines showed a marked increase of the IC<sub>50</sub> values and significant changes in cell morphology as compared to the non-treated counterparts. Increased relative expression of ABC transporters, stemness, EMT and DNA damage markers were found in chemoresistant cells. Transcriptome analysis showed differential gene expression profiles associated to drug resistance mechanisms that differed depending on the cytotoxic drug used in the distinct cell line models. *in vitro* results revealed the irreversible EGFR inhibitor canertinib as the most sensitive RTK tested in NB cells. Combination of the EGFR inhibitor with cytotoxic drugs was significantly more toxic than treatment with single drugs. *in vivo* treatment with canertinib showed a significant decrease in the tumor growth for the chemoresistant PDX model.

### **Summary/Conclusions:**



Canertinib in combination with chemotherapy represents a promising therapeutic strategy for chemoresistant HR NB. Ongoing experimental work will also provide further insights into the molecular mechanisms and signaling pathways involved in drug resistance in NB.

## **P285 Metformin can enhance the anti-neuroblastoma effect of ketogenic diet**

*Kofler, B.*<sup>2</sup>, *Catalano, L.*<sup>3</sup>, *Aminzadeh-Gohari, S.*<sup>4</sup>, *Weber, D.*<sup>5</sup>, *Vidali, S.*<sup>6</sup>, *Feichtinger, R.*<sup>7</sup>, *Felder, T.*<sup>8</sup>, *Tevini, J.*<sup>9</sup>

Department of Pediatrics, Paracelsus Medical University, Research Program for Receptor Biochemistry and Tumor Metabolism, Salzburg, Austria<sup>2</sup>, Department of Pediatrics, Paracelsus Medical University, Research Program for Receptor Biochemistry and Tumor Metabolism, Salzburg, Austria<sup>3</sup>, Department of Pediatrics, Paracelsus Medical University, Research Program for Receptor Biochemistry and Tumor Metabolism, Salzburg, Austria<sup>4</sup>, Department of Pediatrics, Paracelsus Medical University, Research Program for Receptor Biochemistry and Tumor Metabolism, Salzburg, Austria<sup>5</sup>, Department of Pediatrics, Paracelsus Medical University, Research Program for Receptor Biochemistry and Tumor Metabolism, Salzburg, Austria<sup>6</sup>, Department of Pediatrics, Paracelsus Medical University, Research Program for Receptor Biochemistry and Tumor Metabolism, Salzburg, Austria<sup>7</sup>, Paracelsus Medical University, Institute for Laboratory Medicine, Salzburg, Austria<sup>8</sup>, Paracelsus Medical University, Institute for Laboratory Medicine, Salzburg, Austria<sup>9</sup>, ,

### **Background/Introduction:**

Neuroblastoma (NB), like other solid tumors, is characterized by a high dependency on glycolysis and low but still functional oxidative phosphorylation (OXPHOS), a biochemical phenomenon also known as Warburg effect. Our previous studies showed that a high-fat, low carbohydrate diet (ketogenic diet; KD) can successfully target NB. The glucose dependency combined with a decreased capacity to utilize alternative substrates by OXPHOS sensitizes NB cells to KD. In addition, owing to the KD's low-carbohydrate content, the KD not only reduces the circulating glucose, but consequently also lowers insulin and IGF-1 levels. Recent studies suggested that the inhibition of residual OXPHOS activity can be beneficial in cancer treatment. Metformin (MET), an anti-diabetic drug, reduces the blood glucose levels and targets complex I of the OXPHOS. In agreement, Kumar et al. have reported that MET inhibits NB tumor growth *in vivo* (Kumar et al. *Oncotarget*, 2014).

### **Aims:**

The aim of this study was to elucidate whether MET can enhance the anti-NB effects of a KD.

### **Methods/Materials:**

The NB cell lines SKNBE (2) (*MYCN*-amplified) and SH-SY5Y (non-*MYCN*-amplified) were treated for 3 days with different concentrations of MET and cell viability was determined by a cell viability assay using 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTT). NB xenografts were established in CD-1 nude mice with SKNBE (2) cells. The NB bearing mice were fed with a control diet (CTRL) and a KD (ketogenic ratio 8:1, supplemented with medium-chain triglycerides) with/without MET (oral gavage, 100 mg/kg body weight). Tumor growth, body weight, blood glucose, and ketone body concentration, were monitored every fourth day. Plasma and tumor samples were collected for molecular analysis.

### **Results:**

*in vitro* treatment of SKNBE(2) and SH-SY5Y cells with MET resulted in a dose dependent decrease of cancer cell proliferation. In contrast to the literature, MET alone did not reduce tumor growth *in vivo*. However, MET enhanced the anti-NB effects of KD and improved the survival rate of SKNBE(2) xenograft bearing mice. The MET concentration in plasma was not influenced by the administration of KD. Only the combination of KD and MET reduced blood glucose levels although after 13 days of therapy they returned to the levels of the CTRL diet group. As expected, KD led to higher levels of ketosis in the KD and KD + MET groups, whereas MET alone had no effect on blood ketone body levels. Moreover, CD31 staining revealed a significant reduction of tumor vessel density in the KD+MET group compared to the CTRL group.

Summary/Conclusions:

In contrast to the study of Kumar et al. (Oncotarget, 2014), MET alone had no effect on NB growth. However, our data suggest that MET could be used to enhance the anti-NB effect of KD and therefore, it could represent a new adjuvant multi modal metabolic therapy.

## **P286 Apoptosis, but not necroptosis is induced by specific aurora A kinase inhibitors in IMR-32 and CHP-134 neuroblastoma cells.**

*Durbas, M.*<sup>2</sup>, *Wisniewska, A.*<sup>3</sup>, *Rokita, H.*<sup>4</sup>, *Horwacik, I.*<sup>5</sup>

Jagiellonian University, Faculty of Biochemistry, Biophysics and Biotechnology, Laboratory of Molecular Genetics and Virology, Krakow, Poland <sup>2</sup>, Jagiellonian University, Faculty of Biochemistry, Biophysics and Biotechnology, Laboratory of Molecular Genetics and Virology, Krakow, Poland<sup>3</sup>, Jagiellonian University, Faculty of Biochemistry, Biophysics and Biotechnology, Laboratory of Molecular Genetics and Virology, Krakow, Poland<sup>4</sup>, Jagiellonian University, Faculty of Biochemistry, Biophysics and Biotechnology, Laboratory of Molecular Genetics and Virology, Krakow, Jagiellonian University, Faculty of Biochemistry, Biophysics and Biotechnology<sup>5</sup>

### Background/Introduction:

Aurora A kinase is highly expressed in multiple human tumors, including neuroblastoma. Previously we have shown that the specific, small-molecule inhibitor of aurora A kinase, MK-5108, combined with the GD2 ganglioside-specific 14G2a mAb significantly enhances cytotoxic effect against IMR-32 and CHP-134 cells [Horwacik et al., 2013, Durbas et al., 2018]. However, molecular mechanism of aurora A kinase inhibition by MK-5108 in neuroblastoma cells needs to be better characterised.

### Aims:

We investigated the cytotoxic potential of MK-5108 in the regulation of apoptosis or necroptosis in neuroblastoma cells.

### Methods/Materials:

To assess the response of neuroblastoma cells to small-molecule inhibitors we measured expression and phosphorylation of various proteins by western blot and cellular ATP levels by luminescence assays.

### Results:

MK-5108 treatment induced the expression of crucial apoptosis markers i.e., cleaved caspase-3 and cleaved-PARP proteins at 72 h in IMR-32 and CHP-134 cells, as assessed by western blot. Additionally, the activities of caspase 3 and 7 were increased in MK-5108-treated IMR-32 and CHP-134 cells, confirming the induction of apoptosis in these neuroblastoma cultures. To investigate the involvement of necroptosis in MK-5108 treatment, we assessed the expression and phosphorylation of key necroptosis-associated proteins i.e., receptor-interacting serine/threonine-protein kinases (RIPs) and mixed lineage kinase domain-like pseudokinase (MLKL). We observed the elevated phosphorylation of RIP3 and significantly increased phosphorylation levels of MLKL in IMR-32 cells. Although, in CHP-134, the phosphorylation level of RIP1 and RIP3 were markedly increased, there was no increase of MLKL phosphorylated form. The expression level of unphosphorylated RIP1 and RIP3 kinases remained unchanged both in IMR-32 and CHP-134. To verify the involvement of necroptosis in MK-5108-stimulated cell death, we used specific inhibitors of RIP1 and RIP3 kinases i.e., necrostatin-1 and GSK'872, respectively. No recovery of cytotoxic effect, as assessed by ATP level, was observed in MK-5108 and necroptosis inhibitors-co-treated cells, as compared to MK-5108 used alone, proving a lack of contribution of necroptosis in the observed cytotoxic effect. Interestingly, inhibition of RIP1 and RIP3 kinases combined with using MK-5108 inhibitor decreased ATP level, advancing the observed cytotoxic effect, as compared to monotherapy with MK-5108 inhibitor. Moreover, the combination of MK-5108 inhibitor and necrosatin-1/GSK'872 significantly increased the activities of caspase 3 and 7, proving better efficacy of the proposed combination therapy. We report that similar results were also obtained for another aurora A kinase inhibitor, MK-8745, which caused increased expression of key apoptosis-associated molecules and increased phosphorylation of RIP3 in both cell lines.

### Summary/Conclusions:

These studies demonstrate that pharmacological inhibition of aurora A kinase by two small molecule inhibitors tested by us, MK-5108 and MK-8745, causes cell death by apoptosis, but not necroptosis. Additionally, inhibitors of RIP kinases might potentiate apoptosis evoked by aurora A kinase inhibitors.

## **P287 Diagnostic significance of SPECT/CT in addition to planar 123I-MIBG-scintigraphy in patients with neuroblastoma**

*Kireeva, E.<sup>2</sup>, Chaurasiya, K.<sup>3</sup>, Shamanskaya, T.<sup>4</sup>, Kachanov, D.<sup>5</sup>, Likar, Y.<sup>6</sup>*

National Medical Research Center of Pediatric Hematology, Oncology & Immunology, Nuclear Medicine, Moscow, Russia <sup>2</sup>, National Medical Research Center of Pediatric Hematology, Oncology & Immunology, Nuclear Medicine, Moscow, Russia<sup>3</sup>, National Medical Research Center of Pediatric Hematology, Oncology & Immunology, Oncology, Moscow, Russia<sup>4</sup>, National Medical Research Center of Pediatric Hematology, Oncology & Immunology, Oncology, Moscow, National Medical Research Center of Pediatric Hematology, Oncology & Immunology<sup>5</sup>, National Medical Research Center of Pediatric Hematology, Oncology & Immunology, Nuclear Medicine, Moscow, Russia<sup>6</sup>

### **Background/Introduction:**

Neuroblastoma is the most common extracranial solid tumor in children, which accounts for 8% of all pediatric malignancies. Metaiodobenzylguanidine radiolabeled with 123I (123I-MIBG) offers high-quality images and is widely used in the evaluation of patients with neuroblastoma. Unfortunately, the assessment of conventional planar 123I-MIBG images presents some difficulties due to the limited resolution of planar images and the lack of anatomical localization, which leads to the appearance of both false-negative as well as false-positive results. In recent years, hybrid imaging has played an important role in radionuclide imaging. Single photon emission tomography/computed tomography (SPECT/CT) provides both functional and anatomical information. SPECT/CT in addition to planar scintigraphy increases the imaging time which may lead to increased sedation in most of the patients.

### **Aims:**

To determine the diagnostic significance of the SPECT/CT protocol in addition to planar 123I-MIBG-scintigraphy in patients with neuroblastoma.

### **Methods/Materials:**

251 patients (118 boys, 133 girls, aged from 0 to 17 years) were included in this retrospective study. 123I-MIBG-scintigraphy including whole body planar images and SPECT/CT of region of interest were performed for all patients using standard protocol. Out of the 251 patients 204 were primary without any type of treatment and 47 - after surgical removal of the primary tumor but without any type of chemotherapy.

### **Results:**

Out of 204 primary patients, 149 results of planar images and SPECT/CT were congruent, however, in 55 cases SPECT/CT played an important role to rule out either false-negative (N=42) or false-positive results (N=13).

Out of 47 postoperative cases the results of planar images and SPECT/CT were congruent in 32 cases, but in 15 cases SPECT/CT ruled out either false-negative (N=3) or false-positive results (N=12).

### **Summary/Conclusions:**

Our study clearly demonstrates that SPECT/CT protocol play an important role in 123I-MIBG-scintigraphy and could significantly impact the patient's management. SPECT/CT provided additional diagnostic information in more than 26% of cases at the time of initial study and more than 30% in postoperative cases.



## **P288 Intracerebroventricular Radioimmunotherapy using <sup>131</sup>I-omburtamab for Neuroblastoma Central Nervous System/Leptomeningeal Metastases, interim results from multi-center trial 101**

*Kramer, K.<sup>2</sup>, Vik, T.<sup>3</sup>, Streby, K.<sup>4</sup>, Lisby, S.<sup>5</sup>, Hyseni, A.<sup>6</sup>, Mora, J.<sup>7</sup>*

Memorial Sloan Kettering Cancer Center, Pediatrics, New York, USA <sup>2</sup>, Riley Hospital for Children, Pediatrics, Indianapolis, USA<sup>3</sup>, Nationwide Children's Hospital, Pediatrics, Columbus, USA<sup>4</sup>, Y-mAbs Therapeutics A/S, Ymabs, Copenhagen, Y-mAbs Therapeutics A/S<sup>5</sup>, Y-mAbs Therapeutics A/S, Ymabs, Copenhagen, Denmark<sup>6</sup>, Hospital Sant Joan de Déu, Pediatrics, Barcelona, Spain<sup>7</sup>

### **Background/Introduction:**

Neuroblastoma metastasis to the central nervous system (CNS) or leptomeninges (LM) carries a dismal prognosis. In a phase 1/2 trial conducted at MSKCC, intraventricular compartmental radioimmunotherapy (cRIT) with <sup>131</sup>I-Omburtamab targeting B7-H3 was safe and improved survival.<sup>1</sup>

### **Aims:**

We report interim results of safety, dosimetry, pharmacokinetics and efficacy of cRIT with <sup>131</sup>I-Omburtamab on the first multi-center, international trial for patients with CNS neuroblastoma.

### **Methods/Materials:**

Y-mAbs trial 101 commenced in December 2018. Eligible patients had CNS neuroblastoma with stable systemic disease, adequate cerebrospinal fluid flow through an indwelling ventricular catheter, and adequate major organ function. A treatment cycle consisted of a 2mCi dosimetry dose and a 50mCi treatment dose, with adjustments for patients under three years or one year of age. Dosimetry by serial imaging, pharmacokinetics by CSF and blood sampling and safety were investigated during a 5-week period. Response and survival were noted by the clinical status and magnetic resonance imaging at 5 and 26 weeks with long term follow-up for overall survival up to 3 years.

### **Results:**

The interim analysis includes 17 patients (10 at MSKCC; 7 at other sites) who received at least one cycle of <sup>131</sup>I-omburtamab before 30-Jun-2019 and have been followed for at least six months. 8 patients received 1 cycle; 9 patients received two cycles. The median follow-up time for these patients is 179 days. 15 patients remain alive at the cut-off date. The OLINDA mean absorbed doses were 3.46 mSv/MBq in the brain and 5.97 mSv/MBq in the liver. Preliminary results on dosimetry level estimates in the cerebrospinal fluid at 0.56 Gray/mCi were orders of magnitude higher than those in the blood at 0.008 Gray/mCi. The most common SAEs were related to myelosuppression. Overall, the administration of <sup>131</sup>I-Omburtamab was well tolerated.

### **Summary/Conclusions:**

Administration of cRIT with <sup>131</sup>I-Omburtamab is feasible and safe in a multi-center setting. <sup>131</sup>I-Omburtamab shows favorable dosimetry.



## **P289 In vivo anti-tumour activity of the orally-bioavailable CDK2/9 inhibitor CYC065 against temozolomide-refractory neuroblastoma**

*Jamin, Y.*<sup>2</sup>, *Poon, E.*<sup>3</sup>, *Liang, T.*<sup>4</sup>, *Barone, G.*<sup>5</sup>, *Fyle, E.*<sup>6</sup>, *Zheleva, D.*<sup>7</sup>, *Lin, C.*<sup>8</sup>, *Chesler, L.*<sup>9</sup>

The Institute of Cancer, London, Division of Radiotherapy and Imaging, Sutton, United Kingdom <sup>2</sup>, The Institute of Cancer, London, Division of Clinical Studies, Sutton, United Kingdom<sup>3</sup>, Baylor College of Medicine, Department of Molecular and Human Genetics, Houston, USA<sup>4</sup>, Great Ormond Street Hospital, Department of Pediatric Oncology, London, Great Ormond Street Hospital<sup>5</sup>, The Institute of Cancer, London, Division of clinical studies, Sutton, United Kingdom<sup>6</sup>, Cyclacel Ltd, Cyclacel Ltd, Dundee, United Kingdom<sup>7</sup>, Baylor College of Medicine, Department of Molecular and Human Genetics, Houston, USA<sup>8</sup>, The Institute of Cancer, London, Division of Clinical Studies, Sutton, United Kingdom<sup>9</sup>

### Background/Introduction:

There is a lack of characterized *in vivo* models that emulate clinical refractory and relapsed high-risk neuroblastoma, potentially affecting the predictability of the mouse hospital approach. The Th-*MYCN* mouse is the most established genetically-engineered model of neuroblastoma, which has been shown to recapitulate the major genetic and pathophysiological features of high-risk *MYCN*-amplified disease and as such, represents a relevant model to provide mechanistic insights for the development and evaluation of *MYCN*-targeted therapeutics. However, its sensitivity to frontline chemotherapy agent such as cyclophosphamide is reminiscent of the chemosensitivity of *MYCN*-amplified neuroblastomas to induction therapy rather than the chemo-resistant nature of refractory/relapsed disease. Temozolomide (TMZ) is a second-line chemotherapy agent, which shows moderate tumour control of refractory/relapsed neuroblastoma and represents, once resistance occurs, the backbone chemotherapy for the introduction of novel therapeutic strategies in European pediatric clinical trials

### Aims:

This study aimed to generate and characterize a TMZ-resistant model using personalised dose escalation in the Th-*MYCN* model to identify novel actionable targets against resistant/refractory disease.

### Methods/Materials:

Homozygous Th-*MYCN* mice with palpable tumours (n=18) were enrolled for initial screening using T<sub>2</sub>-weighted MRI. When tumour volume reached ~ 1000 mm<sup>3</sup>, mice were enrolled on the first cycle consisting of a 6 mg/kg daily dose of TMZ (5 days on/ 2 days off), known to only cause partial response (RECIST). If progressive disease was detected within a cycle, the dose was increased by 50% at the start of the next cycle, until tumour growth could not be controlled anymore. On day 5 of the last cycle, mice were either <sup>i</sup>) sacrificed and tumours excised for molecular and pathological analysis, <sup>ii</sup>) treated with a high dose of TMZ (50 mg/kg p.o., n=4) or <sup>iii</sup>) treated with 50mg/kg CYC065 p.o. (5 days on/2 days off, n=4).

### Results:

We demonstrate that the Th-*MYCN* model acquired resistance to cyclic treatment with TMZ (established at 13.5mg/kg TMZ and confirmed by progressive disease during a further cycle of 50mg/kg). Resistant tumours recapitulated the known molecular and pathological hallmarks associated with TMZ resistance, including increased expression of O6-methylguanine DNA methyltransferase (<sup>MGMT</sup>), a DNA repair enzyme, which removes the TMZ-mediated cytotoxic O6-methylguanine DNA lesions. Interestingly, we identified deregulation of the Rb pathway as an actionable target using kinome profiling. We subsequently demonstrate that the potent CDK2/9 inhibitor CYC065 showed anti-tumour activity in TMZ-resistant Th-*MYCN* tumours, consistent with downregulation of the Rb pathways and induction of apoptosis.

### Summary/Conclusions:

Here, we presented a model of resistance to TMZ using the Th-*MYCN* model and demonstrated its potential as a clinically-relevant platform to evaluate novel therapeutic strategies against high-risk relapsed/refractory neuroblastoma. The anti-tumour activity of the orally-bioavailable CDK2/9 inhibitor CYC065 in this model further highlights CYC065 clinical potential in the treatment of high-risk neuroblastoma.

## **P290 Overcoming Drug Resistance with Enhanced Delivery of a Novel Camptothecin to Treat High-Risk Neuroblastoma (NB)**

*Nguyen, F.*<sup>2</sup>, *Alferiev, A.*<sup>3</sup>, *Guan, P.*<sup>4</sup>, *Guerrero, D.*<sup>5</sup>, *Naraparaju, K.*<sup>6</sup>, *Kolla, V.*<sup>7</sup>, *Chorny, M.*<sup>8</sup>, *Brodeur, G.*<sup>9</sup>

Children's Hospital of Philadelphia, Pediatrics, Philadelphia, USA <sup>2</sup>, Children's Hospital of Philadelphia, Pediatrics, Philadelphia, USA<sup>3</sup>, Children's Hospital of Philadelphia, Pediatrics, Philadelphia, USA<sup>4</sup>, Children's Hospital of Philadelphia, Pediatrics, Philadelphia, Children's Hospital of Philadelphia<sup>5</sup>, Children's Hospital of Philadelphia, Pediatrics, Philadelphia, USA<sup>6</sup>, Children's Hospital of Philadelphia, Pediatrics, Philadelphia, USA<sup>7</sup>, Children's Hospital of Philadelphia, Pediatrics, Philadelphia, USA<sup>8</sup>, Children's Hospital of Philadelphia, Pediatrics, Philadelphia, USA<sup>9</sup>

### Background/Introduction:

Topoisomerase I inhibitors of the camptothecin family are among the most potent anticancer agents, effective against a broad range of pediatric solid tumors. However, they often show poor efficacy against high-risk NBs characterized by intrinsic or acquired drug resistance. Low drug concentrations, drug efflux by ABC transporters and glucuronidation are key mechanisms behind compromised response to camptothecins and other drugs in high-risk NB patients.

### Aims:

We have developed a multivalent, polymer-based prodrug of an experimental camptothecin (SN99), designed to achieve sustained intratumoral drug levels, overcome ABC-mediated drug efflux and avoid elimination by glucuronidation. We tested the hypothesis that enhanced drug design combined with prodrug-based delivery will result in significant and lasting antitumor effects of SN99 in models of high-risk NB with intrinsic and acquired chemoresistance.

### Methods/Materials:

We overexpressed ABCG2 in a null NB cell line (NLF) and exposed these cells to SN99 vs. SN38 (the active form of the clinically used camptothecin, irinotecan). We also determined tumor uptake and biodistribution of SN99 administered systemically as a polymer-based prodrug compared to irinotecan in spontaneous NBs from transgenic *TH-MYCN* mice. Tissue samples collected at predetermined times were analyzed for drug levels using HPLC. Antitumor efficacy of the prodrug was evaluated in NBs exhibiting intrinsic, ABCG2-driven multidrug resistance from homozygous *TH-MYCN* mice. We also used a model of recurrent NB by inoculating mice orthotopically with luciferase-expressing SK-N-BE(2)C cells with mutated P53. Disease progression and response to therapy were determined by monitoring the bioluminescent signal. We compared the effectiveness of the prodrug administered by tail vein injection once a week over 4 weeks to that of irinotecan.

### Results:

As ABCG2 expression in NLF cells increased, SN38 was progressively less effective at inhibiting cell growth, whereas SN99 remained equally effective. SN99 delivery as a prodrug resulted in sustained intratumoral drug concentrations, significantly higher than those of irinotecan/SN38 at all time points. SN99 levels were ~50 times greater at 4 hours and 12 times greater at 168 hours compared to the highest measured SN38 level (4 hours post-delivery), with only trace amounts of SN38 remaining at 24 hours. Irinotecan modestly prolonged survival in tumor-bearing transgenic mice, but the SN99 prodrug caused complete tumor regression that persisted over 6 months after the 4-week treatment in *TH-MYCN* animals, and no tumor was detected at necropsy. Irinotecan had no effect on tumor growth in the chemoresistant orthotopic model, whereas treatment with the SN99 prodrug resulted in a rapid shrinkage and protracted suppression of the tumor regrowth for at least 14 weeks.

### Summary/Conclusions:

SN99 administered as a multivalent polymeric prodrug resulted in increased and protracted intratumoral drug exposure compared to irinotecan. Consistent with these results, treatment with the SN99 prodrug

led to long-term 'cures' of primary drug-resistant tumors in the transgenic model, and to complete regression and lasting regrowth inhibition in the chemoresistant orthotopic model. We conclude that the SN99 prodrug is extremely effective in treating high-risk NBs with intrinsic or acquired drug resistance, and warrants further development for a phase 1 clinical trial.

## **P291 Temporal clustering of neuroblastoma in children and young adults from Ontario, Canada**

*Hayes, L.*<sup>2</sup>, *Basta, B.*<sup>3</sup>, *Muirhead, C.R.*<sup>4</sup>, *Gibson, P.*<sup>5</sup>, *Di Monte, B.*<sup>6</sup>, *Irwin, M.S.*<sup>7</sup>, *Greenberg, M.*<sup>8</sup>, *Tweddle, D.A.*<sup>9</sup>, *McNally, R.M.*<sup>10</sup>

Newcastle University, Population Health Sciences, Newcastle Upon Tyne, United Kingdom <sup>2</sup>, Newcastle University, Population Health Sciences, Newcastle Upon Tyne, United Kingdom<sup>3</sup>, Newcastle University, Population Health Sciences, Newcastle Upon Tyne, United Kingdom<sup>4</sup>, McMaster Children's Hospital, Division of Hematology and Oncology, McMaster, McMaster Children's Hospital<sup>5</sup>, Pediatric Oncology Group of Ontario, Pediatric Oncology Group of Ontario, Toronto, Canada<sup>6</sup>, The Hospital for Sick Children, Division of Hematology/Oncology,, Totonto, Canada<sup>7</sup>, Pediatric Oncology Group of Ontario, Paediatric Oncology Group, Toronto, Canada<sup>8</sup>, Wolfson Childhood Cancer Research Centre, Northern Institute for Cancer Research, Wolfson Childhood Cancer Centre, Newcastle Upon Tyne, United Kingdom<sup>9</sup>, Newcastle University, Population Health Sciences, Newcastle Upon Tyne, United Kingdom<sup>10</sup>

### **Background/Introduction:**

The aetiology of neuroblastic tumours is likely to involve both environmental and genetic factors. A number of possible environmental risk factors have been suggested, including infection. If an irregular temporal pattern in incidence of cases of a disease is found, this might suggest that a transient agent, such as infection, is implicated. Previous work has found that such an agent might have been involved in the aetiology of neuroblastic tumours in children and young adults living in Northern England, where evidence of temporal clustering of cases between months and between fortnights was found.

### **Aims:**

The aim of this analysis was to see if there is evidence of similar temporal clustering of neuroblastic tumours in children and young adults living in Ontario, Canada.

### **Methods/Materials:**

We examined data from the population-based Pediatric Oncology Group of Ontario Networked Information System (POGONIS) registry. All cases of neuroblastic tumours diagnosed in children and adults aged 0-19 years between 1985 and 2016 were extracted from the registry, which covers a population of 3.1 million young people resident in Ontario, Canada at the time of diagnosis. A modified version of the Potthoff-Whittinghill method was used to test for temporal clustering. Estimates of extra-Poisson variation and standard errors were obtained.

### **Results:**

876 cases of neuroblastoma (481 [55%] males) were diagnosed during the study period. No evidence of temporal clustering was found between fortnights within months, between months within quarters or between quarters within years in the study period. However, significant extra-Poisson variation was found between quarters within the 32 year study period (extra-Poisson variation=0.41 SE =0.13; p=0.002) and between years within the study period (extra-Poisson variation =1.05 SE =0.25; p=0.005). When the analysis was repeated for males and females separately extra-Poisson variation was found between quarters within the study period (0.31 SE =0.13; p=0.012) and between years within the study period (0.79 SE =0.25; p=0.005) in females, but no extra-Poisson variation was apparent in males (extra-Poisson variation between quarters -0.11 SE =0.13; p=0.81 and between years -0.05 SE=0.25; p=0.54).

### **Summary/Conclusions:**

The findings are consistent with the possibility that a transient agent, such as an infection, that is characterised by 'peaks and troughs' in its occurrence, might be implicated in the aetiology of neuroblastic tumours. It is notable that in this population temporal clustering arose over longer time periods than was reported in the previous work undertaken in Northern England, A putative explanation for this is the large geography of Ontario in comparison to Northern England, which might

impact on the time it takes for infectious agents to spread. Further work is required to explore why temporal clustering was apparent in females but not males and to examine potential differences between children diagnosed in infancy and those diagnosed later in childhood.

## **P292 ABYSMAL OUTCOME OF HIGH RISK NEUROBLASTOMA : EXPERIENCE FROM A CENTRE IN INDIA**

*Mathew, L.<sup>2</sup>, John, R.<sup>3</sup>, Boddu, D.<sup>4</sup>*

Christian Medical College Hospital, Pediatric Hematology- Oncology, Vellore, India <sup>2</sup>, Christian Medical College Hospital, Pediatric Hematology-Oncology, Vellore, India<sup>3</sup>, Christian Medical College Hospital, Pediatric Hematology-Oncology, Vellore, India<sup>4</sup>

### Background/Introduction:

The outcome of children with high risk neuroblastoma has improved dramatically in the developed countries with introduction of immunotherapy. Such treatment is not available and is prohibitively expensive to patients from LMIC. This study was done to look at the overall and disease free survival of children who were treated in a centre in India, where even myeloablative chemotherapy is too expensive for majority of our patients.

### Aims:

The aims and objectives of this analysis was to assess response to rapid COJEC chemotherapy in children treated for high risk neuroblastoma (HR-NBL) and the final outcome after surgery, radiotherapy, MAT and differentiation therapy.

### Methods/Materials:

Children >18 months who were diagnosed to have HR-NBL and completed rapid COJEC chemotherapy from 2008- 2019 were included in the analysis. Standard criteria was used to assess response as complete or good partial response.

### Results:

40 children, 26 boys and 14 girls, aged 19 months to 13 years were included. Primary site of tumour was adrenal gland in 22, thoracic 6, retroperitoneal 4, paravertebral 4 and unknown in 4. Metastatic site of involvement were bone marrow -36 and bone-28, lymphnodes-12, sphenothmoid sinuses-2 and liver in one. Myc-N was tested in 28 patients; amplified- 5, negative 19, test failed in 2 and report not available in 2.

Response assessment after COJEC complete/good partial response in 32 children; 30/36 bone marrow negative, 20/28 bone metastasis showed partial response. 25 children underwent excision of tumour; debulking was done in 3 of them. 8 had inoperable tumour, 2 opted against further treatment, 2 had no detectable residual disease and another 2 progressive disease, hence were not operated. 30 children had radiation to primary site and 3 had MIBG therapy. 9 children had MAT and 30 received cis-retinoic acid.

Mean follow up period of 19 months (median of 24.7m), 10 children are alive, 29 died and there was no information about one child who was well at end of treatment. Among the 10 alive, only 4 are disease free. Mean time to relapse post rapid COJEC was 12.48 months (3-34 months).

### Summary/Conclusions:

With rapid COJEC 80% of patients achieved complete /good partial response. However, the overall survival of this group was only 25% and disease free survival was only 10%. Immunotherapy and tumour vaccine if made available will improve survival of children with high risk neuroblastoma in India.

## **P293 Rapid in vivo validation of HDAC inhibitor-based treatments in pediatric nervous system tumors**

*Najafi, S.<sup>2</sup>, Wrobel, J.<sup>3</sup>, Peterziel, H.<sup>4</sup>, Krunic, D.<sup>5</sup>, Ridinger, J.<sup>6</sup>, Milde, T.<sup>7</sup>, Westermann, F.<sup>8</sup>, Meder, B.<sup>9</sup>, Distel, M.<sup>10</sup>, Witt, O.<sup>11</sup>, Oehme, I.<sup>12</sup>*

Hopp Children's Cancer Center Heidelberg (KITZ), Heidelberg, Germany; Clinica, Pediatric oncology, Heidelberg, Germany <sup>2</sup>, Hopp Children's Cancer Center Heidelberg (KITZ), Heidelberg, Germany, Pediatric oncology, Heidelberg, Germany<sup>3</sup>, Clinical Cooperation Unit Pediatric Oncology, German Cancer Research Center (DKFZ, Pediatric oncology, Heidelberg, Germany<sup>4</sup>, Light Microscopy Facility, German Cancer Research Center (DKFZ), Heidelberg, Ger, Pediatric oncology, Heidelberg, Light Microscopy Facility, German Cancer Research Center (DKFZ), Heidelberg, Ger<sup>5</sup>, Clinical Cooperation Unit Pediatric Oncology, German Cancer Research Center (DKFZ, Pediatric oncology, Heidelberg, Germany<sup>6</sup>, Department of Pediatric Oncology, Hematology and Immunology, University of Heide, Pediatric oncology, Heidelberg, Germany<sup>7</sup>, Neuroblastoma Genomics, German Cancer Research Center (DKFZ), Heidelberg, German, Pediatric oncology, Heidelberg, Germany<sup>8</sup>, Genome Technology Center, Stanford University, California, USA, Cardiomyopathies, California, USA<sup>9</sup>, Innovative Cancer Models, St. Anna Children's Cancer Research Institute, Vienna,, Innovative Cancer Models, Vienna, Austria<sup>10</sup>, Department of Pediatric Oncology, Hematology and Immunology, University of Heide, Pediatric oncology, Heidelberg, Germany<sup>11</sup>, Clinical Cooperation Unit Pediatric Oncology, German Cancer Research Center (DKFZ, Pediatric oncology, Heidelberg, Germany, <sup>12</sup>

### Background/Introduction:

High-risk neuroblastoma patients respond poorly to multimodal chemotherapy, thus the development of novel drug combinations and improved preclinical drug testing models are necessary to effectively target the disease. Histone deacetylase (HDAC) activity has an important role in numerous cellular processes, and aberrant expression of HDACs has been associated with a variety of malignancies, including neuroblastoma. We have previously reported selective inhibition of HDACs as a promising approach to sensitize aggressive neuroblastomas to chemotherapy.

### Aims:

Establishment of zebrafish neuroblastoma, glioblastoma and medulloblastoma xenograft models, and *in vivo* evaluation of combination treatments involving standard chemotherapy and selected HDAC inhibitors (HDACis).

### Methods/Materials:

A suspension of fluorescently labeled tumor cells is injected two days post fertilization (dpf) into the yolk sac (tumor volume studies), perivitelline space (cell dissemination studies) or brain of zebrafish larvae. At 3dpf compounds of interest are added to the maintenance water and tumor volume or disseminated cells are monitored by confocal microscopy 48h after treatment start (5dpf). Tumor cells were also co-stained with, tetramethylrhodamine ethyl ester (TMRE) and DiD to detect the amount of viable cells as well as the total amount of injected cells. Moreover, cell proliferation was assessed by quantifying the expression of Ki67 and phospho-histone H3, immunomarkers specific for cells undergoing mitosis, on sections of the xenografted tumors.

### Results:

On average, 40-60% of tumor cells remain mitotically active following injection, and we are able to monitor cancer progression, including disseminating of single tumor cells, with single-cell resolution in an intact environment. HDACis proved on-target activity via substrate acetylation and induced the shrinkage of the xenografted tumors. HDACis in combination with doxorubicin substantially affected tumor growth as well as tumor cell dissemination to the fish tails.

### Summary/Conclusions:



Our findings indicate that engraftment of pediatric tumor cells into zebrafish larvae can facilitate fast results on drug responsiveness, before moving on to more costly and time-consuming mammalian studies.

## **P294 Epigenetic Differentiation Therapy: A preclinical study and methylation analysis in phase I study**

Ushijima, T.<sup>2</sup>, Hattori, N.<sup>3</sup>, Yamashita, S.<sup>4</sup>, Kawamoto, H.<sup>5</sup>, Kumamoto, H.<sup>6</sup>, Ogawa, C.<sup>7</sup>, Souzaki, R.<sup>8</sup>, Taguchi, T.<sup>9</sup>, Nitani, C.<sup>10</sup>, Hara, J.<sup>11</sup>

National Cancer Center Research Institute, Epigenomics, Tokyo, Japan<sup>2</sup>, National Cancer Center Research Institute, Division of Epigenomics, Tokyo, Japan<sup>3</sup>, National Cancer Center Research Institute, Division of Epigenomics, Tokyo, Japan<sup>4</sup>, National Cancer Center Hospital, Division of Pediatric Oncology, Tokyo, National Cancer Center Hospital<sup>5</sup>, National Cancer Center Hospital, Division of Pediatric Oncology, Tokyo, Japan<sup>6</sup>, National Cancer Center Hospital, Division of Pediatric Oncology, Tokyo, Japan<sup>7</sup>, Graduate School of Medical Sciences, Kyushu University, Department of Pediatric Surgery, Fukuoka, Japan<sup>8</sup>, Graduate School of Medical Sciences, Kyushu University, Department of Pediatric Surgery, Fukuoka, Japan<sup>9</sup>, Osaka City General Hospital, Department of Pediatric Hematology/Oncology, Osaka, Japan<sup>10</sup>, Osaka City General Hospital, Department of Pediatric Hematology/Oncology, Osaka, Japan<sup>11</sup>

### Background/Introduction:

The CpG island methylator phenotype (CIMP) in neuroblastoma (NBL) is strongly associated with poor prognosis, giving larger HR than *MYCN* amplification [Abe, Cancer Res, 65:828, 2005; Abe, Cancer Lett, 247:253, 2007; Banelli, Lab Invest, 92:458, 2011; Kiss, BMC Med Genet, 13:83, 2012]. Therapeutically, treatment with a DNA demethylating agent, decitabine (DAC), and 13-*cis*-retinoic acid had synergistic effect on differentiation of NB-39nu cells [Abe, Oncology, 74:50, 2008].

### Aims:

We aimed to demonstrate the potential of a combination therapy of DAC and a synthetic differentiation agent, tamibarotene (TBT) *in vitro* and *in vivo*. We also aimed to monitor the effect of DNA demethylation in peripheral leucocyte in phase 1 study.

### Methods/Materials:

Gene expression was analyzed by qRT-PCR, and DNA methylation was analyzed by quantitative MSP in cell lines. Demethylation in peripheral leucocytes was analyzed by pyrosequencing of LINE1 sequence.

### Results:

DAC suppressed the growth of 12 NBL cell lines by inducing demethylation and expression of genes related to cell death and neurological processes. TBT induced neural extension and upregulation of differentiation markers, such as *HOXD4*, *NGFR*, and *NTRK1*, in five NBL cell lines. Pretreatment of the five cell lines with DAC enhanced the upregulation levels of differentiation markers, showing that DAC enhanced TBT-induced differentiation. The tumor suppression effect of DAC and TBT was finally confirmed *in vivo* using a mouse xenograft model of NB-1 cell line.

Based upon these preclinical findings, an investigator-initiated phase I trial of TBT as a single agent was completed in 2017, determining a recommended dose of TBT for pediatric patients. Then, a phase I trial of a combination of DAC and TBT was started in 2018, detail of which will be reported elsewhere. Successful demethylation of LINE1 sequence in peripheral leukocytes was observed in three of five patients at level 0, and in three to four of four patients at level -1 of DAC.

### Summary/Conclusions:

A combination therapy of DAC and TBT was effective *in vitro* and that DAC and TBT could suppress growth of NBL cells as a single agent *in vivo*. In our phase I study involving DAC and TBT, demethylation in peripheral leukocytes was confirmed.



## **P295 Optimizing sustained release local therapy: single versus dual chemotherapy for the treatment of neuroblastoma**

*Chiu, B.<sup>2</sup>, Taylor, J.<sup>3</sup>, Yavuz, B.<sup>4</sup>, Zeki, J.<sup>5</sup>, Ikegaki, N.<sup>6</sup>, Coburn, J.<sup>7</sup>, Harrington, K.<sup>8</sup>, Shimada, H.<sup>9</sup>, Kaplan, D.<sup>10</sup>*

Stanford University, Surgery, Stanford, USA<sup>2</sup>, Stanford University, Surgery, Stanford, USA<sup>3</sup>, Tufts University, Biomedical Engineering, Medford, USA<sup>4</sup>, Stanford University, Surgery, Stanford, Stanford University<sup>5</sup>, University of Illinois at Chicago, Anatomy and Cell Biology, Chicago, USA<sup>6</sup>, Worcester Polytechnic Institute, Biomedical Engineering, Worcester, USA<sup>7</sup>, Tufts University, Biomedical Engineering, Medford, USA<sup>8</sup>, Stanford University, Pathology, Stanford, USA<sup>9</sup>, Tufts University, Biomedical Engineering, Medford, USA<sup>10</sup>

### **Background/Introduction:**

Neuroblastoma is the most common pediatric extracranial solid malignancy with limited effective treatment. We have shown that single drugs delivered locally in a sustained release manner through a silk-based biomaterial are effective in decreasing orthotopic neuroblastoma xenograft growth. We further optimized this approach and hypothesized that increasing doses of local chemotherapy or delivering two chemotherapeutic agents simultaneously could inhibit additional tumor growth.

### **Aims:**

To investigate the effectiveness of local sustained released therapy using two chemotherapeutic agents.

### **Methods/Materials:**

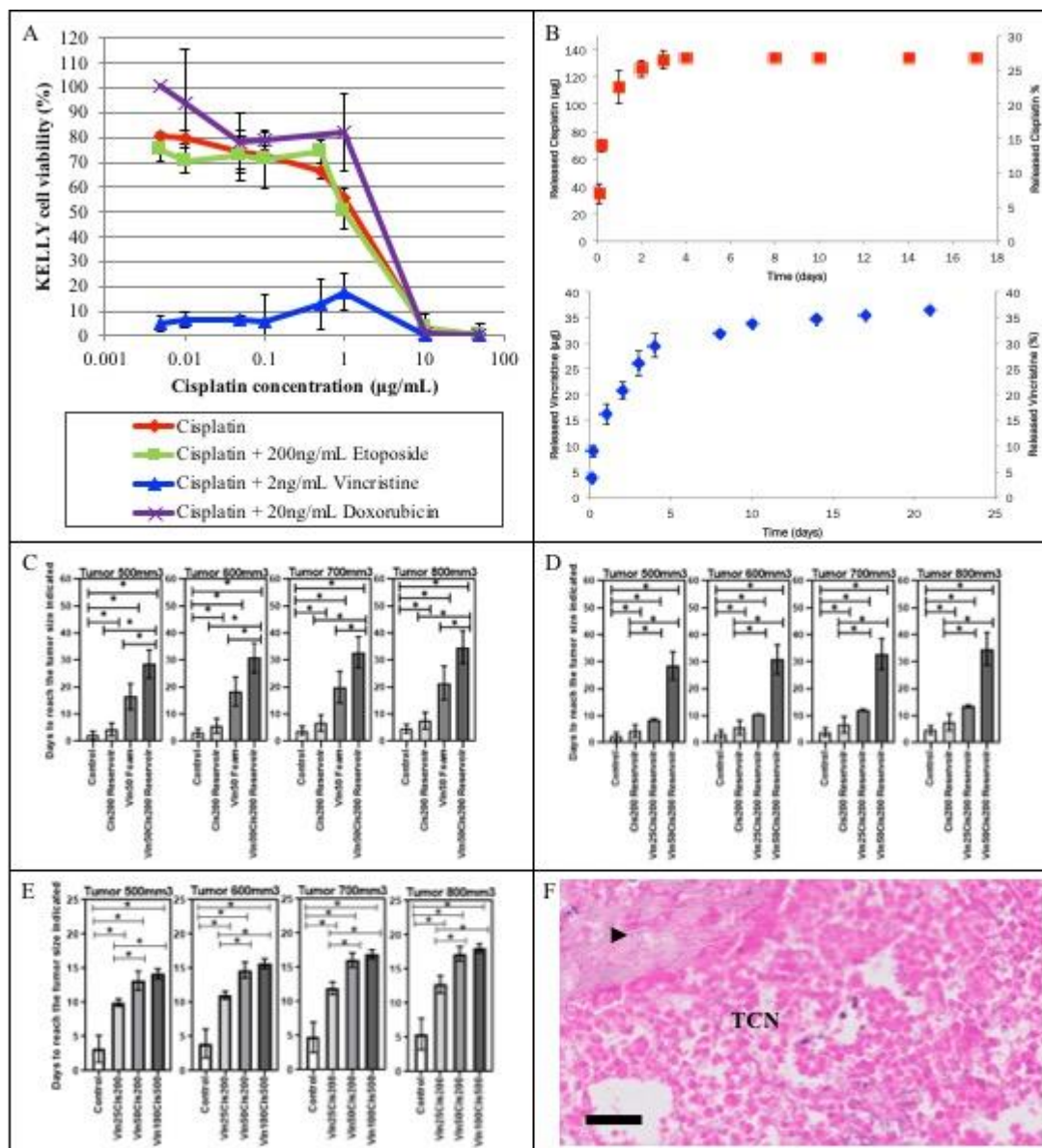
*MYCN*-amplified (KELLY) and non-*MYCN*-amplified (SKNAS) neuroblastoma cells were treated with combinations of cisplatin, vincristine, doxorubicin, and etoposide to determine cytotoxicity and synergy. Drug-loaded silk platforms were created, and the amounts of drug released from the material over time was recorded. Murine orthotopic neuroblastoma xenografts were generated and tumors were implanted with silk platforms loaded with single or dual chemotherapies. Ultrasound monitored tumor growth. Histologic evaluation of tumors was performed.

### **Results:**

*In vitro*, vincristine/cisplatin combination was synergistic, significantly decreasing cell viability relative to other combinations. After loading onto silk platform, both drugs could be released effectively. Locally implanted vincristine/cisplatin silk platform induced increased tumor growth suppression compared to either agent alone in KELLY tumors ( $p < 0.05$ ). The dose-dependent effect seen in KELLY tumors treated with combination therapy diminished at higher doses in SKNAS tumors, with little benefit with doses  $> 50/500\mu\text{g}$  for vincristine/cisplatin, respectively. H&E staining of tumor sections demonstrated tumor cell necrosis adjacent to drug-loaded silk platforms.

### **Summary/Conclusions:**

Local delivery of sustained release chemotherapy can suppress tumor growth especially at high doses or with two synergistic drugs. Customized dual therapy is a promising approach for future clinical testing.



**Figure 1.** (A) Cytotoxicity profile of KELLY cells treated with cisplatin and doxorubicin, etoposide or vincristine. (B) Amount ( $\mu\text{g}$ ) and percentage of drug released from silk platform loaded with a combination of 100  $\mu\text{g}$  vincristine and 500  $\mu\text{g}$  cisplatin. (C) Growth kinetics, (displayed as the number of days for tumor to reach a given size) for KELLY tumors treated with single vs dual agent silk reservoir; (D) Substantial Kelly tumor growth inhibition caused by increasing doses of dual therapy. (E) Growth kinetics of SKNAS tumor treated with increasing doses of vincristine and cisplatin demonstrate limited therapeutic benefit at the highest dose range. (F) KELLY tumors treated with local combination therapy were sectioned and stained with H&E. The histology showed tumor cell necrosis adjacent to the drug-loaded silk platform. Asterisk (\*) indicates p-value < 0.05; scale bar = 100  $\mu\text{m}$ ; arrow (▶) indicates drug loaded silk platform; TCN, tumor cell necrosis.

## **P296 Successful treatment of central nervous system relapses in metastatic high-risk neuroblastoma after KIR-ligand-mismatched allogeneic cord blood transplantation without anti-GD2 antibody therapy**

*Imaya, M.<sup>2</sup>, Yamamori, A.<sup>3</sup>, Wakamatsu, M.<sup>4</sup>, Yoshida, T.<sup>5</sup>, Miwata, S.<sup>6</sup>, Narita, K.<sup>7</sup>, Kitazawa, H.<sup>8</sup>, Rieko, T.<sup>9</sup>, Ichikawa, D.<sup>10</sup>, Hamada, M.<sup>11</sup>, Nishikawa, E.<sup>12</sup>, Kawashima, N.<sup>13</sup>, Narita, A.<sup>14</sup>, Okuno, Y.<sup>15</sup>, Muramatsu, H.<sup>16</sup>, Nishio, N.<sup>17</sup>, Kojima, S.<sup>18</sup>, Kramer, K.<sup>19</sup>, Takahashi, Y.<sup>20</sup>*

Nagoya University Hospital, Pediatrics, Nagoya, Japan <sup>2</sup>, Nagoya University Hospital, Pediatrics, Nagoya, Japan<sup>3</sup>, Nagoya University Hospital, Pediatrics, Nagoya, Japan<sup>4</sup>, Nagoya University Hospital, Pediatrics, Nagoya, Nagoya University Hospital<sup>5</sup>, Nagoya University Hospital, Pediatrics, Nagoya, Japan<sup>6</sup>, Nagoya University Hospital, Pediatrics, Nagoya, Japan<sup>7</sup>, Nagoya University Hospital, Pediatrics, Nagoya, Japan<sup>8</sup>, Nagoya University Hospital, Pediatrics, Nagoya, Japan<sup>9</sup>, Nagoya University Hospital, Pediatrics, Nagoya, Japan<sup>10</sup>, Nagoya University Hospital, Pediatrics, Nagoya, Japan<sup>11</sup>, Nagoya University Hospital, Pediatrics, Nagoya, Japan <sup>12</sup>, Nagoya University Hospital, Pediatrics, Nagoya, Japan <sup>13</sup>, Nagoya University Hospital, Pediatrics, Nagoya, Japan <sup>14</sup>, Nagoya University Hospital, Pediatrics, Nagoya, Japan <sup>15</sup>, Nagoya University Hospital, Pediatrics, Nagoya, Japan <sup>16</sup>, Nagoya University Hospital, Pediatrics, Nagoya, Japan <sup>17</sup>, Nagoya University Hospital, Pediatrics, Nagoya, Japan <sup>18</sup>, Memorial Sloan-Kettering Cancer Center, Pediatric Oncology, New York, USA <sup>19</sup>, Nagoya University Hospital, Pediatrics, Nagoya, Japan <sup>20</sup>

### Background/Introduction:

Although intensive care has improved the survival of high-risk neuroblastoma (NB) patients, recurrent NB has a poor prognosis. In particular, central nervous system (CNS) relapse of NB is difficult to rescue. In previous reports, 14 cases of CNS relapse of NB were fatal, with a median survival duration of 3.6 months from relapse (range: 10 days to 25 months) (Kramer et al. Cancer. 2001; Astigarraga et al. Med Pediatr Oncol. 1996). We previously reported that KIR-ligand-incompatible allogeneic umbilical cord blood transplantation (CBT) reduces bone marrow relapse and improves survival in children with high-risk metastatic NB because NB is susceptible to KIR-mismatched NK cells (Nishio et al. Advances in Neuroblastoma Research. 2018).

### Aims:

We report the successful treatment of three children with CNS relapse of NB after KIR-ligand-mismatched allogeneic CBT without anti-GD2 antibody treatment.

### Methods/Materials:

Case 1: A 2-year-old boy with stage 4 NB received multiple courses of combined chemotherapy, BU/L-PAM followed by auto-peripheral blood stem cell transplantation (PBSCT), Flu/L-PAM/TBI (2 Gy) followed by KIR-ligand-mismatched CBT, and focal radiotherapy (19.8 Gy). Ten months after CBT, isolated CNS relapse was diagnosed. He underwent metastatectomy, proton beam craniospinal irradiation (CSI; 19.8 Gy), and focal radiotherapy (10.8 Gy). He has been living without disease progression for 4 years after CNS relapse.

Case 2: A 3-year-old boy with stage 4 NB received multiple courses of combined chemotherapy, BU/L-PAM followed by auto-PBSCT, L-PAM/TBI (12 Gy) followed by KIR-ligand-mismatched CBT, and focal radiotherapy (18 Gy). Eleven months after CBT, CNS relapse was diagnosed. He underwent metastatectomy, proton beam CSI (18 Gy), and focal radiotherapy (12.6 Gy), and a second KIR-ligand-mismatched CBT with Flu/L-PAM/TBI (2 Gy) was performed because of positive bone marrow minimal residual disease (BM-MRD). He also received intrathecal radioimmunotherapy involving 131I-omburtamab. He has been living without disease progression for 22 months after CNS relapse.

Case 3: A 3-year-old girl with stage 4 NB received multiple courses of combined chemotherapy, IFO/L-PAM followed by auto-PBSCT, and Flu/L-PAM/VP-16 followed by KIR-ligand-mismatched CBT. Seven months after CBT, isolated CNS relapse was diagnosed. She underwent metastatectomy, CSI

(21 Gy), and focal radiotherapy (10 Gy). She also received three cycles of TMZ/CPT11 and intrathecal 131I-omburtamab. She has been living without disease progression for 10 months after CNS relapse.

#### Results:

We reported three children who developed CNS relapse of NB at 10, 11, and 7 months after KIR-ligand-mismatched CBT. Tumor resection and CSI were performed in all cases. One patient underwent a second KIR-ligand-mismatched CBT because of positive BM-MRD, and two patients received intrathecal omburtamab. Without anti-GD2 antibody treatment, bone marrow relapse was controlled with KIR-ligand-mismatched CBT. The three children have been living without disease progression for 4 years, 22 months, and 10 months after CNS relapse.

#### Summary/Conclusions:

Our findings suggest that KIR-ligand-mismatched CBT could control bone marrow relapse without anti-GD2 antibody treatment, and CNS relapse could be rescued by local control therapy involving metastectomy, CSI, and intrathecal omburtamab.

## P297 Submyeloablative Doses of [<sup>131</sup>I]MIBG Therapy for Relapsed/Refractory Neuroblastoma with Segmental Chromosomal Alterations in Taiwan

*Liu, Y.<sup>2</sup>, Wang, S.C.<sup>3</sup>, Wang, L.Y.<sup>4</sup>, Lu, M.Y.<sup>5</sup>, Hsu, W.M.<sup>6</sup>, Yen, H.J.<sup>7</sup>, Yeh, T.C.<sup>8</sup>, Liu, H.C.<sup>9</sup>, Hou, J.Y.<sup>10</sup>, Miser, J.S.<sup>11</sup>*

Taipei Medical University and Taipei Medical University Hospital, Pediatric Hematology/Oncology, Taipei, Taiwan<sup>2</sup>, Changhua Christian Children's Hospital, Pediatric Hematology/Oncology, Changhua, Taiwan<sup>3</sup>, Changhua Christian Hospital, Nuclear Medicine, Changhua, Taiwan<sup>4</sup>, National Taiwan University Children's Hospital, Pediatric Hematology/Oncology, Taipei, National Taiwan University Children's Hospital<sup>5</sup>, National Taiwan University Children's Hospital, Pediatric Surgery, Taipei, Taiwan<sup>6</sup>, Taipei Veterans General Hospital, Pediatric Hematology/Oncology, Taipei, Taiwan<sup>7</sup>, Mackay Children's Hospital, Pediatric Hematology/Oncology, Taipei, Taiwan<sup>8</sup>, Mackay Children's Hospital, Pediatric Hematology/Oncology, Taipei, Taiwan<sup>9</sup>, Mackay Children's Hospital, Pediatric hematology/Oncology, Taipei, Taiwan<sup>10</sup>, Taipei Medical University, College of Medical Science and Technology, Taipei, Taiwan<sup>11</sup>

### Background/Introduction

Catecholamine synthesis and metabolism are hallmarks of neuroblastoma (NB). Iodine-131-labeled meta-iodobenzylguanidine ([<sup>131</sup>I]MIBG) targets the Norepinephrine Transporter, accumulates in NB cells, and serves as a retrieval therapy in NB.

### Aims

To evaluate the feasibility and clinical benefit of [<sup>131</sup>I]MIBG therapy at submyeloablative doses (<12 mCi/kg) for patients with relapsed/refractory, *MYCN*-non-amplified NB with segmental chromosomal alterations (SCAs) in Taiwan.

### Methods/Materials

In 2015, an [<sup>131</sup>I]MIBG Treatment Facility was established at Changhua Christian Hospital, Changhua, Taiwan with a private room with lead shields to maintain area dose rates  $\leq 0.5$  uSv/hr outside the room, independent sanitary pipelines & tank, and patient isolation until dose rate < 50 uSv/hr at 1 meter. The maximal allowable dose was 300 mCi/infusion. Parents were isolated outside/behind lead shields, trained in radiation safety principles and given real-time radiation monitors. The genomic type of NB was determined by Array-based Chromogenic Genomic Hybridization (CityoChip™ Oligo 8x60k; BlueGnome, Cambridge, UK). *MYCN* status was confirmed by chromogenic *in situ* hybridization or droplet digital PCR.

### Results

During 2015–19, four doses of [<sup>131</sup>I]MIBG were given to three male patients with MIBG-avid NB aged 13, 24, and 3 years, respectively. All patients tolerated the isolation & infusion without complications.

Case #1 was diagnosed with retroperitoneal NB with bony metastases, *MYCN* non-amplified with SCAs (loss at 1q42.2q44 and gains at 1p13q42.12 and Xp) and numerical chromosomal alterations (NCAs; +13, +14, -Y) at 11 years of age (y/o); he was refractory to TPOG-N2002-HR2 (N6 induction) and Topotecan/Cyclophosphamide/Etoposide. [<sup>131</sup>I]MIBG of 150 mCi (3 mCi/kg) was given 25 months after initial diagnosis. Stable Disease was observed for 1 month.

Case #2 was diagnosed with retroperitoneal NB with marrow & nodal metastases at 12 y/o. After CR to TPOG-N2002-HR2 including high-dose CEM + ASCR and Focal Radiotherapy, he suffered multiple relapses since 6 years after diagnosis. Tumor at relapse showed *MYCN* low-level gain (5 signals/cell) with SCAs (losses at 1p, 3p, 9p, 11q, 14q, 15q, 19q, 22q and gains at 1p, 1q, 2p, 2q, 5p, 5q, 7p, 8p, 8q, 12q, 13q, 17q, 18p) and NCAs (+12, -Y). [<sup>131</sup>I]MIBG of 300 mCi (5 mCi/kg) × 2 tandem doses were given at 24 y/o. Stable Disease was observed for 4 months before Progression with Liver Metastases. The patient died of tumor progression 17 months after [<sup>131</sup>I]MIBG.

Case #3 was diagnosed with retroperitoneal NB with bone & marrow metastases at 9 m/o. He achieved Complete Response after TPOG-N2002-HR1 Induction (Cyclophosphamide/Cisplatin/Epirubicin/Etoposide) but relapsed at 10 months after diagnosis. Biopsy at relapse showed *MYCN* non-amplification (2.03x), *MYC* low-level gain (2.7x), and *ALK*



F1174L (MAF=51.4%) and *TERT* promoter -124C>T (MAF=49.4%) mutations by targeted nextgeneration sequencing. After failing ICE & Irinotecan/Temozolomide/Dinutuximab beta, he received Palliative Radiotherapy followed by [<sup>131</sup>I]MIBG of 100 mCi (9.5 mCi/kg) at 3 y/o. Clinical Response was observed for 3 weeks. Further targeted therapy with an ALK inhibitor was initiated.

#### Summary/Conclusions

Submyeloablative doses of [<sup>131</sup>I]MIBG have been successfully administered to Taiwanese patients with relapsed/refractory NB with SCAs, showing short-term clinical benefit. Doseescalation of [<sup>131</sup>I]MIBG and/or combination with chemotherapy or other radiosensitizers may further improve treatment response.

## **P298 Preclinical evaluation of HDAC inhibitor-mediated modulation of MIBG avidity in neuroblastoma**

*Koumarianou, E.,<sup>1</sup> Loh, A.H.P.<sup>2</sup>, Ong, J.W.E.<sup>3</sup>, Wong, M.K.<sup>4</sup>, Hee, E.X.Y.<sup>5</sup>, Wong, M.R.E.<sup>6</sup>, Tham, J.Y.<sup>7</sup>, Novera, W.<sup>8</sup>, Reolo, M.J.Y.<sup>9</sup>, Chacko, A.<sup>10</sup>, Loh, A.H.P.<sup>11</sup>*

Duke NUS Medical School, Laboratory for Translational and Molecular Imaging, Singapore, Singapore<sup>1</sup>, KK Women's and Children's Hospital, VIVA-KKH Paediatric Brain and Solid Tumour Programme, Singapore, Singapore<sup>2</sup>, Duke NUS Medical School, Laboratory for Translational and Molecular Imaging, Singapore, Singapore<sup>3</sup>, KK Women's and Children's Hospital, VIVA-KKH Paediatric Brain and Solid Tumour Programme, Singapore, Singapore<sup>4</sup>, KK Women's and Children's Hospital, VIVA-KKH Paediatric Brain and Solid Tumour Programme, Singapore, KK Women's and Children's Hospital<sup>5</sup>, KK Women's and Children's Hospital, VIVA-KKH Paediatric Brain and Solid Tumour Programme, Singapore, Singapore<sup>6</sup>, Duke NUS Medical School, Laboratory for Translational and Molecular Imaging, Singapore, Singapore<sup>7</sup>, Duke NUS Medical School, Laboratory for Translational and Molecular Imaging, Singapore, Singapore<sup>8</sup>, Duke NUS Medical School, Laboratory of Molecular and Translational Imaging, Singapore, Singapore<sup>9</sup>, Duke NUS Medical School, Laboratory for Translational and Molecular Imaging, Singapore, Singapore<sup>10</sup>, KK Women's and Children's Hospital, VIVA-KKH Paediatric Brain and Solid Tumour Programme, Singapore, Singapore<sup>11</sup>

### **Background/Introduction:**

Norepinephrine transporter (NET) is differentially over-expressed in most human neuro-ectodermal tumors, and is a highly specific mechanism for directing uptake of meta-iodobenzylguanidine (MIBG). Phase 2 trials incorporating <sup>125</sup>I-MIBG have shown promising responses, but unfortunately 40% of neuroblastoma tumors are <sup>125</sup>I-MIBG-non-avid and cannot benefit from this treatment. While this may be due to low surface NET protein expression, histone deacetylase inhibitors (HDACi's) have been shown to increase total and surface NET expression and afford a potential mechanism to positively modulate MIBG avidity in neuroblastoma.

### **Aims:**

We hypothesized that NET expression and surface localization could be enhanced pharmacologically with HDAC inhibitors to increase preclinical <sup>125</sup>I-MIBG avidity, within human-equivalent dose limits. We sought to characterize the MIBG uptake and cellular cytotoxicity in neuroblastoma cell lines, and modulation of MIBG avidity in neuroblastoma xenograft models, following exposure to vorinostat (SAHA) at preclinical concentrations equivalent to human plasma C<sub>max</sub> at current pediatric maximum-tolerated dose (MTD) of 180mg/m<sup>2</sup>.

### **Methods/Materials:**

SK-N-SH, SK-N-BE2, and SK-N-BE2C cell lines were exposed to SAHA at 0-4.00μM from 8-24h. Cell viability was evaluated with MTS assay, and <sup>125</sup>I-MIBG uptake was measured normalized to number of viable cells. To verify corresponding modulation of protein expression following 24h of treatment at MTD C<sub>max</sub>-equivalent concentration, total NET protein expression was evaluated by western blot and SLC6A2 (hNET) transcript expression was evaluated by qPCR in lysates of cell lines and xenograft tumors (cell line xenografts and patient-derived xenografts (PDXs)). Finally, to evaluate *in vivo* modulation of MIBG avidity, orthotopic SK-N-SH cell line xenografts and PDXs were treated with allometrically scaled MTD-equivalent dose of SAHA, and <sup>125</sup>I-MIBG and NET biodistribution evaluated in tumor and organs after 24h.

### **Results:**

Greatest increase in <sup>125</sup>I-MIBG uptake was seen with SK-N-SH cells at higher SAHA concentrations after 24h exposure, however there was concomitant cytotoxicity with decreased viability of SK-N-SH cells under these conditions. At 8 and 16h, across all SAHA concentrations, <sup>125</sup>I-MIBG uptake was 2.5 fold higher in SK-N-BE2C cells than SK-N-SH, consistent with higher NET expression as per reported

data ; while at 24h, at 0.73-4.00 $\mu$ M SAHA,  $^{125}$ I-MIBG uptake increased by 2-fold in SK-N-SH and decreased by 2-fold in SK-N-BE2C. Correspondingly, following 24h exposure to SAHA at MTD Cmax-equivalent concentration, normalized NET protein expression increased median 5-fold in SK-N-SH cells. However, in xenografts, similar upregulation was only seen in PDX tumors but not SK-N-SH cell line xenograft tumors, with only the former demonstrating increase in normalized NET protein and hNET expression 24h following 50mg/kg per-oral SAHA. Yet, following the same dose of SAHA, biodistribution studies showed no significant increase in  $^{125}$ I-MIBG avidity of PDX or SK-N-SH cell line xenografts.

#### Summary/Conclusions:

NET protein expression and  $^{125}$ I-MIBG uptake increased in neuroblastoma cell lines after 24h exposure to SAHA concentrations equivalent to expected human plasma Cmax at MTD. Following *in-vivo* dosing, NET protein and hNET expression was upregulated only in PDX tumors. However this did not result in increase in  $^{125}$ I-MIBG avidity *in-vivo*. Our findings suggest that at current pediatric MTD, SAHA may have limited clinical utility in positively modulating MIBG avidity of neuroblastoma tumors.

## **P299 Meta-[<sup>211</sup>At]astatobenzylguanidine ([<sup>211</sup>At]MABG) increases overall survival in a preclinical model of disseminated neuroblastoma**

Batra, V.<sup>2</sup>, Samanta, M.<sup>3</sup>, Makvandi, M.<sup>4</sup>, Martinez, D.<sup>5</sup>, Groff, D.<sup>6</sup>, Schnepf, R.<sup>7</sup>, Pryma, D.<sup>8</sup>, Maris, J.<sup>9</sup>

Perelman School of Medicine at the University of Pennsylvania, Pediatric Oncology, Philadelphia, USA<sup>2</sup>, Children's Hospital of Philadelphia, Pediatric Oncology, Philadelphia, USA<sup>3</sup>, Perelman School of Medicine at the University of Pennsylvania, Radiology, Philadelphia, USA<sup>4</sup>, Children's Hospital of Philadelphia, Pathology, Philadelphia, Children's Hospital of Philadelphia<sup>5</sup>, Children's Hospital of Philadelphia, Pediatric Oncology, Philadelphia, USA<sup>6</sup>, Emory University School of Medicine, Pediatric Oncology, Atlanta, USA<sup>7</sup>, Perelman School of Medicine at the University of Pennsylvania, Radiology, Philadelphia, USA<sup>8</sup>, Perelman School of Medicine at the University of Pennsylvania, Pediatric Oncology, Philadelphia, USA<sup>9</sup>

### Background/Introduction:

The alpha particle emitting targeted radiotherapeutic [<sup>211</sup>At]MABG has superior radiobiological properties (shorter path length and higher energy to effect double strand breaks) compared to the currently used [<sup>131</sup>I]MIBG. Responses to [<sup>131</sup>I]MIBG therapy are typically short-lived, at least in part due to re-emergence of micrometastatic disease within the bone marrow compartment.

### Aims:

We sought to define the anti-tumor activity of [<sup>211</sup>At] MABG in the setting of disseminated minimal residual disease (MRD).

### Methods/Materials:

We used lentiviral transduction in IMR-5 cells to generate *hNET* overexpressing neuroblastoma cell line models and performed *in vitro* cytotoxicity studies using [<sup>211</sup>At]MABG. Dose finding studies were performed with [<sup>211</sup>At]MABG (n=10 at each dose, cumulative dose range 1-1.8 mCi/kg) to establish the maximum tolerated dose (MTD) in NOD *scid* gamma (NSG) mice. Disseminated MRD models were established in NSG mice by intravenous tail vein injection of dual GFP/luciferase and *hNET* transfected IMR-5 cells (1x 10<sup>6</sup> cells per mouse). Mice (n=10) were treated with [<sup>211</sup>At]MABG intraperitoneally (IP) in four fractionated doses of 300 uCi/kg each administered twice weekly after adequate thyroid protection and followed subsequently with twice weekly bioluminescence (BLI) imaging and toxicity assessment for 60 days.

### Results:

NET overexpressing IMR-5 cell lines showed higher uptake of NET ligands and differential superior cytotoxicity compared to parental isogenic IMR-5 cells (EC50's in transfected lines were ~0.001 uCi/ml compared to 0.01 uCi/ml in wild type). In pilot studies, all NSG mice injected with IMR-5-Luc/GFP-*hNET* cells showed tumor engraftment, with bioluminescence arising in liver, spleen, adrenals, ovaries and abdominal cavity at a range of 12-15 days. Thus, intervention trials began at day 4 post injection of tumor cells. Onset of disseminated disease was significantly delayed in the [<sup>211</sup>At]MABG treated animals (median 40.5 ± 11.5 days versus 15 ± 16.4 days), and at day 60, eight of nine of the [<sup>211</sup>At]MABG treated mice were alive as compared to 2/10 of the control mice. Therapy was well tolerated with no evidence of significant weight loss or other toxicity.

### Summary/Conclusions:

[<sup>211</sup>At]MABG is a safe and effective monotherapy for neuroblastoma MRD, and thus has the potential for synergistic anti-tumor efficacy with [<sup>131</sup>I]MIBG due to their complementary radiobiologic properties.

## **P300 Pretreatment 18F-FDG PET/CT May Have Prognostic Value in Neuroblastoma**

Weiss, B.<sup>2</sup>, Sung, A.<sup>3</sup>, Zhang, B.<sup>4</sup>, Trout, A.<sup>5</sup>

Cincinnati Children's Hospital Medical Center, Oncology, Cincinnati, USA <sup>2</sup>, Cincinnati Children's Hospital Medical Center, Radiology, Cincinnati, USA<sup>3</sup>, Cincinnati Children's Hospital Medical Center, Biostatistics & Epidemiology, Cincinnati, USA<sup>4</sup>, Cincinnati Children's Hospital Medical Center, Radiology, Cincinnati, Cincinnati Children's Hospital Medical Center<sup>5</sup>

### Background/Introduction:

Despite advances in therapy, survival for patients presenting with metastatic neuroblastoma remains poor. While <sup>123</sup>I-metaiodobenzylguanidine (MIBG) remains the dominant scintigraphic imaging modality for staging and follow-up of neuroblastoma, <sup>18</sup>F-FDG PET/CT plays a role in patients with MIBG negative disease. The role of <sup>18</sup>F-FDG PET/CT in patients with MIBG-avid disease continues to be explored.

### Aims:

The objective of this study was to report quantitative FDG PET findings in a cohort of children with neuroblastoma (irrespective of MIBG avidity) and to assess for associations with *MYCN* amplification status, as a prognostic marker, and with survival.

### Methods/Materials:

This was a single institution retrospective review of patients with newly diagnosed neuroblastoma for whom a pre-therapy <sup>18</sup>F-FDG PET/CT was obtained between July 2006 and July 2019. FDG-PET examinations had been obtained with 0.1-0.14 mCi/kg of FDG with imaging at 1 hour after radiopharmaceutical administration. Using the PET-edge tool in MIM (MIM Software; Cleveland, OH), a single observer drew regions of interest around the primary tumor to measure SUVmax, SUVmean, tumor volume (TV), and total lesional glycolysis (TLG, SUVmean x tumor volume). Event free survival (EFS) was defined as the time from diagnosis to tumor recurrence or death. Overall survival (OS) was defined as the time from diagnosis to death. Univariate and multivariate (age-adjusted) analysis via a Cox proportional hazards regression analysis was used to assess the predictive performance of PET indices for EFS and OS. Optimal thresholds for the PET indices were determined using receiver operating characteristic (ROC) curve analysis, and the survival significance of these thresholds were determined using a log-rank test.

### Results:

A total of 55 patients were identified, 45 of whom had *MYCN* FISH performed. The median age at diagnosis was 2.92 years (range 0.011-10.4 years). Average follow-up was 1654 days (range 27-4433 days). Nine patients (16%) experienced disease recurrence, and eleven patients (20%) expired during the study period. Thirteen patients (29%) had *MYCN* amplification. SUVmax ranged from 1.1 to 11.2 (mean  $4.38 \pm 2.23$ ). SUVmean ranged from 0.78 to 4.67 (mean  $2.05 \pm 0.84$ ). TV ranged from 0.80 to 1039 mL (mean  $179 \pm 232$  mL). TLG ranged from 0.77 to 2681 (mean  $418 \pm 601$  mL). Patients with *MYCN* amplification were older than those without *MYCN* amplification ( $3.6 \pm 2.3$  vs  $2.4 \pm 1.6$  years,  $p = 0.04$ ). Mean SUVmax ( $5.9 \pm 2.4$  vs  $4.2 \pm 2.1$ ,  $p = 0.028$ ), tumor volume ( $438 \pm 335$  mL vs  $95 \pm 96$  mL,  $p < 0.0001$ ), and TLG ( $1056 \pm 845$  vs  $226 \pm 329$ ,  $p < 0.0001$ ) were significantly higher in *MYCN* amplified tumors. SUVmax ( $p = 0.028$ ) and SUVmean ( $p = 0.045$ ) were significantly associated with overall survival. An SUVmax threshold of 4.77 ( $p = 0.028$ ) best predicted OS with a median OS of 2604 days for patients with SUVmax above this threshold versus >2957 days for patients with SUVmax below this threshold.

### Summary/Conclusions:

On average, *MYCN* amplified neuroblastomas are larger and more metabolically active than *MYCN* non-amplified tumors. Additionally, higher pretreatment SUVmax and SUVmean are associated with shorter overall survival.



## **P301 Pattern and predictors of sites of relapse in neuroblastoma: a report from the International Neuroblastoma Risk Group (INRG) project**

*Vo, K.<sup>2</sup>, DuBois, S.<sup>3</sup>, Neuhaus, J.<sup>4</sup>, Braunstein, S.<sup>5</sup>, Weil, B.<sup>6</sup>, Matthay, K.<sup>7</sup>*

UCSF Benioff Children's Hospital, University of California, San Francisco, Pediatrics, San Francisco, USA <sup>2</sup>, Dana-Farber/Boston Children's Hospital and Harvard Medical School, Pediatrics, Boston, USA<sup>3</sup>, University of California, San Francisco, Epidemiology and Biostatistics, San Francisco, USA<sup>4</sup>, University of California, San Francisco, Radiation Oncology, San Francisco, University of California, San Francisco<sup>5</sup>, Boston Children's Hospital and Harvard Medical School, Surgery, Boston, USA<sup>6</sup>, UCSF Benioff Children's Hospital, University of California, San Francisco, Pediatrics, San Francisco, USA<sup>7</sup>

### Background/Introduction:

As relapse remains the major obstacle to cure for patients with high-risk neuroblastoma, it is imperative to assess the clinical and biologic predictors of patterns of failure.

### Aims:

To perform a detailed analysis of the biologic, clinical, and prognostic differences according to the pattern of failures at the time of first relapse.

### Methods/Materials:

Children < 21 years of age diagnosed with neuroblastoma or ganglioneuroblastoma between 1989-2017 with available data regarding site of first relapse (isolated local vs. isolated distant vs. combined local and distant sites) were identified from the INRG database. Data were compared between sites of relapse according to clinical features (age, tumor type, INSS stage, serum ferritin, and LDH); biologic features (*MYCN* status, histology, pooled segmental chromosomal aberrations); initial treatment; and overall survival from time of first relapse. Univariate analyses evaluating potential differences in any of the above variables according to the pattern of relapse were performed using ANOVA and chi-square tests. A competing risk regression was used to evaluate predictors of specific patterns of failure.

### Results:

The pattern of first relapse among 1,833 children was: 19% isolated local; 65% isolated distant; and 16% combined failure sites. All evaluated clinical and biologic variables differed statistically by relapse site, with exception of LDH and INPC diagnostic category. High-risk features and receipt of initial intensive therapy were more frequent in patients with isolated distant and combined relapse sites ( $p < 0.05$ ). Patients with INSS stage 3 disease were more likely to have isolated local failures (88/181 relapses, 49%) compared to patients with all other stages (258/1,627 relapses, 16%;  $p < 0.001$ ). The overall survival from time of first relapse was significantly different by site of relapse (5-year OS  $\pm$  standard error): isolated local, 64%  $\pm$  3%; isolated distant, 23  $\pm$  2%; and combined sites, 26  $\pm$  4% ( $p < 0.001$ ).

### Summary/Conclusions:

Patients with isolated distant and combined failure sites have a higher proportion of unfavorable clinical and biological features. Understanding differences based upon sites of relapse may inform our understanding of the failure of therapy at these sites.

## **P302 New strategies to reprogram dysfunctional tumor-infiltrating T cells in high-risk neuroblastoma**

Lucarini, V.<sup>2</sup>, Melaiu, O.<sup>3</sup>, De Ninno, A.<sup>4</sup>, Businaro, L.<sup>5</sup>, Fruci, D.<sup>6</sup>

Ospedale Pediatrico Bambino Gesù, Paediatric Haematology/Oncology, Rome, Italy <sup>2</sup>, Ospedale Pediatrico Bambino Gesù, Paediatric Haematology/Oncology, Rome, Italy<sup>3</sup>, CNR, Institute of photonic and nanotechnology, Rome, Italy<sup>4</sup>, CNR, Institute of photonic and nanotechnology, Rome, CNR<sup>5</sup>, Ospedale Pediatrico Bambino Gesù, Paediatric Haematology/Oncology, Rome, Italy<sup>6</sup>

### Background/Introduction:

The tumor microenvironment (TME) plays a crucial role in cancer progression. The presence of tumor-infiltrating T cells (TILs) and the absence of immunosuppressive elements have been associated with favorable prognosis in high-risk NB patients. Recently, a subset of intratumoral dendritic cells (DC) has been found crucial for anthracycline-induced anticancer immune responses, suggesting that they could be exploited to improve NB therapy.

### Aims:

Herein, we investigated the modulation of the immune infiltrate in tumors derived from the TH-*MYCN* transgenic mice, both *in vivo* and *ex vivo* models and in patient-derived organotypic tumor spheroids (PDOTS) collected from high-risk NB tumors, following treatment with chemotherapeutic drugs in combination with antibodies blocking the immune checkpoints.

### Methods/Materials:

Luciferase-expressing NB cell lines derived from spontaneous tumors of TH-*MYCN* transgenic mice were injected subcutaneously or orthotopically into syngeneic mice. Mice bearing established tumors were sacrificed and the functional status of the TILs was evaluated by flow cytometry. The crosstalk between TILs and tumor cells was evaluated in murine-derived organotypic tumor spheroids (MDOTS), culture systems resembling to native three-dimensional (3D) TME. PDOTS from high-risk NB tumors were also collected and analyzed. Specifically, MDOTS and PDOTS were treated with chemotherapeutic drugs, alone or in combination with immune checkpoint inhibitors (ICI) and then co-cultured with TILs. The ability to recall immune cells to tumor spheroids and the functional status of TILs have been evaluated with microfluidic devices and flow cytometry, respectively.

### Results:

The orthotopic tumor model showed a more immunosuppressive TME than the subcutaneous ones, predominantly infiltrated by macrophages, myeloid-derived suppressor cells and T regulatory cells. *Ex vivo* experiments showed that treatment with chemotherapeutic drugs in combination with ICI caused a decreased in the diameter of MDOTS and an increase in TIL recruitment. Flow cytometry analysis revealed that CD8<sup>+</sup> T cells co-cultured with MDOTS expressed higher levels of IFN and TNF $\alpha$  and reduced levels of Tbet and Tigit. Remarkably, drug treatment suppressed tumor growth *in vivo* and most interestingly induced a strong recruitment of IFN-expressing CD8<sup>+</sup> T cells in drug-treated PDOTS microfluidic devices.

### Summary/Conclusions:

Overall, our findings provide insights for investigating novel immunotherapeutic approaches in NB.



## **P303 A Novel Near-infrared Fluorescent Protein, iRFP720, Facilitates Transcriptional Profiling of Neuroblastoma Metastasis in Mice**

*Huang, T.,<sup>1</sup> Shohet, J.<sup>2</sup>, Rozen, E.<sup>3</sup>, Wu, J.<sup>4</sup>, Wigglesworth, K.<sup>5</sup>, Shohet, J.<sup>6</sup>*

Umass Medical School, Dept of Pediatrics, Worcester, USA<sup>1</sup>, Umass Medical School, Dept of Pediatrics, Worcester, USA<sup>2</sup>, Umass Medical School, Dept of Pediatrics, Worcester, USA<sup>3</sup>, Umass Medical School, Dept of Pediatrics, Worcester, USA<sup>4</sup>, Umass Medical School, Dept of Pediatrics, Worcester, Umass Medical School<sup>5</sup>, Umass Medical School, Dept of Pediatrics, Worcester, USA<sup>6</sup>

### **Background/Introduction:**

Metastatic relapse is a major cause of high risk neuroblastoma mortality and improved approaches to study in vivo metastasis will advance evaluation of novel therapeutics.

### **Aims:**

Here we describe a novel system for tracking metastasis by using a dual marker (bioluminescence and near infrared fluorescent protein 720 - iRFP 720) to label human neuroblastoma cell lines.

### **Methods/Materials:**

Tumor growth and metastasis was evaluated in vivo using orthotopic (renal capsule) or tail vein injection-derived xenografts in NOD scid gamma (NSG) mice.

### **Results:**

We compared the performance of this dual marker in whole-body imaging experiments using combined CT scans with bioluminescent and fluorescent imaging. Tumors from a panel of NB cells (CHLA20, SH-SY5Y, NGP and GIMEN) stably expressing the construct for luciferase and iRFP720 were evaluated. The high sensitivity of bioluminescence and iRFP720 as probes for in vivo imaging enabled detection of tumors at initial stages and the precise isolation of malignant cell subpopulations from metastatic sites. We monitored the growth of the tumors by epifluorescent whole-body imaging for up to six weeks after cell injection. The brightness of the tumors expressing iRFP720 correlated with the brightness levels of the bioluminescent levels for whole body imaging. In the tail vein injection model, the Lung and liver are the primary sites for NB metastasis. In the orthotopic model, human NB cancer cells reliably metastasize to distant organs from primary tumors grown within kidney renal capsule.

We evaluate and quantify the brightness of bioluminescence and iRFP720 fluorescence for ex vivo organ images to obtain an accurate picture of the overall distribution of the primary and metastatic NB cells in each organ. We also used iRFP720 for flow cytometry to quantify metastatic propensity for NB cell lines and FACS to isolate metastatic cell populations.

### **Summary/Conclusions:**

Overall, this approach permits us to precisely localize and continuously monitor the growth of the tumor by dual labeling - luciferase and iRFP 720 markers, and rapidly isolate distinct metastatic populations from different locations. Ongoing transcriptomic analysis comparing bone marrow to lung and liver metastasis will provide additional insight into mechanisms regulating metastasis in NB and the mechanisms of action for novel therapeutics targeting metastasis.

## **P304 Combined inhibition of EZH2 and EGFR results in neuroblastoma cell death**

Mateo-Lozano, S.<sup>2</sup>, Goncalves-Alves, E.<sup>3</sup>, Garcia, M.<sup>4</sup>, Rodriguez-Hernandez, C.J.<sup>5</sup>, Lavarino, C.<sup>6</sup>, Mora, J.<sup>7</sup>

Institut de Recerca. Hospital Sant Joan de Déu, Molecular Biology of Developmental Tumors Lab, Esplugues, Spain <sup>2</sup>, Institut de Recerca. Hospital Sant Joan de Déu, Molecular Biology of Developmental Tumors Lab, Esplugues, Spain<sup>3</sup>, Institut de Recerca. Hospital Sant Joan de Déu, Molecular Biology of Developmental Tumors Lab, Esplugues, Spain<sup>4</sup>, Institut de Recerca. Hospital Sant Joan de Déu, Molecular Biology of Developmental Tumors Lab, Esplugues, Institut de Recerca. Hospital Sant Joan de Déu<sup>5</sup>, Institut de Recerca. Hospital Sant Joan de Déu, Molecular Biology of Developmental Tumors Lab, Esplugues, Spain<sup>6</sup>, Institut de Recerca. Hospital Sant Joan de Déu, Molecular Biology of Developmental Tumors Lab, Esplugues, Spain<sup>7</sup>

### **Background/Introduction:**

The outcome of high-risk neuroblastoma (HR-NB), despite multimodal therapy, remains unsatisfactory at 50% at best. Thus, there is an urgent need to develop novel therapeutic strategies to increase the survival of these patients. Enhancer of zeste homolog 2 (EZH2), the catalytic subunit of Polycomb repressive complex 2 (PRC2), silences transcription through trimethylation of histone H3 lysine 27 (H3K27me3). EZH2 is frequently overexpressed in HR-NB and predicts clinical outcome. Moreover, EZH2 mediates epigenetic silencing of NB suppressor genes and inhibition of histone H3K27 demethylation is effective against HR-NB. Preclinical EZH2 inhibition or depletion has been reported effective but *in vivo* studies suggest that EZH2 inhibition alone may not be sufficient. Expression of human epidermal growth factor receptor (EGFR) has been correlated in a variety of tumors including NB with cancer progression, poor survival, response to therapy and resistance to cytotoxic agents. Due to the vital roles in tumor biology and progression, many EGFR inhibitors have been developed. However, monotherapy of EGFR inhibitors rapidly produces resistance.

### **Aims:**

We explored the putative synergistic effect of targeting EZH2 with GSK126 and EGFR with Canertinib in NB, both *in vitro* and *in vivo*.

### **Methods/Materials:**

The anti-proliferative effect induced by GSK126, Canertinib, and their combination was evaluated by MTS cell proliferation and clonogenic assays with crystal violet staining. The apoptotic effect of these drugs was evaluated by Western blot and annexin V staining by flow cytometry. Xenograft mouse models were generated to evaluate the *in vivo* effect.

### **Results:**

Treatment with either GSK126 or Canertinib alone in two NB cell lines caused a decrease in cell viability, after 8 days of treatment. The combined treatment induced a more pronounced effect. Accordingly, the combined treatment also decreased the ability to form colonies more effectively. Moreover, after 48h of treatment, the combination of GSK126 and Canertinib synergistically induced cleavage of PARP, strongly suggesting induction of apoptosis. The apoptotic effect was confirmed by annexin V staining. *in vivo*, although the inhibition of EZH2 or EGFR alone did not induce a significant delay of tumor growth, the combined treatment synergistically induced tumor growth inhibition.

### **Summary/Conclusions:**

Our findings suggest that the combined use of GSK126 and Canertinib exerts a synergistic effect on tumor growth inhibition both *in vitro* and *in vivo* by inducing apoptosis. This data provides a novel therapeutic opportunity in neuroblastoma.



## **P305 A Nationwide Phase II Study of Extremely Delayed Local Control Strategy in Children with High Risk Neuroblastoma : Japan Children's Cancer Group - Neuroblastoma Committee Trial (JN-H-11)**

*Shichino, H.<sup>2</sup>, Matsumoto, K.<sup>3</sup>, Hishiki, T.<sup>4</sup>, Iehara, T.<sup>5</sup>, Yoneda, A.<sup>6</sup>, Soejima, T.<sup>7</sup>, Takimoto, T.<sup>8</sup>, Takahashi, H.<sup>9</sup>, Yokota, I.<sup>10</sup>, Teramukai, S.<sup>11</sup>, Kamijo, T.<sup>12</sup>, Nakazawa, A.<sup>13</sup>, Fukushima, T.<sup>14</sup>, Hosoi, H.<sup>15</sup>, Mugishima, H.<sup>16</sup>, Nakagawara, A.<sup>17</sup>, Tajiri, T.<sup>18</sup>*

National Center for Global Health and Medicine, Pediatrics, Tokyo, Japan<sup>2</sup>, National Center for Child Health and Development, Children's Cancer Center, Tokyo, Japan<sup>3</sup>, Chiba University School of Medicine, Pediatric Surgery, Chiba, Japan<sup>4</sup>, Kyoto Prefectural University of Medicine Graduate School of science, Pediatrics, Kyoto, Kyoto Prefectural University of Medicine Graduate School of science<sup>5</sup>, Osaka City General Hospital, Children's Medical Center, Pediatric Surgery, Osaka, Japan<sup>6</sup>, Kobe Proton Center, Radiation Oncology, Kobe, Japan<sup>7</sup>, National Center for Child Health and Development, Clinical Epidemiology Research Center for Pediatric Cancer, Tokyo, Japan<sup>8</sup>, Fukushima Medical University, Biostatistics, Fukushima, Japan<sup>9</sup>, Hokkaido University Graduate School of Medicine, Biostatistics, Sapporo, Japan<sup>10</sup>, Kyoto Prefectural University of Medicine Graduate School of science, Biostatistics, Kyoto, Japan<sup>11</sup>, Saitama Cancer Center, Research Institute for Clinical Oncology, Saitama, Japan,<sup>12</sup> Saitama Children's Medical Center, Department of Clinical Research, Saitama, Japan<sup>13</sup>, Tsukuba University Faculty of Medicine, Pediatrics, Tsukuba, Japan<sup>14</sup>, Kyoto Prefectural University of Medicine Graduate School of science, Pediatrics, Kyoto, Japan<sup>15</sup>, Nihon University School of Medicine, Pediatrics, Tokyo, Japan<sup>16</sup>, Saga Heavy Ion Medical Accelerator, Director, Tosu, Japan<sup>17</sup>, Kyoto Prefectural University of Medicine Graduate School of science, Pediatric Surgery, Kyoto, Japan<sup>18</sup>

### Background/Introduction:

The survival rates of high risk neuroblastoma (HR-NB) patients are still unacceptable. The estimated 3-year progression-free and overall survival rates for 50 patients of JNBSG phase II clinical trial (JN-H-07) were 36.5±7.0% and 69.5±6.6% respectively. Increasing the time intensity and dose intensity of chemotherapy are both key factors for the treatment for HR-NB. In Japan the restart of chemotherapy after surgery tended to be significantly delayed, sometimes up to more than one month, due to surgical complications. Therefore, increasing the time intensity of chemotherapy had been an important issue to be addressed.

### Aims:

JNBSG conducted a nationwide prospective single-arm clinical trial of time-intensive multimodal treatment with extremely delayed local control strategy (EDLC).

### Methods/Materials:

Between May 2011 and September 2015, seventy-five patients with newly diagnosed HR-NB patients were enrolled. EDLC consisted of induction chemotherapy (IC), myeloablative chemotherapy (MAC), and local control treatment after MAC. IC consisted with cisplatin (100mg/m<sup>2</sup>), pirarubicin (40mg/m<sup>2</sup>), vincristine (1.5mg/m<sup>2</sup>), and cyclophosphamide (1,200mg/m<sup>2</sup> in the 1st course and 2,400mg/m<sup>2</sup> in the 2nd-5th course). After 5 courses of IC, all patients were immediately followed by MAC with melphalan (200mg/m<sup>2</sup>), etoposide (800mg/m<sup>2</sup>) and carboplatin (1600mg/m<sup>2</sup>) (MEC). After MAC local tumor eradication with surgery and irradiation (21Gy) were performed.

### Results:

A total of 75 patients were enrolled, 64 were evaluable: 40 male, 2.6 y/o average age, 56 stage 4, 51 Neuroblastoma, 21 MYCN amplified, 51 Unfavorable histology. 46 patients completed the protocol treatment, while the treatment was discontinued in 18 patients. The estimated 3-year event-free survival rates and overall survival rates (95%CI) of 64 patients were 44.4% [31.8% - 56.3%] and 80.7% [68.5% - 88.5%], respectively. The surgeries were performed safely in 56 patients. The response rates of INRC after MAC were 20.3%(13/64; 1 CR, 12 VGPR) and 59.3%(38/64; 1 CR, 12

VGPR,25 PR), respectively. The response rates of INRC after completion of protocol treatment were 51.6%(33/64; 23 CR, 10 VGPR) and 62.5% (33/64; 23 CR,10 VGPR, 9PR), respectively. Pathological response rates (Ef2+Ef3) were 74.4% in 43 patients evaluated. No patient died during the protocol treatment nor within 30 days after completion of the protocol treatment. Grade 4 adverse effects, excluding hematological adverse effects, occurred in 31 cases. EDLC was performed without affecting early relapse or progression in many cases.

Summary/Conclusions:

Local control treatment could be postponed until after MAC in a notable number of cases. We considered that EDLC strategy is feasible. In the currently ongoing JNBSG HR-NB study (JN-H-15), EDLC is performed after BU/MEL treatment.

## **P306 Have fever and infections during induction chemotherapy in HR-neuroblastoma an impact on outcome?**

*Ernst, A.J.*<sup>2</sup>, *Eggert, A.*<sup>3</sup>, *Klingebl, T.*<sup>4</sup>, *Borkhardt, A.*<sup>5</sup>, *Niemeyer, C.*<sup>6</sup>, *Rutkowski, S.*<sup>7</sup>, *Hero, B.*<sup>8</sup>, *Simon, T.*<sup>9</sup>, *Berthold, F.*<sup>10</sup>

University of Cologne, Institute of Medical Statistics and Computational Biology (IMSB), Cologne, Germany<sup>2</sup>, Charité Universitätsmedizin Berlin and Berlin Institute of Health, Department of Pediatric Oncology and Hematology, Berlin, Germany<sup>3</sup>, Goethe University Frankfurt (Main), Department of Children and Adolescents, Frankfurt (Main), Germany<sup>4</sup>, Heinrich-Heine-University, Department of Pediatric Oncology, Hematology and Clinical Immunology, Duesseldorf, Heinrich-Heine-University<sup>5</sup>, University of Freiburg, Department of Pediatric Hematology and Oncology, Freiburg, Germany<sup>6</sup>, University Medical Center Hamburg-Eppendorf, Department of Pediatric Hematology and Oncology, Hamburg, Germany<sup>7</sup>, University of Cologne, Department of Pediatric Oncology and Hematology, Cologne, Germany<sup>8</sup>, University of Cologne, Department of Pediatric Oncology and Hematology, Cologne, Germany<sup>9</sup>, University of Cologne, Department of Pediatric Oncology and Hematology, Cologne, Germany<sup>10</sup>

### Background/Introduction:

Fever and infections during induction chemotherapy indicate an activation of the endogenous defense system against the tumor, however, the necessary delay of the subsequent chemotherapy may be associated with some tumor revival.

### Aims:

The impact of inflammatory reactions and prolonged recovery time on outcome in neuroblastoma was investigated.

### Methods/Materials:

A retrospective data analysis of high-risk patients included in the GPOH trials NB2004-HR and NB97 (high-dose chemotherapy arm) was performed.

Eligibility criteria were neuroblastoma diagnosis according to the INSS criteria, stage 4 and age  $\geq 18$  months or non-stage 4 with *MYCN* amplification, and at least 6 courses of chemotherapy before their first progress/residual tumor/death.

Standard induction chemotherapy (SIC) consisted of six alternating courses N5 (cisplatin, etoposide, vindesine) and N6 (vincristine, dacarbazine, ifosfamide, doxorubicine). Patients of the NB2004-HR experimental arm received two additional courses consisting of topotecan, cyclophosphamide and etoposide before SIC.

Event free (EFS) and overall survival (OS) were measured from day one of the sixth chemotherapy course. The adverse effects were graded according the CT-CAE system. The number of febrile episodes and infections, respectively, was limited to a maximum of one per course.

### Results:

484 patients were eligible and received 3318 courses of chemotherapy between March 1997 and December 2016. Fifteen percent had 0 infections throughout all courses, 9% 1, 12% 2, 14% 3, 14% 4, 15% 5, 13% 6. No febrile episode was seen in 5%, 8% had 1 febrile episode, 11% 2, 15% 3, 16% 4, 14% 5, 19% 6, 6% 7, 6% 8. In total, 1657 infections (50%; grade 1-2: 41%, 3-4: 8%, unknown: 1%) and 2001 febrile episodes (60%; grade 1-2: 56%, 3-4: 2%, unknown: 2%) were registered. No differences were detected with regard to sex, age < vs.  $\geq 18$  months) and *MYCN*-status. The N5 courses 3, 5, and 7 had lower frequencies of infections and/or fever. The median time between two successive courses (day 1 to day 1) was 1 day longer with infections (26 days, IQR 23-30) compared to those without (25 days, IQR 22-28,  $P < 0.001$ ). Febrile episodes were also associated with a longer interval (26 days IQR 23-30 vs. 24 days, IQR 22-28,  $P < 0.001$ ).

Patients who did not experience any infection (grades 1-4) had a better EFS and OS (10y-EFS: 44%, 95%-CI: 32-56 vs. 30%, 95%-CI: 25-35, log-rank  $P = 0.013$ ; 10y-OS: 52%, 95%-CI: 40-64 vs. 41%, 95%-CI: 36-46, log-rank  $P = 0.073$ ). The cumulative number of infections (grades 1-4) was not relevant. More severe infections (grades 3-4) had no impact on EFS and OS compared to no and less severe infections (grades 0-2).

An association between febrile episodes (grades 1-4 vs. none; grades 3-4 vs. 0-2) and EFS or OS was not detected.

#### Summary/Conclusions:

Only a minority of high-risk neuroblastoma patients did not experience infections and/or febrile episodes. Patients without infections during induction chemotherapy had a better EFS and OS. Febrile episodes were not associated with outcome differences.

## **P307 Relapsed Refractory Neuroblastoma – Retrospective Review in a Single Centre**

*Koh, M.<sup>2</sup>, Tan, E.<sup>3</sup>, K, V.<sup>4</sup>, Lim, K.<sup>5</sup>, Tan, A.M.<sup>6</sup>, Soh, S.Y.<sup>7</sup>*

KK Women's and Children's Hospital, Paediatrics, Singapore, Singapore <sup>2</sup>, KK Women's and Children's Hospital, Paediatrics, Singapore, Singapore<sup>3</sup>, KK Women's and Children's Hospital, Paediatrics, Singapore, Singapore<sup>4</sup>, KK Women's and Children's Hospital, Paediatrics, Singapore, KK Women's and Children's Hospital<sup>5</sup>, KK Women's and Children's Hospital, Paediatrics, Singapore, Singapore<sup>6</sup>, KK Women's and Children's Hospital, Paediatrics, Singapore, Singapore<sup>7</sup>

### **Background/Introduction:**

Neuroblastoma is an aggressive paediatric tumour which accounts for approximately 15% of cancer-related mortalities in children. While children diagnosed with low- or intermediate-risk neuroblastomas have excellent long-term survival, more than 50% of high-risk neuroblastomas have tumour relapse or regrowth. About 50% of the patients present with stage 4 (metastatic) disease and while the survival rates of these patients have improved, treatment for these patients remains a difficult task with the risk of relapse still prevalent.

### **Aims:**

To study the epidemiological factors and clinical profile of patients in a single centre with relapse of neuroblastomas.

### **Methods/Materials:**

A total of 33 children with relapsed and refractory neuroblastoma in KKH, Singapore from 1997 - 2017 were studied. Data for patient's initial disease characteristics - demographics (age of diagnosis, gender, race); biochemical data (urine HVA/VMA, serum ferritin and LDH); tumour characteristics (primary site, metastases); biological marker (NMYC); and final risk group according to the Children's Oncology Group neuroblastoma risk strata - were collected. Data was primarily studied with comparisons between the high risk and non-high risk group. Initial treatment details whether the patient underwent surgery, chemotherapy, radiotherapy and retinoic acid were also traced. Analysis of pattern of relapse was also done by collecting the time from first diagnosis and sites of relapse.

### **Results:**

A brief analysis of the initial characteristics shows a large percentage (97.0%) of them were of age more than 18 months during the diagnosis of neuroblastoma with the mean age of 4.6 years. According to the INSS stage, almost all the patients presented initially with Stage 3 or 4 (n=31 (93.9%)) but there was one stage 2B and one stage 1. Out of 33 patients, the NMYC status of 21 were traced and 1/3 showed amplification (n=7 (33.3%), total known=21). It was found that none of the non-high risk patients had NMYC amplification compared to the high risk group with had 26.9% of known NMYC amplified (n=7; total known high risk=18). The time of diagnosis to relapse between the high risk and non-high risk groups showed no significant difference with mean time in months 17.05 ( $\pm 10.66$  SD) for high risk and 17.08 ( $\pm 5.51$  SD) for non-high risk. In the high risk group, 23.1% (n=6; total = 26; unknown = 2) relapsed to primary while the remaining 69.2% (n=18) to metastatic. It was found that 80.8% (n=21) of high risk and 71.4% (n=5) of non-high risk patients current disease status was dead. Only 1 (3.8%) out of 26 high risk patients and 2 (28.6%) non-high risk patients are in complete remission.

### **Summary/Conclusions:**

Neuroblastomas continue to be a challenging tumour to treat especially in higher age group patients and high risk patients. Fortunately, progress have been made over the years with intensive research and clinical trials and hopefully the overall survival risk of high risk group patients will improve.





### **P308 Circulating adrenergic neuroblastoma mRNAs predict outcome in stage M children during treatment: a SIOPEN study.**

*Burchill, S.A.<sup>2</sup>, Corrias, M.V.<sup>3</sup>, Tchirkov, A.<sup>4</sup>, Lammens, T.<sup>5</sup>, Vicha, A.<sup>6</sup>, Träger, C.<sup>7</sup>, Yáñez, Y.<sup>8</sup>, Viprey, V.F.<sup>9</sup>, Wheatley, K.<sup>10</sup>, Gregory, W.M.<sup>11</sup>, Pötschger, U.<sup>12</sup>, Elliot, M.<sup>13</sup>, Luksch, R.<sup>14</sup>, Papadakis, V.<sup>15</sup>, Trahair, T.A.<sup>16</sup>, Canete, A.<sup>17</sup>, Laureys, G.<sup>18</sup>, Valteau-Couanet, D.<sup>19</sup>, Ladenstein, R.<sup>20</sup>*

University of Leeds, Leeds Institute of Medical Research, Leeds, United Kingdom <sup>2</sup>, Gaslini Institute, Gaslini Institute, Genoa, Italy<sup>3</sup>, Service de Cytogénétique Médicale and Clermont Université, CHU Clermont-Ferrand, Clermont-Ferrand, France<sup>4</sup>, University Hospital Ghent, University Hospital Ghent, Ghent, University Hospital Ghent<sup>5</sup>, 2nd Faculty of Medicine Charles University and University Hospital Motol, 2nd Faculty of Medicine Charles University and University Hospital Motol, Prague, Czech Republic<sup>6</sup>, Karolinska Institutet, Karolinska Institutet, Stockholm, Sweden<sup>7</sup>, Hospital Universitari i Politècnic La Fe, Oncología Pediátrica, Valencia, Spain<sup>8</sup>, University of Leeds, Leeds Institute of Medical Research, Leeds, United Kingdom<sup>9</sup>, University of Birmingham, Clinical Trials Unit, Birmingham, United Kingdom<sup>10</sup>, University of Leeds, Clinical Trials Research Unit, Leeds, United Kingdom<sup>11</sup>, CCRI/St. Anna Children's Hospital, Statistics, Vienna, Austria, <sup>12</sup>, Leeds General Infirmary, Paediatric Oncology and Haematology, Leeds, United Kingdom <sup>13</sup>, Fondazione IRCCS Istituto Nazionale dei Tumori, S.C. Pediatria Oncologica, Milan, Italy <sup>14</sup>, Agia Sofia Children's Hospital, Pediatric Oncology, Athens, Greece <sup>15</sup>, Sydney Children's Hospital, Kids Cancer Centre, Sydney, Australia <sup>16</sup>, Hospital Universitari i Politècnic La Fe, Pediatric Oncology, Valencia, Spain <sup>17</sup>, University Hospital Ghent, Pediatric oncology, Ghent, Belgium <sup>18</sup>, Institut Gustave Roussy, Département de Cancérologie de l'Enfant et de l'Adolescent, Paris, France <sup>19</sup>, CCRI/St. Anna Children's Hospital, Clinical Trial Unit for Studies & Statistics for Integrated Research & Projects, Vienna, Austria <sup>20</sup>

#### **Background/Introduction:**

Twenty percent of children with high-risk neuroblastoma present with tumours that are resistant to current induction treatment and 40% of those who attain complete or partial remission will relapse and die from disease within 5 years of diagnosis. Early identification of these children could mean modification of their treatment at diagnosis or during early treatment, with the goal of improving outcomes. High levels of adrenergic neuroblastoma mRNAs in bone marrow aspirates and blood in stage M children receiving frontline treatment for high-risk neuroblastoma predict poor outcome<sup>1</sup>, making them attractive candidates as circulating biomarkers of risk.

#### **Aims:**

To prospectively evaluate the predictive power of the adrenergic neuroblastoma mRNAs paired-like homeobox 2B (*PHOX2B*) and tyrosine hydroxylase (TH) in peripheral blood from children with stage M neuroblastoma at sequential time-points of the HR-NBL-1/SIOPEN protocol (<http://www.SIOPEN-NET.org>).

#### **Methods/Materials:**

Peripheral blood samples from 271 children were analysed by reverse transcriptase polymerase chain reaction (RTqPCR) for PHOX2B and TH mRNAs as previously described<sup>1</sup>. The predictive power of PHOX2B and TH mRNAs was evaluated using Kaplan-Meier survival curves and Cox regression analyses. Event-free survival (EFS) was calculated from the date that the blood sample was taken to the date of an event (relapse, death) or censored alive at the last clinic evaluation.

#### **Results:**

Consistent with previous results<sup>1</sup>, high levels of TH and PHOX2B mRNA in blood at diagnosis identified 1 in 5 children with rapidly progressing disease. At the end of induction treatment

(COJEC) detection of PHOX2B and TH mRNA in blood identified 11% (30/271,  $\chi^2= 4.76$ , HR=1.57,  $p=0.029$ ) of children with an increased risk of an event. In blood taken pre- and post- treatment for

minimal disease PHOX2B and TH mRNA remained associated with an increased risk of an event. This was most striking when evaluated at the end of treatment, the detection of TH and PHOX2B mRNAs identifying 8% (10/121) of children with a more than 6-fold increased risk of an event ( $\chi^2=32.0$ , HR=6.6,  $p=0.000$ ). Five year event-free survival (EFS) for children with PCR positive blood was 10% compared to 61% for those with PCR negative blood. In a multivariate analysis the detection of high levels of TH and PHOX2B mRNAs in blood at diagnosis dominated and predicted those children that had PCR positive blood at the end of induction treatment. In the minimal disease setting, children with both TH and PHOX2B mRNAs in blood were also at greater risk of death from disease than children with PCR negative blood samples; 11% (15/131) children had an 8-fold increased risk of death ( $\chi^2=56.8$ , HR=8.1,  $p=0.000$ ). This was independent of MYC-N status, age, type of myeloablative treatment and whether immunotherapy was given.

**Summary/Conclusions:**

TH and PHOX2B mRNAs in blood identify children with stage M disease at greatest risk of relapse. This simple blood test could be used to save specific children from ineffective toxic treatment and fast-track them for alternative possibly more effective investigational treatments.

<sup>1</sup>Viprey et al, 2014, JCO,32;1074-83.

	Number of children	Number PCR negative	Number PCR positive	% positive	Log rank Chi <sup>2</sup>	P value	Hazard Ratio	95% CI	5 Year EFS PCR negative (Std. Error)	5 Year EFS PCR positive (Std. Error)
End of induction	271	241	30	11%	4.76	0.029	1.57	1.04-2.36	34% (0.03)	17% (0.07)
Pre-MRD treatment	173	156	17	10%	6.77	0.009	2.20	1.20-4.04	49% (0.04)	29% (0.11)
Post-MRD treatment	121	111	10	8%	31.89	0.000	6.56	3.10-13.88	61% (0.05)	10% (0.1)

Caption 1: The detection of TH and PHOX2B mRNAs in blood predicts EFS

## **P309 Differentiation of Residual Neuroblastoma Manifestations in Patients with Neuroblastoma Stage 4S**

*Hero, B.*<sup>2</sup>, *Berthold, F.*<sup>3</sup>, *Koerber, F.*<sup>4</sup>, *Plum, G.*<sup>5</sup>, *Butzer, S.*<sup>6</sup>, *Decarolis, B.*<sup>7</sup>, *Eggert, A.*<sup>8</sup>, *Fischer, M.*<sup>9</sup>, *Simon, T.*<sup>10</sup>

University Children's Hospital, Pediatric Oncology, Cologne, Germany <sup>2</sup>, University Children's Hospital, Pediatric Oncology, Cologne, Germany<sup>3</sup>, Institute for Diagnostic and Interventional Radiology, University Hospital, Pediatric Radiology, Cologne, Germany<sup>4</sup>, University Children's Hospital, Pediatric Oncology, Cologne, University Children's Hospital<sup>5</sup>, University Children's Hospital, Pediatric Oncology, Cologne, Germany<sup>6</sup>, University Children's Hospital, Pediatric Oncology, Cologne, Germany<sup>7</sup>, Children's Hospital, Charite - Universitaetsmedizin, Pediatric Oncology, Berlin, Germany<sup>8</sup>, University Children's Hospital, Experimental Pediatric Oncology, Cologne, Germany<sup>9</sup>, University Children's Hospital, Pediatric Oncology, Cologne, Germany<sup>10</sup>

### Background/Introduction:

Neuroblastoma stage 4S is known for its excellent prognosis. Spontaneous regression is regularly observed, but differentiation of neuroblastoma manifestations has also been reported.

### Aims:

We here analysed data of a large cohort of patients with neuroblastoma stage 4S with special attention to differentiation of residual neuroblastoma manifestations and outcome.

### Methods/Materials:

Patients with stage 4S metastatic pattern were included if registered in the national trials NB97 and NB2004 between 1997 and 2016; diagnosed in the first year of life and presenting with *MYCN*-non-amplified tumors. For event-free-survival, progression to stage 4 metastatic pattern, any progression after the first year of life, secondary malignancy and death of any cause were considered, but not progression of primary or liver, skin, or soft tissue metastases during the first year of life typically known for patients with stage 4S.

### Results:

A total of 253 patients met the inclusion criteria and were included in the analysis. Median follow up was 9.1 years (range: 0.13-22 years).

The patients presented at a median of 3.0 months of age (range 0-11.7 months) with metastases in the bone marrow (68%), liver (75%) and/or skin (11%). Chemotherapy was only given to 118 out of 253 patients (47%) either at initial presentation or for typical stage 4S progression of primary or metastases (with and without symptoms) in the first year of life. In median two cycles (range 1-10) were applied. Ninety-seven patients underwent resection of the primary. Of interest, the primary regressed completely in 45 out of 57 patients (79%) never treated with chemotherapy or resected.

For the whole cohort, 5-year-EFS was  $0.82 \pm 0.03$  and 5-year-OS  $0.93 \pm 0.02$ . Eleven patients (4.4%) died from early fatal progression of liver metastases. One patient each developed a second malignancy, died from chemotherapy or died from other causes.

Progression to stage 4 was observed in 22 patients (8.7%) in median after 11.2 months (range 1.5-83 months). All 22 patients received chemotherapy for progression. Nine patients died, but only one patient diagnosed with progression within the first year of life.

After the first year of life, 11 patients (4.4%) presented either with progression of residual primary (n=6), relapse at the primary site (n=2) or progression of metastases (n=3, skin or testes). Progression occurred in median after 14.1 months (range 8.3-51.6 months). Only two out of 11 patients underwent chemotherapy for progression/relapse. Histologic assessment at relapse/progression revealed differentiation in 4 out of 11 patients (ganglioneuroma, n=3; ganglioneuroblastoma intermixed, n=1).

Similarly, in seven out of 15 patients where a residual primary was resected beyond the first year of life, differentiation was observed (ganglioneuroma, n=6; ganglioneuroblastoma intermixed, n=1).

At last follow, 231 patients were alive either in complete remission (n=177; 77%) or presenting with minor or major residuals (n=54; 23%).

Summary/Conclusions:

This analysis confirms the excellent prognosis of neuroblastoma stage 4S and the potential for regression. Besides progression, differentiation was observed as well in residual tumors as in tumors progressing after the first year of life.

## P310 Antibody-drug conjugate efficacy in neuroblastoma

Buongervino, S.<sup>2</sup>, Lane, M.<sup>3</sup>, Garrigan, E.<sup>4</sup>, Pascual-Pasto, G.<sup>5</sup>, Martinez, D.<sup>6</sup>, Zhelev, D.<sup>7</sup>, Dimitrov, D.<sup>8</sup>, Bosse, K.<sup>9</sup>

Children's Hospital of Philadelphia, Division of Oncology, Philadelphia, USA <sup>2</sup>, Children's Hospital of Philadelphia, Division of Oncology, Philadelphia, USA<sup>3</sup>, Children's Hospital of Philadelphia, Division of Oncology, Philadelphia, USA<sup>4</sup>, Children's Hospital of Philadelphia, Division of Oncology, Philadelphia, Children's Hospital of Philadelphia<sup>5</sup>, Children's Hospital of Philadelphia, Pathology, Philadelphia, USA<sup>6</sup>, University of Pittsburgh School of Medicine, Chemical Engineering, Pittsburgh, USA<sup>7</sup>, University of Pittsburgh School of Medicine, Chemical Engineering, Pittsburgh, USA<sup>8</sup>, Children's Hospital of Philadelphia, Division of Oncology, Philadelphia, USA<sup>9</sup>

### Background/Introduction:

The efficacy of antibody-drug conjugates (ADCs) in neuroblastoma relies on several target independent (payload sensitivity, drug transporter expression, drug resistance mutations) and target dependent (cell surface target density, antibody/target internalization kinetics) factors.

### Aims:

Here we aim to define the complex interplay of the multiple elements driving the efficacy of ADCs in neuroblastoma using the GPC2-directed D3-GPC2-PBD ADC (*Cancer Cell*, 2017) as an example.

### Methods/Materials:

Drug transporter expression, drug resistance mutations, GPC2 cell-surface density, D3-GPC2-IgG1 internalization kinetics and ADC payload sensitivity were quantified in a panel of 11 neuroblastoma cell lines utilizing RNA/DNA sequencing, flow cytometry, a fluorescent D3-GPC2-IgG1 antibody and ADC payload IC<sub>50</sub> assays. Payloads tested included tubulin (MMAE, DM1, DM4, dolastatin, HTI-286) and DNA (PNU-159682, PBD, duocarmycin, and N-acetyl-calicheamicin  $\gamma$ 1) interacting drugs. Cell lines were also treated with the D3-GPC2-PBD ADC to define the role of target molecule expression and antibody internalization kinetics in ADC efficacy.

### Results:

Neuroblastoma cell lines were significantly more sensitive to DNA vs. tubulin interacting ADC payloads (n=4 vs. 5; mean IC<sub>50</sub> = 48 pM, range = 14 - 79 pM vs. mean IC<sub>50</sub> = 1,453 pM, range = 137 - 2,784 pM,  $p = 0.04$ ). PNU-159682 was the most potent payload overall (mean IC<sub>50</sub> = 14 pM, range 3.2 - 44 pM), followed by N-acetyl-calicheamicin  $\gamma$ 1 (mean IC<sub>50</sub> = 37 pM, range 5-116 pM) and duocarmycin (mean IC<sub>50</sub> = 61 pM, range 23 - 170 pM). The most potent tubulin interacting payload was dolastatin (mean IC<sub>50</sub> = 137 pM, range 44 - 435 pM). DNA interacting payload treatment induced upregulation of  $\gamma$ H2AX, cleaved PARP and cleaved caspase-3 consistent with on-target DNA damage and apoptosis. MDR1 overexpression and TP53 mutations explained some of the ADC payload resistance patterns observed across this panel which will be reported.

D3-GPC2-IgG1 antibody internalization was significantly correlated with GPC2 cell-surface density ( $r = 0.7$ ,  $p = 0.02$ ) but not with D3-GPC2-PBD IC<sub>50</sub> ( $r = -0.1$ ,  $p = 0.55$ ). In fact, cell lines that were sensitive to PBD (n = 7 of 11; PBD IC<sub>50</sub> <40 pM), with over a 6-fold-range in D3-GPC2-IgG1 internalization, all responded robustly to the GPC2 ADC (IC<sub>50</sub> range 1 - 6 pM), except the very low D3-GPC2-IgG1 antibody internalizing NBL5 cell line (mean IC<sub>50</sub> >5,000 pM). However, the CHP134 cell line similarly internalized a low amount of antibody, yet was remarkably susceptible to PBD (mean IC<sub>50</sub> = 6 vs. 27 pM for NBL5), and thus was also sensitive to the ADC (mean IC<sub>50</sub> = 1.6 pM). Conversely, the NB-1643 cell line internalized the greatest amount of the D3-GPC2-IgG1 antibody and was only modestly susceptible to PBD (mean IC<sub>50</sub> = 49 pM), yet was also very sensitive to the ADC (mean IC<sub>50</sub> = 3 pM).

Taken together, these data reveal the critical interplay between antibody internalization and payload sensitivity in ADC efficacy.

Summary/Conclusions:

DNA interacting ADC payloads are significantly more cytotoxic to neuroblastoma cells than payloads that bind tubulin. Both target dependent and independent elements contribute to the efficacy of ADCs in neuroblastoma.

## **P311 PARP and CHK1 inhibitors synergize in killing *MYCN* amplified neuroblastoma via an ATM-dependent pathway**

*Di Giulio, S.*<sup>2</sup>, *Petroni, M.*<sup>3</sup>, *Colicchia, V.*<sup>4</sup>, *Pastorino, F.*<sup>5</sup>, *Pedretti, F.*<sup>6</sup>, *Fabretti, F.*<sup>7</sup>, *Ramponi, V.*<sup>8</sup>, *Nicolis Di Robilant, V.*<sup>9</sup>, *Coppa, A.*<sup>10</sup>, *Scafetta, G.*<sup>11</sup>, *Goffredo, B.M.*<sup>12</sup>, *Moretti, M.*<sup>13</sup>, *Canettieri, G.*<sup>14</sup>, *Belardinilli, F.*<sup>15</sup>, *Peruzzi, G.*<sup>16</sup>, *Infante, P.*<sup>17</sup>, *Bartolazzi, A.*<sup>18</sup>, *Ponzoni, M.*<sup>19</sup>, *Giannini, G.*<sup>20</sup>

La Sapienza University, Molecular Medicine, Rome, Italy <sup>2</sup>, La Sapienza University, Molecular Medicine, Rome, Italy<sup>3</sup>, La Sapienza University, Molecular Medicine, Rome, Italy<sup>4</sup>, IRCCS Giannina Gaslini Institute, U.O.S.D. Laboratorio Terapie Sperimentali in Oncologia, Genoa, IRCCS Giannina Gaslini Institute<sup>5</sup>, La Sapienza University, Molecular Medicine, Rome, Italy<sup>6</sup>, La Sapienza University, Molecular Medicine, Rome, Italy<sup>7</sup>, La Sapienza University, Molecular Medicine, Rome, Italy<sup>8</sup>, La Sapienza University, Molecular Medicine, Rome, Italy<sup>9</sup>, La Sapienza University, Experimental Medicine, Rome, Italy<sup>10</sup>, Sant'Andrea Hospital, UOC Anatomia patologica morfologica e molecolare, Rome, Italy<sup>11</sup>, I.R.C.C.S. Ospedale Pediatrico Bambino Gesù, Laboratorio di Patologia Metabolica, Rome, Italy, <sup>12</sup>, La Sapienza University, Molecular Medicine, Rome, Italy <sup>13</sup>, La Sapienza University, Molecular Medicine, Rome, Italy <sup>14</sup>, La Sapienza University, Molecular Medicine, Rome, Italy <sup>15</sup>, Center for Life Nanoscience@Sapienza –IIT, Nanotechnologies for Neurosciences, Rome, Italy <sup>16</sup>, La Sapienza University, Molecular Medicine, Rome, Italy <sup>17</sup>, Sant'Andrea Hospital, UOC Anatomia patologica morfologica e molecolare, Rome, Italy <sup>18</sup>, IRCCS Giannina Gaslini Institute, U.O.S.D. Laboratorio Terapie Sperimentali in Oncologia, Genoa, Italy <sup>19</sup>, La Sapienza University, Molecular Medicine, Rome, Italy <sup>20</sup>

### Background/Introduction:

*MYCN* amplification (MNA) is strongly correlated with poor prognosis and treatment failure in patients with neuroblastoma. Despite intense multimodal therapies, about 50% of these patients succumb to their disease, making the search for effective and less toxic therapies an absolute priority.

Activation of oncogenes such as *MYCN* is associated with high replication stress (RS) and DNA damage, making *MYCN*-driven tumors addicted to the activity of molecules involved in RS and DNA damage responses. Prompted by the relevant role of PARPs in controlling RS and DNA repair, we previously demonstrated that olaparib (a pharmacological inhibitor of PARP) enhanced *MYCN*-induced RS and caused cell death via mitotic catastrophe, in high-risk MNA and overexpressing neuroblastoma cells. Inhibition of the CHK1-dependent S-phase checkpoint raised by olaparib anticipated and increased the occurrence of mitotic catastrophe.

### Aims:

Together with the observation that both PARP and CHK1 expression is significantly increased in MNA compared to *MYCN* single copy (MNSC) neuroblastomas, our data suggested that *MYCN*-induced RS could make MNA neuroblastoma strongly addicted to PARP and CHK1 activity, providing a rationale to test their combined inhibition for therapeutic purposes.

### Methods/Materials:

We used a large panel of neuroblastoma cell lines to address the effectiveness of multiple PARP and CHK1 inhibitors to induce DNA damage, cell growth inhibition and cell death, as measured by comet assay, MTS, trypan blue exclusion test, immunoblot assay and mRNA quantification. For *in vivo* experiments, mice were subcutaneously or orthotopically injected with IMR32 cells and treated with vehicle, olaparib, the CHK1 inhibitor MK-8776 or their combination. Tumor growth was measured by caliper or bio-luminescent imaging. Genetic correction of mutant ATM in IMR32 cells was obtained by CRISPR/Cas9 technology.

### Results:

PARP inhibitors sharply increased the sensitivity of neuroblastoma cells to CHK1 inhibitors. The efficacy of this combination was far higher in MNA cell lines than in MNSC cells, *in vitro*. Indeed, the



MK-8776+olaparib combination led to accumulation of DNA damage and cell death in a synergistic and *MYCN*-dependent manner, allowing the use of suboptimal doses of MK-8776. Consistently, suboptimal doses of MK-8776 plus olaparib significantly reduced the growth of MNA neuroblastoma subcutaneous and orthotopic xenografts, and the growth of a SHH-*MYCN* medulloblastoma model, with no major toxicities.

IMR32, the most responsive cell line, bears a putatively pathogenic ATM mutation. Since it has been reported that genetic loss of homologous recombination repair genes, including ATM, may predict sensitivity to PARPi, in the 11q-deleted neuroblastoma, we asked whether ATM was involved in the responses to PARPi+CHK1i combination in this MNA neuroblastoma cell line. CRISPR/Cas9-based genetic correction of mutant ATM showed that this mutation did not fully abolish ATM kinase function and was irrelevant for the biological responses to PARPi+CHK1i combination. Rather, ATM pharmacological inhibition revealed that its kinase activity was required for the proapoptotic effect of PARPi+CHK1i combination, in MNA neuroblastoma cells.

#### Summary/Conclusions:

Our data highlight a new potential chemo-free strategy to treat MNA neuroblastoma and shed light on the involvement of ATM in shaping cell responses to PARPi+CHK1i in the MNA background.

**P312 Long-term evaluation of a combined polychemotherapy, MIBGI131 in vivo purging, autologous bone marrow transplantation (ABMT) and dendritic cell vaccine (DCV) program for the treatment of high-risk (HR) neuroblastomas (NB)**

*Hally Neves, N.S.<sup>2, 3</sup>, VINCE, C.S.<sup>4</sup>, AZAMBUJA, A.M.P.<sup>5</sup>, BRUMATTI, M.<sup>6</sup>, PEREIRA, P.L.<sup>7</sup>, DUTRA, M.<sup>8</sup>, FILIPPI, R.Z.<sup>9</sup>, SIQUEIRA, S.A.C.<sup>10</sup>, WELTMAN, E.<sup>11</sup>, NOVAK, E.<sup>12</sup>, GIMENEZ, T.<sup>13</sup>, KREPISCHI, A.C.V.<sup>14</sup>, BENDIT, I.<sup>15</sup>, BORBA, C.<sup>16</sup>, CRISTOFANI, L.M.<sup>17</sup>, ODONE-FILHO, V.<sup>18</sup>*

ITACI - Instituto de Tratamento do Câncer Infantil, Pediatric Hematology Oncology - São Paulo University Medical School, Sao Paulo, Brazil <sup>2</sup>, Brazil<sup>3</sup>, ITACI - Instituto de Tratamento do Câncer Infantil, Pediatric Hematology Oncology - São Paulo University Medical School, Sao Paulo, Brazil<sup>4</sup>, ITACI - Instituto de Tratamento do Câncer Infantil, Pediatric Hematology Oncology - São Paulo University Medical School, Sao Paulo, ITACI - Instituto de Tratamento do Câncer Infantil<sup>5</sup>, Hospital Israelita Albert Einstein, HEMATOLOGY ONCOLOGY, Sao Paulo, Brazil<sup>6</sup>, Hospital Israelita Albert Einstein, HEMATOLOGY ONCOLOGY, Sao Paulo, Brazil<sup>7</sup>, ITACI - Instituto de Tratamento do Câncer Infantil, Pediatric Hematology Oncology - São Paulo University Medical School, Sao Paulo, Brazil<sup>8</sup>, Hospital Israelita Albert Einstein, Pathology, Sao Paulo, Brazil<sup>9</sup>, HOSPITAL DAS CLÍNICAS DA FACULDADE DE MEDICINA DA UNIVERSIDADE DE SÃO PAULO, PATHOLOGY, São Paulo, Brazil<sup>10</sup>, Hospital Israelita Albert Einstein, RADIOTHERAPY, Sao Paulo, Brazil<sup>11</sup>, FUNDAÇÃO PRÓ-SANGUE HEMOCENTRO, Tumor Biology, Sao Paulo, Brazil, <sup>12</sup>, ITACI - Instituto de Tratamento do Câncer Infantil, TUMOR BIOLOGY, Sao Paulo, Brazil <sup>13</sup>, São Paulo University Medical School, Genetics and Evolutive Biology, São Paulo, Brazil <sup>14</sup>, FUNDAÇÃO PRÓ-SANGUE HEMOCENTRO, Tumor Biology, Sao Paulo, Brazil <sup>15</sup>, ITACI - Instituto de Tratamento do Câncer Infantil, Pediatric Hematology Oncology - São Paulo University Medical School, Sao Paulo, Brazil <sup>16</sup>, ITACI - Instituto de Tratamento do Câncer Infantil, Pediatric Hematology Oncology - São Paulo University Medical School, Sao Paulo, Brazil <sup>17</sup>, ITACI - Instituto de Tratamento do Câncer Infantil, Pediatric Hematology Oncology - São Paulo University Medical School, Sao Paulo, Brazil <sup>18</sup>

**Background/Introduction:**

HR NB represent an enormous problem for Pediatric Oncology. The impact of combinations including platinum derivatives (PD), the value of stem-cell (SC) transplants and more recently of anti-GD2, are the most significant steps for their treatment. Not all resources are easily available for institutions throughout the world. To establish a program that can truly add potential curative objectives, considering all these difficulties, is a true challenge.

**Aims:**

To provide a feasible and useful program for Brazilian institutions, considering the disparity of resources existent within the country.

**Methods/Materials:**

From 2000 to 2017, 439 children with NB were referred for treatment to the Institute for the Treatment of Pediatric Cancer (ITACI). 150/439 (34%) had been previously treated in other institutions. Only 357/439 (81.30%) had enough data allowing adequate analysis. This study enclosed 252/357 (70.6%) children over 18 months of age (median: 34.5 months) at diagnosis, either stage 4 or 3 with regional node involvement and/or amplified *MYCN*. 27 months was their median follow-up. Treatment: i = induction with cycles of: A) Topotecan/Cyclophosphamide; B) Vincristine/Cyclophosphamide/Adriamycin & Carboplatin/Etoposide (up to 2010: A-A-B-B-B; after 2010: A-A-B-B-A-A-B-B); ii = delayed surgery (primary site); iii = collection of peripheral blood SC (starting in 2002, after previous exposure to therapeutic MIBGI<sup>131</sup> [10 mci/kg, as previously defined in a phase I study, allowing enough peripheral stem-cell collection {PBSCC}, capable of recovering marrow function]); iv = ABMT, conditioning regimen until 2012: Carboplatin/Etoposide/Melphalan; Busulfan/Melphalan afterwards); v = individualized radiotherapy; vi = cis-retinoic acid and; vii = DCV (tumor cells from the patient's tumor and allogeneic dendritic cells). Response criteria: at the time of PBSCC the children should have: absence of any sign of progressive disease (PD), absence of bone

marrow (BM) involvement (9 BM aspirations & 2 biopsies & negative immunohistochemical study for NB84 and synaptophysin), normal lactate dehydrogenase levels and total absence of progression in images. Exclusion criteria: any sign of PD at the time of PBSCC.

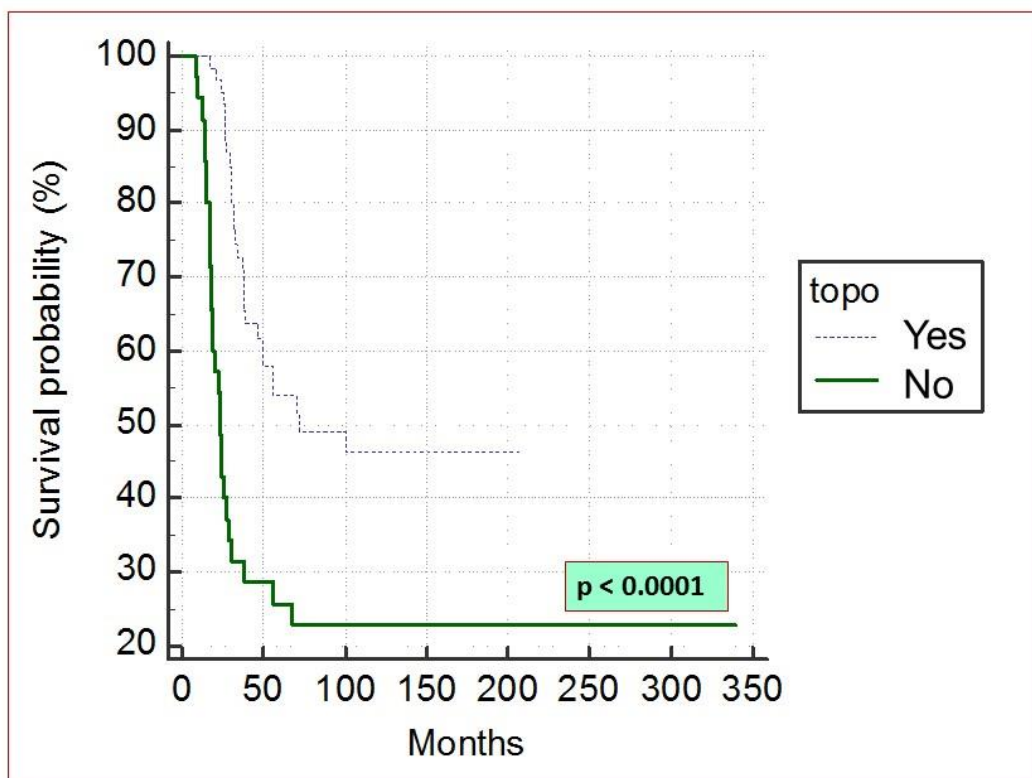
Results:

Overall results revealed (all patients had PD):  $35.9 \pm 5.08\%$ , without significant interferences with the use of ABMT or not and, for the transplanted patients, the use of MIBG purging or DCV. The only significant difference found was regarding the favorable use of CYCLOPHOSPHAMIDE/EETOPOSIDE (figure 1). When the analysis was restricted to patients entirely followed at ITACI, the overall survival was of  $55.8 \pm 7.65\%$  for the patients who did not experience either recurrences or progression during their management, without any change of the original chemotherapy program, compared to the ones who had modifications in their original programs due to progression or recurrences, before the point where ABMT was to be done (figure 2).

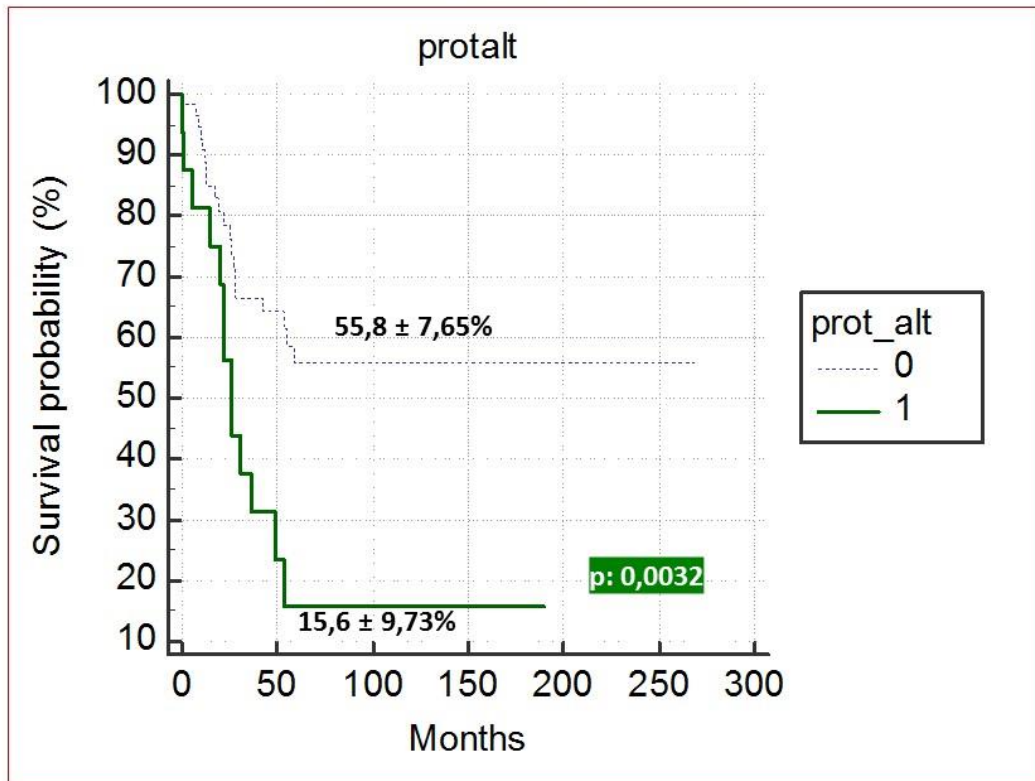
Summary/Conclusions:

The above described program may be able to confer results parallel to the best ones in the literature. However, the objective of a general application in Brazil revealed impossible, being essentially restricted to one or very few institutions. Although the definite role of each component of the program cannot be established, DCV are to be maintained for future propositions. The regular addition of anti-GD2 is the key point of these new possibilities.

## OS, PATIENTS AFTER ABMT, CYCLO+ TOPO X NO



OS, ABMT, up-front treatment X alternative chemo, single institution treatment



**P313 Delayed local treatment and Busulfan/Melphalan as a myeloablative chemotherapy for high-risk neuroblastoma (JN-H-15): a report from Neuroblastoma Committee of Japan Children's Cancer Group (JCCG) Trial**

*Matsumoto, K.<sup>2</sup>, Shichino, H.<sup>3</sup>, Yoneda, A.<sup>4</sup>, Hishiki, T.<sup>5</sup>, Hara, J.<sup>6</sup>, Iehara, T.<sup>7</sup>, Soejima, T.<sup>8</sup>, Nozawa, K.<sup>9</sup>, Okita, H.<sup>10</sup>, Takimoto, T.<sup>11</sup>, Yokota, I.<sup>12</sup>, Teramukai, S.<sup>13</sup>, Kamijo, T.<sup>14</sup>, Watanabe, K.<sup>15</sup>, Hino, M.<sup>16</sup>, Fukushima, T.<sup>17</sup>, Hiyama, E.<sup>18</sup>, Nakagawara, A.<sup>19</sup>, Tajiri, T.<sup>20</sup>*

National Center for Child Health and Development, Children's Cancer Center, Tokyo, Japan <sup>2</sup>, National Center for Global Health and Medicine, Pediatrics, Tokyo, Japan<sup>3</sup>, Osaka City General Hospital, Pediatric Surgery, Osaka, Japan<sup>4</sup>, National Center for Child Health and Development, Children's Cancer Center, Tokyo, National Center for Child Health and Development<sup>5</sup>, Osaka City General Hospital, Pediatric Hematology and Oncology, Osaka, Japan<sup>6</sup>, Kyoto Prefectural University of Medicine, Pediatrics, Kyoto, Japan<sup>7</sup>, Kobe Proton Center, Radiation Oncology, Hyogo, Japan<sup>8</sup>, Kanagawa Children's Medical Center, Radiological Technology, Kanagawa, Japan<sup>9</sup>, Keio University Hospital, Pathology, Tokyo, Japan<sup>10</sup>, National Center for Child Health and Development, Children's Cancer Center, Tokyo, Japan<sup>11</sup>, Graduate School of Medicine, Hokkaido University, Biostatistics, Hokkaido, Japan, <sup>12</sup>, Kyoto Prefectural University of Medicine, Biostatics, Kyoto, Japan <sup>13</sup>, Saitama Cancer Center, Research Institute for Clinical Oncology, Saitama, Japan <sup>14</sup>, Shizuoka Children's Hospital, Hematology and Oncology, Shizuoka, Japan <sup>15</sup>, Chiba University Hospital, Pediatrics, Chiba, Japan <sup>16</sup>, University of Tsukuba Hospital, Pediatrics, Tochigi, Japan <sup>17</sup>, Hiroshima University Hospital, Pediatric surgery, Hiroshima, Japan <sup>18</sup>, SAGA Heavy Ion Medical Accelerator in Tosu, CEO, Saga, Japan <sup>19</sup>, Kyoto Prefectural University of Medicine, Pediatric surgery, Kyoto, Japan <sup>20</sup>

**Background/Introduction:**

Neuroblastoma Committee (JNBSG) of Japan Children's Cancer Group (JCCG) conducted a phase II nation-wide clinical trial, JN-H-15, for high-risk neuroblastoma. This protocol was characterized by "delayed local treatment" in which tumor resection was performed after completing all chemotherapeutic courses including myeloablative high-dose chemotherapy (HDC), and by using busulfan/ melphalan (180 mg/m<sup>2</sup>) as HDC.

**Aims:**

To reveal the feasibility and effectiveness of JN-H-15 protocol.

**Methods/Materials:**

Between February 2015 and March 2018, 66 patients with high-risk neuroblastoma enrolled. One patient was excluded because of misdiagnosis of staging. All were diagnosed pathologically according to the central review as ganglioneuroblastoma (7), neuroblastoma poorly differentiated (57) and neuroblastoma undifferentiated (2). The construction of JN-H-15 protocol was similar to previous JN-H-11 protocol except for the following courses; two courses of 05A3 regimen in the induction therapy were substituted to two courses of ICE regimen, and MEC regimen for myeloablative chemotherapy was substituted to busulfan/melphalan (BuMel) regimen (busulfan on days -7 to -4 (0.8 to 1.2 mg/kg according to body weight) and melphalan (90 mg/m<sup>2</sup>) on day -3, -4).

**Results:**

Sixty-five patients evaluated were INRG stage M metastatic neuroblastoma (n=59) or *MYCN* amplified INRG stage L2 neuroblastoma (n=6). *MYCN* amplification was observed in 29 patients in total. Median age at diagnosis was 3.1 years old (range 0-10). Forty-seven patients (72.3%) completed the protocol and 18 patients discontinued because of progressive disease (1), parents' desire (8) or physicians' decision (6) due to poor response of induction chemotherapies.

The grade 4 adverse effects except for hematological adverse effects were seen in 5 cases which was fewer than JN-H-11; liver dysfunction (2), anaphylaxis (1), acute respiratory distress (1) and

coagulopathy (1). VOD after BuMel was experienced in 5 patients but all resolved including grade 3 hyperbilirubinemia in 2 patients. No patients died of treatment-related complication.

At the end of September 2019, thirty-eight out of 47 therapy-completed patients survived without disease. The response rates before HDC by INRC was 49.2% (32/65; CR(2), VGPR(7), PR(23), MR(8), NR(7) ) and that at 30 days after HDC was 53.9% (35/65; CR(2), VGPR(6), PR(27), MR(5), NR(7) ). The response rate after completion of protocol treatment were 46.2%(CR+VGPR) and 64.6%(CR+VGPR+PR) (30/65 and 42/65 respectively; CR(27), VGPR(3), PR(12), MR(1), NR(1), PD(1) ), which was comparable to that in JN-H-11 protocol. Delayed resections of primary tumors were performed in 41 patients, and pathological response rate (Ef2+Ef3) was 73.7% in 38 patients evaluated, which was similar to the result of JN-H-11.

#### Summary/Conclusions:

JN-H-15 protocol, which included “delayed local treatment”, ICE and BuMel regimen to improve the outcome, appears to be feasible and effective without anti-GD2 for high-risk neuroblastoma. Response rate was similar to JN-H-11 protocol, but the severe adverse events caused in JN-H-15 were fewer than that in JN-H-11, which were due to mostly using BuMel as HDC. Long-term effectiveness of JN-H-15 protocol should be evaluated.

## **P314 Neuroblastoma between 1990 and 2014 in the Netherlands: Increased incidence and improved survival of high-risk neuroblastoma**

*Tas, M.L.<sup>2</sup>, Reedijk, A.M.J.<sup>3</sup>, Karim-Kos, H.E.<sup>4</sup>, Kremer, L.C.M.<sup>5</sup>, Van de Ven, C.P.<sup>6</sup>, Dierselhuis, M.P.<sup>7</sup>, Van Eijkelenburg, N.K.A.<sup>8</sup>, Van Grotel, M.<sup>9</sup>, Kraal, K.C.J.M.<sup>10</sup>, Peek, A.M.L.<sup>11</sup>, Coebergh, J.W.W.<sup>12</sup>, Janssens, G.O.R.<sup>13</sup>, De Keizer, B.<sup>14</sup>, De Krijger, R.R.<sup>15</sup>, Pieters, R.<sup>16</sup>, Tytgat, G.A.M.<sup>17</sup>, Van Noesel, M.M.<sup>18</sup>*

Princess Máxima Center for Pediatric Oncology, Pediatric Oncology, Utrecht, Netherlands <sup>2</sup>, Princess Máxima Center for Pediatric Oncology, Pediatric Oncology, Utrecht, Netherlands<sup>3</sup>, Netherlands Comprehensive Cancer Organisation, Research, Utrecht, Netherlands<sup>4</sup>, Princess Máxima Center for Pediatric Oncology, Pediatric Oncology, Utrecht, Princess Máxima Center for Pediatric Oncology<sup>5</sup>, Princess Máxima Center for Pediatric Oncology, Pediatric Oncology, Utrecht, Netherlands<sup>6</sup>, Princess Máxima Center for Pediatric Oncology, Pediatric Oncology, Utrecht, Netherlands<sup>7</sup>, Princess Máxima Center for Pediatric Oncology, Pediatric Oncology, Utrecht, Netherlands<sup>8</sup>, Princess Máxima Center for Pediatric Oncology, Pediatric Oncology, Utrecht, Netherlands<sup>9</sup>, Princess Máxima Center for Pediatric Oncology, Pediatric Oncology, Utrecht, Netherlands<sup>10</sup>, Princess Máxima Center for Pediatric Oncology, Pediatric Oncology, Utrecht, Netherlands<sup>11</sup>, Erasmus Medical Center, Public Health, Rotterdam, Netherlands, <sup>12</sup>, Princess Máxima Center for Pediatric Oncology, Pediatric Oncology, Utrecht, Netherlands <sup>13</sup>, Princess Máxima Center for Pediatric Oncology, Pediatric Oncology, Utrecht, Netherlands <sup>14</sup>, Princess Máxima Center for Pediatric Oncology, Pediatric Oncology, Utrecht, Netherlands <sup>15</sup>, Princess Máxima Center for Pediatric Oncology, Pediatric Oncology, Utrecht, Netherlands <sup>16</sup>, Princess Máxima Center for Pediatric Oncology, Pediatric Oncology, Utrecht, Netherlands <sup>17</sup>, Princess Máxima Center for Pediatric Oncology, Pediatric Oncology, Utrecht, Netherlands <sup>18</sup>

### Background/Introduction:

Trends of longitudinal incidence of neuroblastoma on a population based scale are not well understood. Similarly, long-term trends in neuroblastoma incidence and survival in unscreened populations are unknown.

### Aims:

In this study we explored trends in incidence, stage and age at diagnosis, treatment and survival of neuroblastoma in the Netherlands from 1990-2014.

### Methods/Materials:

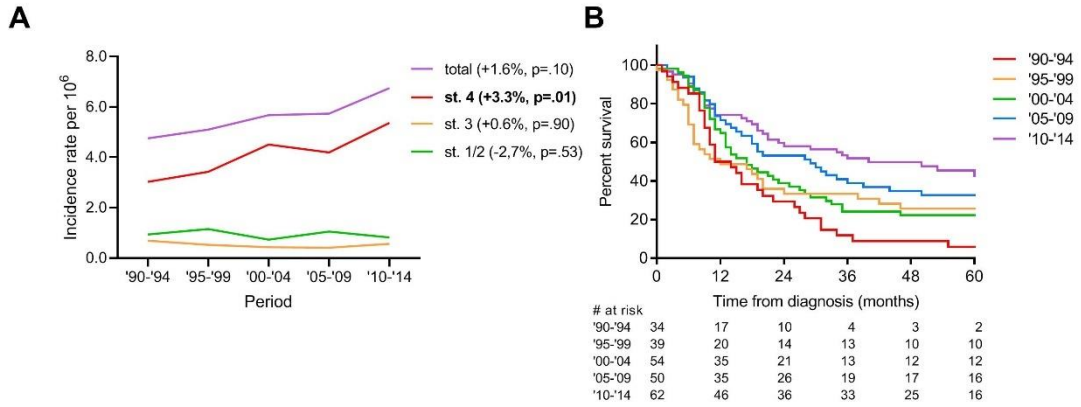
Data on all patients aged <18 years diagnosed with a neuroblastoma between 1990 and 2014 were provided by the Netherlands Cancer Registry (NCR). Trends in incidence by age group and stage at diagnosis were evaluated by calculating the average annual percentage change (AAPC). Univariate and multivariable survival analyses were performed for stage 4 disease to test whether changes in treatment are associated with survival.

### Results:

The cohort consisted of 593 newly diagnosed neuroblastoma cases. Forty-five percent was <18 months of age at diagnosis and 52% had stage 4 disease. The age-standardized incidence rate of stage 4 disease increased from 3.2 to 5.3 per million children per year (AAPC + 2.9%,  $p < .01$ ). This increase in incidence of stage 4 disease was exclusively caused by patients aged  $\geq 18$  months (3.0 to 5.4; AAPC +3.3%,  $p = .01$ , Figure 1A). Five-year OS of all patients improved from 44 $\pm$ 5% to 61 $\pm$ 4% from 1990 to 2014 ( $p < .01$ ) and from 19 $\pm$ 6% to 44 $\pm$ 6% ( $p < .01$ ) for patients with stage 4 disease. For patients  $\geq 18$  months old with stage 4 disease the 5-yr OS improved from 6 $\pm$ 4% to 43 $\pm$ 7% from 1990 to 2014 ( $p < .01$ , Figure 1B). Multivariable analysis revealed that high-dose chemotherapy followed by autologous stem cell rescue and anti-GD2 based immunotherapy were associated with the improved survival of stage 4 neuroblastoma patients (HR 0.46,  $p < .01$  and HR 0.37,  $p < .01$  respectively).

Summary/Conclusions:

Incidence of stage 4 neuroblastoma has increased exclusively in patients aged  $\geq 18$  months since 1990, while the incidence of other stages has remained stable. The 5-year OS of stage 4 patients improved, mostly due to the introduction of high-dose chemotherapy followed by stem cell rescue and immunotherapy.



Caption 1: A) Time trends of incidence rates per million children  $\geq 18$  months B) Five year OS by period for patients with stage 4 neuroblastoma  $\geq 18$  months.



## **P315 Establishing zebrafish xenografts for pediatric High Risk Neuroblastoma**

*Bozatzj, P.<sup>2</sup>, Rifatbegovic, F.<sup>3</sup>, Tarlungeanu, D.<sup>4</sup>, Pascoal, S.<sup>5</sup>, Sturtzel, C.<sup>6</sup>, Scheuringer, E.<sup>7</sup>, Westermann, F.<sup>8</sup>, Distel, M.<sup>9</sup>, Taschner-Mandl, S.<sup>10</sup>*

St Anna Children's Cancer Research Institute, Tumor biology, Vienna, Austria <sup>2</sup>, St Anna Children's Cancer Research Institute, Tumor biology, Vienna, Austria<sup>3</sup>, St Anna Children's Cancer Research Institute, Tumor biology, Vienna, Austria<sup>4</sup>, St Anna Children's Cancer Research Institute, Innovative cancer models, Vienna, St Anna Children's Cancer Research Institute<sup>5</sup>, St Anna Children's Cancer Research Institute, Innovative cancer models, Vienna, Austria<sup>6</sup>, St Anna Children's Cancer Research Institute, Innovative cancer models, Vienna, Austria<sup>7</sup>, Dkfz, German Cancer Research Center, Heidelberg, Germany<sup>8</sup>, St Anna Children's Cancer Research Institute, Innovative cancer models, Vienna, Austria<sup>9</sup>, St Anna Children's Cancer Research Institute, Tumor biology, Vienna, Austria<sup>10</sup>

### **Background/Introduction:**

High Risk neuroblastoma (HR-NB) accounts for approximately 50 % of neuroblastoma cases. These patients show aggressive metastatic tumors with a very poor response to therapy. More than 90 % of HR-NB patients present with disseminated tumor cells in the bone marrow and other parts of the body already at the time of diagnosis. To date the survival rate for HR-NB patients is below 40 % despite intensive multimodal treatment. HR-NB patients are treated according to the European HR protocol regardless of their response to induction chemotherapy and genetic makeup. This is in part due to the lack of detailed knowledge of the driver genes as well as the lack of reliable disease models for pre-clinical drug testing. In recent years, zebrafish has developed into a novel powerful tool to study cancer via xenotransplantation of human cell lines. However, xenotransplantation of human neuroblastoma derived from primary cells into the zebrafish larvae has been challenging and automated screening methods were not available.

### **Aims:**

The aim of this study is to establish zebrafish xenografts as a reliable, fast and personalized model for studying HR-NB. This will allow us to perform high-throughput image-based drug screens for addressing patients that are not responsive to current therapies.

### **Methods/Materials:**

We characterized three neuroblastoma cell lines, derived from HR-NB patients with different genetic makeup by whole genome sequencing, tagged with GFP, sorted and injected them into the perivitelline space of 2-days-post-fertilisation zebrafish larvae. Xenografts were treated with a combination of drugs that have been approved in clinical trials for HR-NB patients.

### **Results:**

In this study we demonstrate the successful engraftment of human neuroblastoma cells into the perivitelline space of 2-days-post-fertilisation zebrafish larvae. Upon transplantation, larvae were kept at 35 °C, a compromised temperature between the optimal temperature for human cell growth (37 °C) and zebrafish physiological development (28.5 °C). The injected larvae did not develop any health defects or malformations. Similarly, we also show that there was no significant difference in viability, apoptosis and migration between human NB cells that grew in culture at 35 °C and 37 °C. We were able to visualize and quantify the tumor cell growth *in vivo* for up to 8 days post injection, using the Operetta high-content live imaging system (PerkinElmer). As a proof-of-principle we estimated the IC50 of two drugs that have been reported to reduce growth of neuroblastoma cell lines *in vitro*. Based on the determined IC50 values we are now testing the drug effects on the xenotransplanted cell lines growth *in vivo*.

### **Summary/Conclusions:**

The successful establishment of a preclinical model for pediatric HR-NB will enable us to address the unmet need of increasing the survival rate of the aggressive disease. Our ultimate aim is to exploit

patient-derived neuroblastoma xenografts for quick identification of novel drug targets and combination therapies for precision medicine.

## **P316 A Cross-Sectional Study of the profile of Neuroblastoma at an Indian Tertiary Cancer Centre**

*Qureshi, S.<sup>2</sup>, Kembhavi, S.<sup>3</sup>, Chinnaswamy, G.<sup>4</sup>, Vora, T.<sup>5</sup>, Laskar, S.<sup>6</sup>, Khanna, N.<sup>7</sup>, Shah, S.<sup>8</sup>, Rangarajan, V.<sup>9</sup>, Prasad, M.<sup>10</sup>, Ramadwar, M.<sup>11</sup>, Shetty, O.<sup>12</sup>*

Tata Memorial Hospital, Pediatric Surgical Oncology, Mumbai, India <sup>2</sup>, Tata Memorial Hospital, Pediatric Radiology, Mumbai, India<sup>3</sup>, Tata Memorial Hospital, Pediatric Oncology, Mumbai, India<sup>4</sup>, Tata Memorial Hospital, Pediatric Oncology, Mumbai, Tata Memorial Hospital<sup>5</sup>, Tata Memorial Hospital, Pediatric Radiation Oncology, Mumbai, India<sup>6</sup>, Tata Memorial Hospital, Pediatric Radiation Oncology, Mumbai, India<sup>7</sup>, Tata Memorial Hospital, Nuclear Medicine, Mumbai, India<sup>8</sup>, Tata Memorial Hospital, Nuclear Medicine, Mumbai, India<sup>9</sup>, Tata Memorial Hospital, Pediatric Oncology, Mumbai, India<sup>10</sup>, Tata Memorial Hospital, Pathology, Mumbai, India<sup>11</sup>, Tata Memorial Hospital, Molecular Pathology, Mumbai, India, <sup>12</sup>

### Background/Introduction:

The tremendous progress in diagnostics and therapy for neuroblastoma worldwide has not ricocheted in most developing countries. Apart from these major lacunae, there is a constant strain to describe the incidence and distribution of neuroblastoma.

### Aims:

Since the information on disease burden in a community is an intricate part of the health care planning to guide quality improvement initiatives and policy formulation process we intended to review the profile and the treatment offered to patients with neuroblastoma presenting at a tertiary referral cancer which caters to essentially entire India.

### Methods/Materials:

All patients under 15 years with histologically confirmed neuroblastic tumours presenting at a tertiary cancer center from July 2006 to December 2018 were identified from the hospital database. Patients without a definite diagnosis of malignancy irrespective of the clinicoradiological features and prior therapy for suspected or proven disease elsewhere were excluded from the analysis. Staging evaluations included cross-sectional imaging for assessment of the primary, core biopsy for confirmation of the histology and assessment of *MYCN* status either by polymerase chain reaction and/or fluorescent in situ hybridization. Metastatic workup included a bone marrow aspirate and biopsy, I 131 meta-iodo-benzyl guanidine scan, bone scan and/or fluorodeoxyglucose positron emission tomogram scan. Patients were risk-stratified according to the COG risk stratification schema into low, intermediate and high-risk. The treatment included surgery alone or with chemotherapy for patients with low and intermediate-risk tumors. For high-risk tumors induction chemotherapy followed by surgery and radiotherapy was utilized. Autologous stem cell transplant (ASCT) was not feasible in all patients due to several reasons and immunotherapy is not available in our country. Overall the treatment was categorized into four groups, prior treatment only, prior treatment and treatment at our institute, treatment only at our institute and no cancer-directed treatment.

### Results:

The median age of the entire cohort of eligible 791 patients was 3 years with the majority (67.3%) in the 0-4 years' age group. The male to female ratio was 1.7:1 with 503 (63.6%) males and 288 (36.4%) females. The majority of patients were categorized as high-risk (51.4%) while low and intermediate-risk was present in 21% and 15% respectively. 12% of the patients could not be assigned risk due to inadequate work-up either due to the fulminant course of the disease or treatment abandonment. Of the 791 patients, 41 had presented with recurrent disease after receiving curative treatment elsewhere. Of the 750 patients with a primary presentation, 55.5% had treatment only at TMH and an additional 14% who had prior incomplete treatment also received treatment in TMH. 12% had prior treatment only and 18.5% had no cancer-directed treatment.

#### Summary/Conclusions:

Predictably, high-risk tumors form the bulk of our patients. A very high incidence of no cancer-directed treatment was observed in our population due to several patients (large burden of disease, suboptimal nutritional status, the toxicity of the treatment and poor family motivation) and administrative factors (non-availability of ASCT, immunotherapy, etc).

## **P317 Autologous stem cell mobilization in paediatric oncology patients; characteristics of Poor Mobilisers and the use of Filgrastim, Pegfilgrastim and Plerixafor.**

*Kraal, K.C.J.M.<sup>2</sup>, Rozeman, M.L.<sup>3</sup>, Jaspers-Bakker, A.<sup>4</sup>, Van den Bos, C.<sup>5</sup>, Zsiros, J.<sup>6</sup>, Tissing, W.<sup>7</sup>, Tytgat, G.A.M.<sup>8</sup>, Westinga, C.<sup>9</sup>, Voermans, C.<sup>10</sup>, Timmerman, I.<sup>11</sup>, Zwaan, C.M.<sup>12</sup>*

Princess Maxima Centre, Pediatric Oncology, Utrecht, Netherlands<sup>2</sup>, Princess Maxima Centre, Pediatric Oncology, Utrecht, Netherlands<sup>3</sup>, Princess Maxima Centre, Pediatric Oncology, Utrecht, Netherlands<sup>4</sup>, Princess Maxima Centre, Pediatric Oncology, Utrecht, Princess Maxima Centre<sup>5</sup>, Princess Maxima Centre, Pediatric Oncology, Utrecht, Netherlands<sup>6</sup>, University Medical Center Groningen, Pediatric Oncology, Groningen, Netherlands<sup>7</sup>, Emma Children's Hospital (EKZ/ AMC), Pediatric Oncology, Amsterdam, Netherlands<sup>8</sup>, Department of Clinical Pharmacy, Cell Therapy Facility, University Medical Center, Department of Clinical Pharmacy, Utrecht, Netherlands<sup>9</sup>, Sanquin Research and Landsteiner Laboratory, Department of Hematopoiesis, Amsterdam, Netherlands<sup>10</sup>, Sanquin Research and Landsteiner Laboratory, Department of hematopoiesis, Amsterdam, Netherlands<sup>11</sup>, Princess Maxima Centre, Pediatric Oncology, Utrecht, Netherlands,<sup>12</sup>

### Background/Introduction:

Mobilization of a sufficient amount of peripheral blood stem cells (PBSC) can be a challenge in paediatric patients with cancer. Recently, Pegfilgrastim and Plerixafor, a CXCR4 antagonist, are used for hematopoietic stem cell (HSC) mobilisation but data on their use in the paediatric population is limited.

### Aims:

To identify characteristics of Poor Mobilisers (PM) and compare success of mobilisation, apheresis yield, quality of stem cells and engraftment between different mobilisation regimens.

### Methods/Materials:

In this retrospective study we analysed medical records of 215 patients who underwent PBSC mobilization from August 2005 to December 2019 at the Princess Maxima Centre and Academic Medical Centre Amsterdam, The Netherlands. Mobilization regimens consisted of either daily injections Filgrastim 10mcg/kg or one single injection Pegfilgrastim (100, 150 or 200mcg/kg) after chemotherapy. Plerixafor was administered when peripheral blood CD34+ (PBCD34) counts failed to reach 20cells/ul. Patients were classified as PM when harvest counts were below  $2 \times 10^6$  CD34+cells/kg (within maximum 3 consecutive days) or in case Plerixafor was used.

### Results:

Two hundred fifteen paediatric oncology patients (age 1-19) scheduled for PBSC collection were consecutively included. One hundred four children were diagnosed with Neuroblastoma (NBL); median age 3 (range 0-19); 61% male, 39% female. Seventy-five NBL-patients (72,1%) received Filgrastim, 8 patients (7,7%) received Pegfilgrastim, 4 patients (3,8%) received Pegfilgrastim + Filgrastim, 6 patients (5,7%) received on-demand Plerixafor during initial mobilization, 9 patients (8,7%) received Plerixafor after initial failed harvest with (Peg)filgrastim. Twenty-one NBL-patients (20%) were classified as PM; NBL-patients accounted for 70% of all PM. Bone marrow (BM) involvement at both diagnosis and start of mobilization was more common in PM (90,5% vs. 60,5%;  $p=0.04$  and 57,1% vs. 26,5%;  $p=0.01$ ). NBL patients had more BM involvement at diagnosis and mobilisation (72,1 vs. 4,5%;  $p<0.01$  and 32,7 vs. 0%;  $p<0.01$ ) and received more cycles of chemotherapy prior to mobilisation compared to other malignancies (4 vs. 3;  $p<0.01$ ). After exclusion of PM, PBCD34+ counts before apheresis (40,46 vs. 99 cells/ul;  $p<0.05$ ), harvest yield (2,69 vs. 6,35  $\times 10^6$ cells/kg;  $p<0.05$ ) and CFU-GM/CD34+ ratio (0,20 vs. 0,30;  $p<0.05$ ) were lower in the NBL group. With the use of Plerixafor, 76% of all PM and 71% of PM with NBL were able to achieve a successful harvest. Successful engraftment was achieved in all PM and 94% of good mobilisers with NBL. No differences in neutrophil ( $>0,5 \times 10^9/L$ ) and platelet ( $>20 \times 10^9/L$ ) engraftment were observed between PM and good mobilisers. Median time to platelet engraftment was 32,5 days in NBL-patients and 19

days for patients with other malignancies ( $p < 0.05$ ). Further analyses will be performed on harvest products of selected patients to compare phenotypes of CD34+ cells mobilised with Filgrastim, PEGfilgrastim and Plerixafor.

Summary/Conclusions:

A significant proportion of NBL patients fails initial mobilisation with (Peg)filgrastim. The use of Plerixafor seems effective in most but not all poor mobilisers. NBL patients showed lower apheresis yield and longer time to platelet engraftment compared to patients with other malignancies. Further testing of harvest material will provide more information on CD34+ cell function and phenotype mobilised using different mobilization agents.

## **P318 Relationship between local control and quality assurance of radiotherapy. A report from the radiotherapy committee of the SIOOPEN High Risk Neuroblastoma Trial (HR-NBL1/SIOOPEN)**

*Boterberg, T.<sup>2</sup>, Magelssen, H.<sup>3</sup>, Ledoux, B.<sup>4</sup>, Kelly, S.M.<sup>5</sup>, Dieckmann, K.<sup>6</sup>, Malinova, B.<sup>7</sup>, Mandeville, H.<sup>8</sup>, Valteau-Couanet, D.<sup>9</sup>, Ladenstein, R.<sup>10</sup>, Gaze, M.N.<sup>11</sup>*

Ghent University Hospital, Department of Radiation Oncology, Ghent, Belgium <sup>2</sup>, Oslo University Hospital, Department of Oncology, Oslo, Norway<sup>3</sup>, Cliniques Universitaires Saint-Luc, Department of Radiation Oncology, Brussels, Belgium<sup>4</sup>, EORTC, Headquarters, Brussels, EORTC<sup>5</sup>, Medical University Of Vienna, Department of Radiation Oncology, Vienna, Austria<sup>6</sup>, University Hospital Motol, Department of Radiation Oncology, Prague, Czech Republic<sup>7</sup>, The Royal Marsden NHS Foundation Trust, Children's & Young Person's Unit & Haemato-oncology Unit, Sutton, United Kingdom<sup>8</sup>, Gustave Roussy, Département de Cancérologie de l'Enfant et de l'Adolescent, Paris, France<sup>9</sup>, St. Anna Hospital, Children's Cancer Research Institute, Vienna, Austria<sup>10</sup>, University College London Hospitals, Department of Clinical Oncology, London, United Kingdom<sup>11</sup>

### Background/Introduction:

In high risk metastatic and *MYCN*-amplified localised neuroblastoma, radiotherapy (RT) is part of the multimodality treatment approach and plays an important role to increase local control. Since inappropriate target volume delineation or dose delivery may have a negative impact on local control in irradiated patients, radiotherapy quality assurance (RTQA) is increasingly recognized as an essential component in the management of clinical trials. A retrospective RTQA analysis of a subset of cases irradiated in the HR-NBL1/SIOOPEN trial has been performed and correlated with local control data.

### Aims:

The aims of this analysis were 1) to perform RTQA based on imaging and RT data in high risk neuroblastoma patients and 2) to investigate and correlate local control data with the RTQA outcome results in these patients.

### Methods/Materials:

135 patients with complete imaging and radiotherapy data sets and local control information treated in the HR-NBL1/SIOOPEN trial between 2002 and 2018 were analyzed. All patients received irradiation according to the HR-NBL1/SIOOPEN trial protocol after induction chemotherapy, surgery, high dose chemotherapy, and autologous stem cell reinfusion. RTQA was performed during sessions attended by at least three SIOOPEN radiotherapy committee radiation oncologists. They assessed delineation of target volume and organs at risk, target dose coverage, and dose to organs at risk for all cases. Local control data were obtained from the HR-NBL1 database and verified in the patients' source files whenever possible.

### Results:

The majority of the patients (70%) were treated with two-field antero-posterior or parallel oblique techniques, although from 2010 onwards an increasing number of patients were treated with more conformal techniques, including intensity modulated radiotherapy and protons. 91% of patients received the per protocol prescribed dose of 21 Gy. In 30 of the 135 patients investigated, deviations from protocol with possible negative impact were found during RTQA. A significantly increased local recurrence rate of 43% (13/30) was found when RT had not been delivered according to the protocol guidelines, compared with 22% local recurrence rate (23/105) in patients treated with the correct target volume and dose ( $p = .02$ , chi-square test).

### Summary/Conclusions:

These results confirm earlier observations from the SIOOPEN radiotherapy committee regarding the importance of RTQA in the treatment of neuroblastoma. Further analysis in a larger patient population

is currently being performed. These data suggest the need for centralized prospective RTQA prior to RT delivery to improve protocol compliance rates and possibly local control.

The SIOPEN HR-NBL2 trial will have mandatory prospective RTQA through the SIOPE Quality and Excellence in Radiotherapy and Imaging for Children and Adolescents with Cancer across Europe in Clinical Trials (QUARTET) platform. Of note, in this trial there is a randomisation for patients with residual disease after surgery to a higher boost dose up to 36 Gy, in comparison with tumour bed irradiation only of 21.6 Gy. In this setting prospective RTQA may be even more important since delineation of residual disease is not always straightforward, and the higher dose is more likely to exceed tolerance of nearby organs at risk.



## **P319 Epigallocatechin-3-gallate is a specific inhibitor of the LIN28B/let-7 axis in neuroblastoma**

*Cocchi, S.*<sup>2</sup>, *Greco, V.*<sup>3</sup>, *Sighel, D.*<sup>4</sup>, *Sidarovich, V.*<sup>5</sup>, *Adami, V.*<sup>6</sup>, *Gatto, P.*<sup>7</sup>, *Lolli, G.*<sup>8</sup>, *Sturlese, M.*<sup>9</sup>, *Moro, S.*<sup>10</sup>, *Mancini, I.*<sup>11</sup>, *Defant, A.*<sup>12</sup>, *Provenzani, A.*<sup>13</sup>, *D'Agostino, V.*<sup>14</sup>, *Quattrone, A.*<sup>15</sup>

University of Trento, Cellular, computational and integrative biology, Trento, Italy <sup>2</sup>, University of Trento, Cellular, computational and integrative biology, Trento, Italy<sup>3</sup>, University of Trento, Cellular, computational and integrative biology, Trento, Italy<sup>4</sup>, University of Trento, Cellular, computational and integrative biology, Trento, University of Trento<sup>5</sup>, University of Trento, Cellular, computational and integrative biology, Trento, Italy<sup>6</sup>, University of Trento, Cellular, computational and integrative biology, Trento, Italy<sup>7</sup>, University of Trento, Cellular, computational and integrative biology, Trento, Italy<sup>8</sup>, University of Padova, Department of Chemical Sciences, Padova, Italy<sup>9</sup>, University of Padova, Department of Chemical Sciences, Padova, Italy<sup>10</sup>, University of Trento, Department of Physics, Trento, Italy<sup>11</sup>, University of Trento, Department of Physics, Trento, Italy, <sup>12</sup>, University of Trento, Cellular, computational and integrative biology, Trento, Italy <sup>13</sup>, University of Trento, Cellular, computational and integrative biology, Trento, Italy <sup>14</sup>, University of Trento, Cellular, computational and integrative biology, Trento, Italy <sup>15</sup>

### Background/Introduction:

LIN28B is an RNA binding protein extensively overexpressed in Neuroblastoma (NB) compared to other tumour types. Its expression in the sympathoadrenergic lineage of mice reproduces the human disease, underlying the importance of LIN28B in NB pathogenesis. One function of LIN28B is to prevent the maturation of miRNAs belonging to the let-7 family, important tumour suppressors able to induce inhibition of proliferation and of terminal differentiation. Therefore, the LIN28B-let7 axis can be considered a novel therapeutic target for NB treatment.

### Aims:

We propose that interfering, using small molecules, with the LIN28B/let-7 miRNAs axis will lead to a block of LIN28B activity and consequently to an increase of let-7miRNA levels. This, given sufficient time, would promote cell differentiation and thus the reduction of NB aggressiveness.

### Methods/Materials:

We performed a screening of small molecules using the amplified luminescent proximity homogeneous assay technology (Alpha Screen). The validation of the hits was carried out using the Alpha Screen and the RNA electrophoretic mobility shift assay (REMSA). We then analysed the activity of the selected molecule on NB cell lines. We firstly constructed dose-response curves to evaluate the cytotoxicity of EGCG and choose different concentrations for the treatments. Therefore, we evaluated through RT-PCR the variation of let-7 miRNAs after the treatment

### Results:

In order to identify molecules able to inhibit the LIN28B/ let-7miRNAs interaction, we screened a library of 5000 compounds. After the validation of the hits, we selected two molecules as candidate inhibitors: epigallocatechin-3-gallate (EGCG) and theaflavin-3-gallate (TFG), the last being also a metabolic derivative of the first in the liver. We synthesized some analogues of the two hits and assayed their activity *in vitro*. All the derivatives presented some variable degrees of activity, but still EGCG resulted to be the most effective compound. Therefore, we decided to validate its effect on NB cell lines. Our results showed a dose and time-dependent effects on cell viability, accompanied by the expected increase the level of let-7 miRNAs. Since it is reported that EGCG presents an unfavourable stability in solution, we attempted to improve the activity of EGCG encapsulating the molecule in PLGA-PEG nanoparticles. Using this vector we detected a significant increase in molecular activity.

### Summary/Conclusions:

EGCG is a well-known natural product already used in different applications, especially for its reported antioxidant activity. However, despite the promising biochemical results obtained, when we tested the

molecule on NB cell lines the activity was lower than expected. This could be due to the low stability of the molecule in solution, as reported in the literature. Therefore, we enclosed the molecule in PLGA-PEG nanoparticles and assessed if its biological activity could be improved. EGCG biological effects in NB cells significantly increased when EGCG was enclosed in nanoparticles. EGCG-nanoparticles induced a higher increase of let-7miRNAs and reduction of cellular growth, even at low concentrations. In the next future, we will perform experiments aimed at investigating the activity of EGCG-nanoparticles in synergism with other drugs. We will then evaluate the efficacy of the molecule in vivo using a suitable mouse model.

## **P320 Combined targeting of the p53 and pRb pathway in neuroblastoma**

*Schubert, N.A.*<sup>2</sup>, *Schild, G.G.*<sup>3</sup>, *Van Oirschot, S.*<sup>4</sup>, *Keller, K.M.*<sup>5</sup>, *Alles, L.K.*<sup>6</sup>, *Vernooij, L.*<sup>7</sup>, *Nulle, M.E.*<sup>8</sup>, *Van den Boogaard, T.L.*<sup>9</sup>, *Molenaar, J.J.*<sup>10</sup>

Princess Máxima Center for Pediatric Oncology, Research, Utrecht, Netherlands<sup>2</sup>, Princess Máxima Center for Pediatric Oncology, Research, Utrecht, Netherlands<sup>3</sup>, Princess Máxima Center for Pediatric Oncology, Research, Utrecht, Netherlands<sup>4</sup>, Princess Máxima Center for Pediatric Oncology, Research, Utrecht, Princess Máxima Center for Pediatric Oncology<sup>5</sup>, Princess Máxima Center for Pediatric Oncology, Research, Utrecht, Netherlands<sup>6</sup>, Princess Máxima Center for Pediatric Oncology, Research, Utrecht, Netherlands<sup>7</sup>, Princess Máxima Center for Pediatric Oncology, Research, Utrecht, Netherlands<sup>8</sup>, Princess Máxima Center for Pediatric Oncology, Research, Utrecht, Netherlands<sup>9</sup>, Princess Máxima Center for Pediatric Oncology, Research, Utrecht, Netherlands<sup>10</sup>

### **Background/Introduction:**

Clinical complete remission is achieved in many stage 4 neuroblastoma patients, but the high risk of relapse and the accompanying treatment-resistant nature of these tumors is still a challenge. We have previously identified higher frequencies of mutations that affect both the p53 and the pRb pathway, such as the homozygous loss of *CDKN2A* or co-amplification of *MDM2* and *CDK4*. In addition, both cyclin D1 and MDM2 overexpression is a common characteristic of primary neuroblastoma. These genes act upstream of Rb and p53, respectively, and hence play a major role in cell cycle regulation.

### **Aims:**

Our aim was to study whether the aberrations in the p53 and pRb pathway render cells more sensitive to CDK4 or MDM2 inhibition. Moreover, we were interested in whether combined inhibition of CDK4 and MDM2 would be of added value.

### **Methods/Materials:**

We exposed a series of 11 cell lines to the most promising CDK4 (ribociclib, palbociclib and abemaciclib) and MDM2 (SAR405838, HDM-201 and idasanutlin) inhibitors for 72 hours. We selected cell lines with well-defined mutational properties: homozygous *CDKN2A* deletion, co-amplification of *CDK4* and *MDM2*, *TP53* mutation and/or *MYCN* amplification. Moreover, CHP134 cells with inducible overexpression of CDK4 and/or MDM2 were generated to test our findings in an isogenic system. The effect of single compound and combined inhibition on cell viability and the cell cycle was studied using 10 different concentrations of abemaciclib and idasanutlin. This combination was also tested in an *in vivo* neuroblastoma xenograft model.

### **Results:**

Sensitivity for the MDM2 inhibitors inversely correlated with *TP53* mutation status, but surprisingly, it did not correlate to other p53 pathway aberrations (*MDM2* amplification/*CDKN2A* deletion). Similarly, there was no clear correlation seen for the CDK4 inhibitors with G1 checkpoint aberrations (*CDK4* amplification/*CDKN2A* deletion). Induced overexpression of CDK4 and/or MDM2 in CHP134 also did not increase sensitivity to CDK4 or MDM2 inhibitors. Bliss Independence values, calculated as a measure of synergy after combined treatment with abemaciclib and idasanutlin, revealed no synergistic effect. Instead, we saw an additive to slightly antagonistic effect in most cell lines. This adverse effect was supported by cell cycle analyses, which showed a lower apoptotic fraction, as well as PARP and caspase 3 cleavage, after combined treatment, as opposed to MDM2 inhibition alone. The *in vivo* study supported these results and showed reduced tumor growth after abemaciclib or idasanutlin treatment. Although combined treatment resulted in stable tumor volumes, the combined effect was not synergistic.

### **Summary/Conclusions:**

As was previously suggested in the first clinical trials with CDK4 inhibitors, neither *CDK4* nor *CDKN2A* status are clear biomarkers for CDK4 inhibitor sensitivity. Our results suggest that *MDM2* and

*CDKN2A* status also fail as biomarkers for MDM2 inhibitor sensitivity. Further testing is necessary to identify (other) biomarkers for these inhibitors. Moreover, the logical rationale to combine CDK4 and MDM2 inhibitors in neuroblastoma patients with both pRb and p53 pathway disturbances, should be taken with precaution.

## **P321 Anti-B7-H3 Antibody MGA271 Induces Antibody-Dependent Cellular Cytotoxicity with Ex Vivo Activated Natural Killer Cells against B7-H3 Positive Neuroblastoma and Tumor Microenvironment Cells and Enhances Immunotherapy of Neuroblastoma Tumors with Adoptive**

Wu, H.W.<sup>2</sup>, Sheard, M.A.<sup>3</sup>, Zobel, M.J.<sup>4</sup>, Robert, R.C.<sup>5</sup>

Children's Hospital Los Angeles, Hematology and Oncology, Los Angeles, USA <sup>2</sup>, Children's Hospital Los Angeles, Hematology and Oncology, Los Angeles, USA<sup>3</sup>, Children's Hospital Los Angeles, Hematology and Oncology, Los Angeles, USA<sup>4</sup>, Children's Hospital Los Angeles, Hematology and Oncology, Los Angeles, Children's Hospital Los Angeles<sup>5</sup>

### Background/Introduction:

Standard treatment of high-risk neuroblastoma (NB) with the anti-disialoganglioside (GD2) monoclonal antibody (mAb) dinutuximab after myeloablative chemotherapy has increased event-free and overall survival of patients. However, 40% of patients still relapse during or after this treatment. Using a second mAb that targets a different antigen on NB and/or tumor microenvironment cells concomitantly with dinutuximab has the potential to improve therapy.

### Aims:

B7-H3 (CD276), a member of the B7 family of molecules, is expressed on many tumors, including neuroblastoma. We determined if MGA271, a humanized anti-B7-H3 mAb, can induce antibody-dependent cellular cytotoxicity (ADCC) with *ex vivo* activated natural killer (aNK) cells against NB and tumor microenvironment cells and so increase the anti-NB efficacy of aNK cells combined with the anti- GD2 mAb dinutuximab.

### Methods/Materials:

Expression of B7-H3 on eight *MYCN*-amplified (*MYCN-A*) and three non-amplified (*MYCN-NA*) neuroblastoma cell lines, on cultured mesenchymal stromal cells (MSCs) from the bone marrow of neuroblastoma patients, and on cultured human endothelial cells (ECs) was determined with flow cytometry. ADCC mediated by aNK cells (1:1 effector:target cell ratio) with MGA271 (Macrogenics) against NB cells, MSCs, and ECs was assessed *in vitro*. The efficacy of aNK cells ( $10^7$  IV, 2x/week x 4 weeks) with dinutuximab (15 µg IV, 2x/week x 4 weeks) without or with MGA271 (100 µg IV, 2x/week x 4 weeks) against established intra-renal NB tumors formed by *MYCN-A*, luciferase-labeled cell lines SMS-KCNR or CHLA-136 in immunodeficient NOD-scid gamma (NSG) mice was evaluated by quantifying tumor growth and by mouse survival.

### Results:

B7-H3 was highly expressed, as demonstrated by flow cytometry, on all of eleven NB cell lines, including LA-N-6 and CHLA-90 which do not express GD2, on MSCs, and on ECs. MGA271 efficiently induced ADCC with aNK cells against NB cell lines, including LA-N-6 and CHLA-90 which were not sensitive to ADCC mediated by dinutuximab. MGA271 also induced ADCC with aNK cells against MSCs and ECs in a dose-dependent manner. NB tumors formed in NSG mice after intra-renal injection of SMS-KCNR or CHLA-136 cells were most effectively treated with aNK cells and dinutuximab when MGA271 was added as evaluated by both tumor growth and mouse survival.

### Summary/Conclusions:

B7-H3 is expressed on NB cells, MSCs, and ECs, and MGA271 induces ADCC with aNK cells against these B7-H3 positive cells. Treatment efficacy of dinutuximab with aNK cells against NB tumors in NSG mice is significantly increased by addition of MGA271.

## **P322 Second high-dose chemotherapy with autologous stem cell rescue in relapsing high risk neuroblastoma patients**

*Hero, B.<sup>2</sup>, Simon, T.<sup>3</sup>, Fischer, M.<sup>4</sup>, Lode, H.N.<sup>5</sup>, Lang, P.<sup>6</sup>, Eggert, A.<sup>7</sup>, Berthold, F.<sup>8</sup>*

University Children's Hospital, Pediatric Oncology, Cologne, Germany <sup>2</sup>, University Children's Hospital, Pediatric Oncology, Cologne, Germany<sup>3</sup>, University Children's Hospital, Experimental Pediatric Oncology, Cologne, Germany<sup>4</sup>, University Children's Hospital, Pediatric Oncology, Greifswald, University Children's Hospital<sup>5</sup>, University Children's Hospital, Pediatric Oncology, Tuebingen, Germany<sup>6</sup>, Children's Hospital, Charite - Universitaetsmedizin, Pediatric Oncology, Berlin, Germany<sup>7</sup>, University Children's Hospital, Pediatric Oncology, Cologne, Germany<sup>8</sup>

### Background/Introduction:

The prognosis of progressing or relapsing high risk neuroblastoma is still poor. Often, further progression is already observed during second-line induction chemotherapy. However, part of the patients respond to second-line induction chemotherapy and are candidates for consolidation treatments such as second high-dose chemotherapy with autologous stem cell rescue (ASCR).

### Aims:

To determine outcome of patients receiving second high-dose chemotherapy with autologous stem cell rescue after first relapse or progression.

### Methods/Materials:

Data of high risk neuroblastoma patients registered in the prospective national trials NB97 and NB2004-HR were analyzed. Inclusion criteria were INRG high risk criteria and age < 21 years at initial diagnosis, presenting with relapse/progression after previous high-dose chemotherapy with ASCR, response or stable disease to second line relapse induction therapy, and consolidated with second ASCR between 2007 and 2018. Only patients treated for first relapse or progression were included. Event-free-survival (EFS) was calculated from second ASCR to subsequent relapse, death or last follow up, overall survival (OS) was calculated from second ASCR to death or last follow up.

### Results:

A total of 21 patients met the inclusion criteria and were included in the analysis. At first diagnosis, 18 were stage 4 > 18 months and three were stratified high risk by *MYCN* amplification. Patients presented at a median age of 2.7 at initial diagnosis (range 0.6-6.3 years) and of 5.8 years at second ASCR (range 3.2-12.6 years). Seven patients were treated for isolated locoregional relapse and 14 for systemic or combined relapse.

At second ASCR, five patients presented in complete remission, eleven in partial remission and one patient with stable disease. The remission status was not available in four patients. High-dose regimens used were heterogeneous and treosulfan-based in eleven patients and busulfan-based in six patients. In eleven patients the second high-dose chemotherapy was combined with mIBG-therapy.

One patient died from transplant-associated toxicity, 13 patients experienced relapses 2.6 to 35 months after the second ASCR, 9 of them died. Seven patients are currently alive without further progression. At three years after second ASCR, EFS was  $0.32 \pm 0.10$  and OS  $0.57 \pm 0.11$ .

### Summary/Conclusions:

In this retrospective analysis of a small cohort of patients with relapse of high-risk neuroblastoma responding to second-line induction chemotherapy, consolidation with second high dose chemotherapy and ASCR seemed to be of benefit. This approach warrants further investigation in prospective trials.



## **P323 Pleuroparenchymal fibroelastosis: a rare complication of neuroblastoma therapy**

*Das, S.<sup>2</sup>, Foster, J.<sup>3</sup>, Melicoff, E.<sup>4</sup>*

Baylor College of Medicine, Pediatrics, Houston, USA <sup>2</sup>, Baylor College of Medicine, Pediatrics, Houston, USA<sup>3</sup>, Baylor College of Medicine, Pediatrics, Houston, USA<sup>4</sup>

### **Background/Introduction:**

Non-infectious pulmonary complications after chemotherapy and/or bone marrow transplant are well described. Pleuroparenchymal fibroelastosis (PPFE) is a rare interstitial lung disease, first reported as an idiopathic pneumonitis in adults in the 1990s. The disease is characterized by pleural thickening and fibrosis, parenchymal fibrosis predominantly in the upper lobes, with peripheral and subpleural elastic deposition. PPFE, as described in adults, has also been associated with prior bone marrow transplantation, lung transplantation, infection, toxic exposures/pollutants, chemotherapy, and in autoimmune disease. Patients with PPFE are at risk for progressive respiratory failure, refractory pneumothorax. Currently, the only known therapeutic option is lung transplant. We present a case series of patients with history of neuroblastoma, referred for lung transplant evaluation for end stage lung disease secondary to PPFE.

### **Aims:**

To describe the association of prior history of neuroblastoma to secondary lung fibrosis, specifically PPFE, occurring years after treatment.

### **Methods/Materials:**

The electronic medical records of eight patients with a history of neuroblastoma referred to Texas Children's Hospital for lung transplant evaluation from 2012-2019 were retrospectively reviewed. Clinical information, including symptoms, age at diagnosis of lung fibrosis, lung function testing and body mass index at time of presentation to the lung transplant service, along with treatment regimen, were extracted. Radiologists with an expertise in chest imaging and experience in diagnosis of PPFE reviewed chest CT scans, and pathology (either via lung biopsy or explant after lung transplant) was reviewed, if available, for confirmation of PPFE.

### **Results:**

The mean age of diagnosis of neuroblastoma was 3.3 years (range 1.8-4.7 years). The mean time between diagnosis of neuroblastoma and presentation of PPFE was 9.4 years (range 6.25-11.75 years). All 8 patients received chemotherapy according to the following protocols: ANBL0532, ANBL00P1, and A3973. Furthermore, all patients received radiation therapy (not to the chest however), along with autologous bone marrow transplant, preceded by varying conditioning regimens. All patients presented with progressively worsening dyspnea and dry cough; four of the eight patients presented with pneumothorax, with persistent air leak. Lung function testing demonstrated a severe restrictive defect, and the mean body mass index z-score was -2.9. Six patients presented with severe malnutrition, which became challenging to manage despite enteral and sometimes parenteral supplementation. Six of the eight patients have been lung transplanted: 4 are alive and doing well >2 years post-transplant; 1 patient was recently transplanted; and 1 patient died 6 years after transplant. Two patients were not eligible for transplant: one patient received talc pleurodesis, which is viewed as a contraindication in most lung transplant centers; the other was removed from the list for non-adherence.

### **Summary/Conclusions:**

Pleuroparenchymal fibroelastosis is a rare but underrecognized and important complication, seen in some patients years after neuroblastoma treatment. We speculate on cause of the progressive lung disease-whether it is an association of certain chemotherapeutic regimens (as thought in adults), or are certain patients predisposed due to other factors (eg, possible telomere shortening or genetic



predisposition). Our future work will include further investigation into those predisposing factors that could lead to PPFE in this population.

## **P324 New diagnostic tool based on label-free Tomographic Phase Microscopy to detect circulating neuroblastoma cells**

*Cimmino, F.*<sup>2</sup>, *Montella, A.*<sup>3</sup>, *Miccio, L.*<sup>4</sup>, *Memmo, P.*<sup>5</sup>, *Pirone, D.*<sup>6</sup>, *Bianco, V.*<sup>7</sup>, *Merola, F.*<sup>8</sup>, *Mugnano, M.*<sup>9</sup>, *Visconte, F.*<sup>10</sup>, *Kurelac, I.*<sup>11</sup>, *Villone, M.*<sup>12</sup>, *Maffettone, P.*<sup>13</sup>, *Iolascon, A.*<sup>14</sup>, *Ferraro, P.*<sup>15</sup>, *Capasso, M.*<sup>16</sup>

Università degli studi di Napoli Federico II, Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Napoli, Italy <sup>2</sup>, CEINGE, Biotecnologie Avanzate, \_\_, Napoli, Italy<sup>3</sup>, Institute of Applied Sciences and Intelligent Systems ISASI-CNR, \_\_, Napoli, Italy<sup>4</sup>, Institute of Applied Sciences and Intelligent Systems ISASI-CNR, \_\_, Napoli, Institute of Applied Sciences and Intelligent Systems ISASI-CNR<sup>5</sup>, Institute of Applied Sciences and Intelligent Systems ISASI-CNR, Dipartimento di Ingegneria Elettrica e delle tecnologie dell'informazione DIETI, Napoli, Italy<sup>6</sup>, Institute of Applied Sciences and Intelligent Systems ISASI-CNR, \_\_, Napoli, Italy<sup>7</sup>, Institute of Applied Sciences and Intelligent Systems ISASI-CNR, \_\_, Napoli, Italy<sup>8</sup>, Institute of Applied Sciences and Intelligent Systems ISASI-CNR, \_\_, Napoli, Italy<sup>9</sup>, CEINGE, Biotecnologie Avanzate, \_\_, Napoli, Italy<sup>10</sup>, Università di Bologna, Dipartimento di Scienze Mediche e Chirurgiche, Bologna, Italy<sup>11</sup>, Università degli studi di Napoli Federico II, Dipartimento di Ingegneria Chimica, dei Materiali e della Produzione Industriale, Napoli, Italy, <sup>12</sup>, Università degli studi di Napoli Federico II, Dipartimento di Ingegneria Chimica, dei Materiali e della Produzione Industriale, Napoli, Italy <sup>13</sup>, Università degli studi di Napoli Federico II, Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Napoli, Italy <sup>14</sup>, Institute of Applied Sciences and Intelligent Systems ISASI-CNR, \_\_, Napoli, Italy <sup>15</sup>, Università degli studi di Napoli Federico II, Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Napoli, Italy <sup>16</sup>

### Background/Introduction:

Circulating tumor cells (CTCs) serve as tumor biomarkers in many types of cancer and their clinical utility is under investigation. Recent evidence suggests that CTCs are detectable in neuroblastoma (NB) and may be useful as predictive biomarkers in early-phase clinical trials of response to chemotherapy. In addition, genome characterization of CTCs will offer the unique potential to design a precise targeting therapy to avoid disease recurrence. Despite the large number of approaches proposed to detect and classify CTCs, e.g. by examining their physical and/or biological properties, the accurate identification of CTCs still remains an unaccomplished goal.

### Aims:

Achieving affordable and accurate liquid biopsy for NB clinical use has so far proven difficult due to two main problems, i.e. technological issues in development of practical devices because of very small number of CTCs with respect the other components of the bloodstream (e.g., red blood cells, white blood cells and platelets) and lack of reliable biomarkers. The development of a method based on label-free microscopy to detect a set of all-optical and morphological features will be useful for NB CTCs detection in blood.

### Methods/Materials:

A new technology is proposed, based on the combination of tomographic phase microscopy (TPM) and microfluidics. TPM is an imaging technique able to supply three-dimensional phase contrast images at single cell level that will be applied on cells that rotate into engineered microfluidic channels in order to accurately control their rotation. The method allows to discern the inner structures of a cell without labelling but measuring the refractive indexes and thicknesses of the cell organelles as nucleus, nucleolus, mitochondrion etc. To provide reference values for nuclei and mitochondria, NB cell lines (SHSY5Y, SKNSH, CHP134, IMR32, SKNBE) were evaluated by using Amnis ImageStream<sup>x</sup> Mk II flow cytometer. Cells previously stained with DAPI (nuclear stain) or MitoTracker (mitochondrial stain) were evaluated by IDEAS software combining fluorescence intensity (DAPI or MitoTracker) and morphometric measures (darkfield or brightfield). In particular, distinct cells subpopulations in the same cell lines were observed based nuclei diameter (DAPI; intensity darkfield) or mitochondria number (mitotracker; area brightfield).

## Results:

Five NB cell lines were evaluated by TPM while rolling into microchannels and by flow cytometry upon fluorescent staining. Tomographic images of single cells were analysed to identify the cell constituents and fluorescence images of single cells were analysed to determine nuclei diameter and mitochondria number. We are now performing computational analyses to define physical features that are peculiar of NB cells and that can be detected by TPM. A comparison between the results obtained by Amnis ImageStream<sup>x</sup> Mk II flow cytometer and TPM will be presented.

## Summary/Conclusions:

High-throughput tomographic imaging of NB single cells in microfluidic platform will define a new diagnostic tool based on morphological parameters as biomarkers to identify NB CTCs. The proposed method can open the way for new label-free diagnostic tool for fast blood analysis and effective advanced NB screening.

## **P325 A Comparative Study of Extracellular Vesicle Encapsulated miRNA from 3D *in vitro* and *in vivo* Neuroblastoma Models.**

Nolan, J.C.<sup>2</sup>, Curtin, C.<sup>3</sup>, Frawley, T.<sup>4</sup>, O'Brien, F.<sup>5</sup>, Piskareva, O.<sup>6</sup>

Royal College of Surgeons in Ireland, Cancer Bioengineering Group, Dept. of Anatomy and Regenerative Medicine, Dublin, Ireland <sup>2</sup>, Royal College of Surgeons in Ireland, Tissue Engineering Research Group, Dept. of Anatomy and Regenerative Medicine, Dublin, Ireland<sup>3</sup>, Royal College of Surgeons in Ireland, Cancer Bioengineering Group, Dept. of Anatomy and Regenerative Medicine, Dublin, Ireland<sup>4</sup>, Royal College of Surgeons in Ireland, Tissue Engineering Research Group, Dept. of Anatomy and Regenerative Medicine, Dublin, Royal College of Surgeons in Ireland<sup>5</sup>, Royal College of Surgeons in Ireland, Cancer Bioengineering Group, Dept. of Anatomy and Regenerative Medicine, Dublin, Ireland<sup>6</sup>

### Background/Introduction:

The main challenge in treating high risk neuroblastoma is the development of multidrug resistance and metastatic spread, resulting in this rare cancer accounting for 15% of childhood cancer deaths. Neuroblastoma cells communicate with a vast array of neighbouring and distant cells via paracrine and endocrine signalling such direct cell-to-cell contact, cell-to-matrix adhesion and extracellular vesicles (EVs). In our pilot studies, EVs isolated from drug sensitive and resistant neuroblastoma cell lines conferred an increased colony forming ability and drug resistance to sensitive neuroblastoma cells, highlighting the ability of EV cargo containing DNA, RNA and protein to alter the behaviour of recipient cells in an oncogenic manner. Interactions between tumour cells and the extracellular matrix (ECM) also influence disease initiation, progression, and outcome. Therefore, the use of a more physiologically reflective preclinical model which facilitates these 3D ECM interactions enables more reliable testing of therapeutic agents, biomarker identification and basic research. Recently, we described a physiologically relevant porous 3D collagen-nanohydroxyapatite scaffold *in vitro* model for neuroblastoma that demonstrated cell growth and chemosensitivity similar to murine models. We hypothesised that this 3D model could be used to study mechanisms of EV communication in neuroblastoma.

### Aims:

We aimed to profile the miRNA cargo of EVs derived from drug resistant and sensitive neuroblastoma cells grown in orthotopic mouse models compared to corresponding cells grown in the physiologically relevant 3D porous collagen-nanohydroxyapatite scaffolds.

### Methods/Materials:

The Kelly (sensitive) and KellyCis83 (resistant) cell lines were grown in 3D *in vitro* scaffold and in murine orthotopic models. Circulating EVs were isolated from murine plasma and from conditioned media surrounding *in vitro* cells by ultracentrifugation, and validated by Western Blot and nanoparticle-tracking analysis. Profiling of EV miRNA cargo was performed using TaqMan™ OpenArray™ Human MicroRNA Panels.

### Results:

Cell growth and viability in the 3D *in vitro* model reflected that seen *in vivo* with resistant cells exhibiting higher growth than sensitive. Both cell lines exhibited a considerably higher tolerance to cisplatin in 3D *in vivo* and *in vitro* models at clinically relevant dose (200µM) compared to 2D (20 µM). We identified several miRNAs including miR-17/-92a/-106a and -483-3p which are abundantly expressed in EVs exported from cancer cells grown in the 3D *in vitro/in vivo* models and those which are altered in response to chemotherapeutic resistance. High expression of these miRNA in high risk neuroblastoma is significantly associated with poor overall and progression free survival indicating their potential role in mediating response and resistance to therapy.

### Summary/Conclusions:

Our study demonstrates the ability of EVs derived from drug resistant neuroblastoma cells to reprogram recipient cells, driving tumour progression and resistance. These results also demonstrate the potential of our 3D *in vitro* scaffold based model for the investigation of tumour behaviour, toxicity testing and its utility for the identification of EV encapsulated miRNA biomarkers for further exploration in liquid biopsies.

## **P326 New dose strategy for omega-3 fatty acids for neuroblastoma maintenance trials using body surface area and establishment of a novel omega-3 index, the EDD-index.**

*Kogner, P.,<sup>1</sup>Hedberg, G.<sup>2</sup>, Ljungblad, L.<sup>3</sup>, Hedberg, G.<sup>4</sup>, Wickström, M.<sup>5</sup>, Eissler, N.<sup>6</sup>, Pickova, J.<sup>7</sup>, Tedroff, K.<sup>8</sup>, Johnsen, J.I.<sup>9</sup>, Strandvik, B.<sup>10</sup>, Gleissman, H.<sup>11</sup>*

Karolinska Institutet, Childhood Cancer Research Unit, Stockholm, Sweden<sup>1</sup>, Karolinska Institutet, Childhood Cancer Research Unit, Stockholm, Sweden<sup>2</sup>, Karolinska Institutet, Childhood Cancer Research Unit, Stockholm, Sweden<sup>3</sup>, Karolinska Institutet, Childhood Cancer Research Unit, Stockholm, Sweden<sup>4</sup>, Karolinska Institutet, Childhood Cancer Research Unit, Stockholm, Karolinska Institutet<sup>5</sup>, Karolinska Institutet, Childhood Cancer Research Unit, Stockholm, Sweden<sup>6</sup>, Swedish University of Agricultural Sciences, Department of Molecular Sciences, Uppsala, Sweden<sup>7</sup>, Karolinska Institutet, Department of Women's and Children's Health, Stockholm, Sweden<sup>8</sup>, Karolinska Institutet, Childhood Cancer Research Unit, Stockholm, Sweden<sup>9</sup>, Karolinska Institutet, Department of Biosciences and Nutrition, Stockholm, Sweden<sup>10</sup>, Karolinska Institutet, Childhood Cancer Research Unit, Stockholm, Sweden<sup>11</sup>

### **Background/Introduction:**

Children surviving cancers, including high-risk neuroblastoma receiving multi-modal therapy, often develop chronic health conditions including late-effects on cognition and metabolism. We have recently shown that the long-chain omega-3 fatty acids, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), have significant therapeutic impact on neuroblastoma *in vitro* and *in vivo* including inhibition of tumor development suggesting a role against minimal residual disease. Omega-3 may have a role as adjuvant therapy showing synergism with several established modalities. In particular, oral DHA and EPA for long-term maintenance after concluded neuroblastoma therapy has been suggested to both limit risk of relapse and modulate risk of late adverse effects. However, the doses needed in clinical trials have not been established.

### **Aims:**

To establish a dose for future phase 2 trials we performed a dose-finding phase 1 study, the OmegaChild study (EPN 2014/120-31/1, NCT02134600).

### **Methods/Materials:**

A single blind, dose-escalating study of n-3 fatty acids supplementation was performed in 40 pediatric patients (21 girls) with median age 9.5 years (range 3-16 years). All children were in clinical remission (3-36 months) after concluded cancer therapy. The omega-3 dose was prescribed per body surface area (BSA) for 90 days. Participants were allocated to one of five daily doses: 400, 800, 1600, 2400 or 3000 mg/m<sup>2</sup> of EPA and DHA (40:60) administered as a daily dose in fruit juice. Fatty acid concentration in plasma phospholipids and red blood cells (RBC) were determined by GC and given as mol%. Omega-3 index ( $\Sigma$ EPA, DHA)), as well as our newly suggested EDD-index which also includes DPA were calculated at day 0, 45 and 90.

### **Results:**

Thirty-three participants completed the study with excellent compliance and no significant side effects. Median adherence was 96%. The omega-3 supplementation correlated with dose dependent increase of EPA and DPA, but less of DHA, indicating that DPA should be included in a novel n-3 index (EDD) ( $P < 0.0001$ ,  $\rho = 0.82$  CI (0.655 - 0.909)). The highest doses influenced both omega-6 and monounsaturated fatty acids, suggesting an optimal dose of about 1500 mg/m<sup>2</sup> BSA. However, doses up to 3448 mg/m<sup>2</sup> were tolerable and without severe adverse events.

### **Summary/Conclusions:**

Adjusting the dose of omega-3 supplementation to BSA resulted in a highly significant dose-dependent increase of the omega-3 fatty acids. We suggest a daily omega-3 dose of about 1500 mg/m<sup>2</sup> BSA for future phase-2 studies in children including long-term maintenance to evaluate impact

on clinical outcome of high-risk neuroblastoma. The marked increase of DPA suggests its inclusion in evaluation of the clinical effects, using our suggested novel EDD-index (E PA, D PA, D HA).

## **P327 Late-effects following high risk neuroblastoma treatment**

*Herd, F.<sup>2</sup>, Skinner, R.<sup>3</sup>, Tweddle, D.A.<sup>4</sup>*

NHS Grampian, Paediatric Oncology, Aberdeen, United Kingdom <sup>2</sup>, Newcastle University, Translational and Clinical Research Institute, Newcastle, United Kingdom<sup>3</sup>, Newcastle University, Translational and Clinical Research Institute,, Newcastle, United Kingdom<sup>4</sup>

### **Background/Introduction:**

Survival from high risk neuroblastoma (HRNB) used to be very poor resulting in escalation of treatment intensity to include radiotherapy, high dose chemotherapy with stem cell rescue and more recently immunotherapy. While survival has improved and is currently reported to be about 50% at 5 years, this improvement comes at a cost. There is emerging data highlighting the physical burden of intense treatment. In survivors of all stages of neuroblastoma the 20yr cumulative incidence of chronic disease has been reported at 41%<sup>1</sup>. This is four times that of siblings with the main systems affected being musculo-skeletal, neurological, endocrine and second malignancy. The ENSG 5 study of high risk patients showed that 77% of patients had one late effect with deafness and endocrine disturbance being the most frequent<sup>2</sup>. Only one study assessed psychological outcomes in survivors of HRNB and used siblings as controls. They demonstrated increased anxiety / depression, reduced attention, increased antisocial behaviour and increased social withdrawal<sup>3</sup>.

### **Aims:**

To identify the number of patients entering the long term follow up phase following high risk treatment for neuroblastoma in 2 UK principal treatment centres.

To assess the incidence and pattern of late effects and the relationship to changing treatment intensity.

### **Methods/Materials:**

Using local cancer registries survivors more than 5 years from treatment for HRNB were identified in 2 CCLG principal treatment centres (Newcastle & Aberdeen). A retrospective review of case notes and electronic records was performed and data collected on age at diagnosis, location of tumour, length of follow up, subsequent diagnoses and need for medical intervention and details about late mortality. Late effects were graded as per CTCAE 4.03.

### **Results:**

A total of 31 patients were identified with 27 patients from Newcastle and 4 from Aberdeen. They were diagnosed between 1987 and 2013 and were treated with a variety of different treatment regimens. All received high dose chemotherapy with stem cell rescue, 60% radiotherapy and 26% anti GD<sup>2</sup> immunotherapy. Mean age at diagnosis was 3.6 years (range 0.16-20.9 years). Length of follow up ranged from 5-25.5 years with mean 13.5yrs. There were 3 late relapses with one death from disease. All survivors had at least one late effect with a median of 2 per person with a maximum of 10. Hearing loss and endocrine disturbance / fertility problems were the most common late effects. One patient is awaiting a lung transplant after developing lung disease secondary to therapy. In those with information available about dental health over 50% had dental issues. All 3 patients to develop scoliosis had paravertebral disease with radiotherapy to spine.

### **Summary/Conclusions:**

Adult survivors of high risk neuroblastoma exist with a life-long burden of treatment. It is important to ascertain the variation in late effects in relation to treatment and the impact the late effects have on daily functioning and quality of life.



## **P328 Long term follow-up of children with Neuroblastoma receiving radiotherapy to metastatic lesions within the German Neuroblastoma Trials NB97 and NB 2004**

*Jazmati, D.<sup>2</sup>, Butzer, S.<sup>3</sup>, Hero, B.<sup>4</sup>, Steinmeier, T.<sup>5</sup>, Doyen, J.<sup>6</sup>, Simon, T.<sup>7</sup>, Timmermann, B.<sup>8</sup>*

University Hospital Essen, Clinic for Particle Therapy, Essen, Germany <sup>2</sup>, University of Cologne, Department of Pediatric Oncology and Hematology, Cologne, Germany<sup>3</sup>, University of Cologne, Department of Pediatric Oncology and Hematology, Cologne, Germany<sup>4</sup>, University Hospital Essen, Clinic for Particle Therapy, Essen, University Hospital Essen<sup>5</sup>, Centre Antoine-Lacassagne, University of Côte d'Azur, Department of Radiation Oncology, Nice, France<sup>6</sup>, University of Cologne, Department of Pediatric Oncology and Hematology, Cologne, Germany<sup>7</sup>, University Hospital Essen, Clinic for Particle Therapy, Essen, Germany<sup>8</sup>, , , ,

### Background/Introduction:

Neuroblastoma (NB) is the most common extracranial solid malignancy in children. Over 50% of patients present with metastatic lesions, and despite a multimodal treatment approach the prognosis of these patients is not satisfactory

Although radiotherapy has become an integral part for treatment of the primary tumor, the role of radiotherapy for osteomedullary lesions is not well defined. When compared to a cohort of children who did not receive RT to metastatic lesions, previous studies did not present any significant benefit of adding focal irradiation to metastatic sites (Polishchuk et al., 2014; Kandula et al., 2015). However, small series have reported promising results (Casey et al., 2018; Mazloom et al., 2014).

### Aims:

We performed a retrospective analysis to assess the impact of RT to osteomedullary lesions in children with NB.

### Methods/Materials:

Clinical records of patients who received irradiation for osteomedullary metastases during first-line treatment included in the prospective, national, multicenter neuroblastoma trials NB97 (EU - 20661) or NB2004-HR (NCT 00410631) were retrospectively analyzed

### Results:

Within the national trials NB97 and NB2004, 18 (57% male, 43% female) patients with residual osteomedullary uptake at the end of induction therapy were irradiated and eligible for this analysis. Seventeen patients received irradiation to MIBG active metastatic lesions at the time of RT, whereas one patient received RT to one metastasis after complete metabolic response due to previous therapy. At the time of RT two or more lesions (Median 3 (2-5)) were present in 12 children. Among 53 lesions detected before starting RT, 23 received RT.

A medium (range) dose of 36Gy (20Gy-45Gy) to one or more (n=1-3) osteomedullary metastases was applied. The irradiated metastatic sites were located either in the skull (29.1%), extremity (41.6%), spine (20.8%) or ribs (8.3%) respectively. The medium follow-up time was 149 months (55-220) in survivors. At the time of last follow up 4 patients were free of relapse or progression. 11 patients had deceased, either due to tumor progression (n=9) or due to complications of the systemic therapy (n=2). An isolated local failure at an irradiated metastatic site was recorded in four patients. In five patients, progressions in the region of the irradiated site together with other progressive osteomedullary sites were reported. Furthermore, 4 patients progressed osteomedullary, but not at the irradiated sites. The global local control rate was 9 of 18 patients.

### Summary/Conclusions:

Due to the limited number of patients and the retrospective nature of this analysis, the impact of RT to residual metastatic sites cannot be proved. In order to provide evidence, a prospective study would be desirable.

In the absence of convincing evidence, the indication for radiotherapy to metastatic lesions has to be reserved to multidisciplinary protocols or applied only after thoughtful individual multidisciplinary tumor board decisions.

## **P329 *In vitro* cytotoxicity and in vivo evaluation of localization and immune sensitivity of human neuroblastoma in xenografts**

Rahavi, S.M.R.<sup>2</sup>, Reid, G.<sup>3</sup>, Lim, C. J.<sup>4</sup>

BCCHR, Experimental Medicine, Vancouver, Canada <sup>2</sup>, BCCHR, Pediatrics, Vancouver, Canada<sup>3</sup>, BCCHR, Pediatrics, Vancouver, Canada<sup>4</sup>

### Background/Introduction:

Neuroblastoma (NB) is the most commonly occurring extra-cranial solid tumor in children and accounts for ~10% of all childhood cancers. Half of NB cases occur in children under two years of age. NB is thought to originate from neural crest precursor cells of the developing sympathetic nervous system, with primary tumors localizing in the abdomen, adrenal gland or lumbar ganglia; however its metastatic spread includes such diverse tissues as the lymph nodes, bone, bone marrow, liver and skin. The high-risk metastatic form that accounts for ~50% of NB has survival outcomes of less than 30% with conventional therapy. Due to the heterogenous genetic landscape inherent to NB, a major focus for therapy development is aimed at harnessing the anti-tumour capabilities of the immune system, in particular that mediated by natural killer (NK) cells.

### Aims:

Approaches to evaluate killing of target cells *in vitro* include flow-based and chromium-based assays. Here we describe a simple and reproducible system that facilitates both *in vitro* and *in vivo* monitoring of NB cell killing by NK cells based on dual color click beetle red (CBR) and green (CBG) luciferase bioluminescence for separate labeling of target and effector cells. *In vitro*, the system is convenient for longitudinal monitoring and optimization of target cell killing, while *in vivo* there is the potential for tracking of activities within specific target tissues.

### Methods/Materials:

A panel of NB cell lines (NB1643, NB1691, NB EBC1, NB SD, SH-SY5Y, SK-N-AS, CHLA136, CHLA79, CHLA90, and IMR32) and NK92 were tagged with CBG and/or CBR using lentivirus. The NK target K562 cell line was also tagged to provide a benchmark evaluation of NB killing. NK92-mediated killing was evaluated *in vitro* and the cell lines were engrafted intravenously (IV) into NSG mice to create an *in vivo* model of NB homing and metastasis for evaluating immune-based therapy.

### Results:

Our results indicate that liver is the most popular homing location when NB cells injected IV; tumors for all NB cell lines formed in the liver. Tumors formed by NB16, CHLA79 and NB1691 initiated both in the liver and BM, while IMR32, NBEBc1, and NBSD BM tumors appeared after full establishment of liver tumors. We never observed CHLA90, SK-N-AS and SH-SY5Y tumors develop or metastasis to BM. Tumor development to humane end point varies among different NB cell lines. SK-N-AS and CHLA136 reach humane endpoint at day 25 and 153 respectively. Bioluminescent and flow cytometry based and cytotoxicity assay results correlate.

### Summary/Conclusions:

This approach could reveal the impact of modulation of patient NK cells on targeting tumor niches and increasing cytolytic activity. This will establish a more stringent pre-clinical testing platform for the development of immune therapies for NB. The luciferase-tagged NB cell line panel allows for collective evaluation of NB tracking and tracking in real-time both *in vitro* and *in vivo*. This also offers a robust platform to evaluate immune cell killing of localized NB cells *in vivo* and could accelerate pre-clinical immunotherapy development for this disease.

Reza S.M. Rahavi <sup>1</sup>, Gregor Reid <sup>2,3</sup>, Chinten James Lim <sup>2,3</sup>

<sup>1</sup>Dept of Medicine, <sup>2</sup>Dept of Pediatrics, University of British Columbia, and

<sup>3</sup>Michael Cuccione Childhood Cancer Research Program, BC Children's Hospital Research

### **P330 Evaluation of ATR inhibitors in preclinical models of high-risk neuroblastoma**

*Stewart, E.*<sup>2</sup>, *Federico, S.F.*<sup>3</sup>, *Blankenship, K.B.*<sup>4</sup>, *Hoffmann, L.H.*<sup>5</sup>, *Freeman, B.F.*<sup>6</sup>, *Dyer, M.D.*<sup>7</sup>

St. Jude Children's Research Hospital, Oncology, Memphis, USA <sup>2</sup>, St. Jude Children's Research Hospital, Oncology, Memphis, USA<sup>3</sup>, St. Jude Children's Research Hospital, Oncology, Memphis, USA<sup>4</sup>, St. Jude Children's Research Hospital, Oncology, Memphis, St. Jude Children's Research Hospital<sup>5</sup>, St. Jude Children's Research Hospital, Preclinical PK Shared Resource, Memphis, USA<sup>6</sup>, St. Jude Children's Research Hospital, Developmental Neurobiology, Memphis, USA<sup>7</sup>

#### **Background/Introduction:**

ATR gene mutations are found in older high-risk neuroblastoma (NB) patients and correlate with poor outcome. ATR is a chromatin remodeling protein important for deposition of histone variant H3.3 at telomeres and G-quadruplex structures in the DNA that can impede DNA replication. Adult cancer cell lines with ATR mutations have been shown to be sensitive to ATR inhibitors (ATR<sub>i</sub>s), but little has been explored in the pediatric setting. ATR is essential for maintenance of telomeres and other functions including repairing damaged DNA. As *MYCN* amplified NB cells incur replicative stress through DNA damage related to elevated levels of reactive oxygen species, targeting ATR in that setting may be beneficial as well.

#### **Aims:**

To evaluate the efficacy of ATR inhibitors (VX-970 and AZD6738) as single agents and in combination with liposomal irinotecan (nal-IRI) in preclinical models of high risk neuroblastoma.

#### **Methods/Materials:**

Orthotopic xenografts (O-PDXs) were created by injecting luciferase labeled NB cells into the para-adrenal space of nude mice. Pharmacokinetic studies on NB tumor-bearing mice were performed for all drugs to determine matched human AUC-guided dosing. A total of 68 O-PDXs derived from 2 high-risk NB patients, one with *MYCN* amplification (SJNBL046\_X) and one with ATRX mutation (SJNBL047443\_X), were randomized and enrolled into 6 treatment groups: control, nal-IRI, VX-970, AZD6738, VX-970 + nal-IRI, AZD6738 + nal-IRI. The combinations with nal-IRI were chosen for comparison as other studies have shown ATR<sub>i</sub>s sensitize cancer cells to topoisomerase I inhibitors. Four courses of blinded placebo-controlled therapy were planned on a clinically relevant schedule. Ultrasounds were done to measure tumor volume with each course, and bioluminescence was measured weekly to evaluate tumor response. Mice were classified as progressive disease if tumor approached 20% body weight at any point in the study.

#### **Results:**

Single agent use of VX-970 or AZD6738 did not show a significant survival advantage in either O-PDX tested compared to control mice that did not receive chemo. Mice receiving ATR<sub>i</sub>s combined with nal-IRI survived longer than mice receiving ATR<sub>i</sub>s alone in both O-PDXs; however there was not a significant difference overall compared to mice receiving single agent nal-IRI. The average survival days for all groups are shown in the table below.

#### **Summary/Conclusions:**

Comprehensive preclinical testing using multiple O-PDX models of NB that represent the clinical and molecular spectrum of high-risk disease is feasible and warranted to explore potential novel agents. While neither ATR<sub>i</sub> tested showed significant survival advantage in the O-PDXs tested, this study serves as a benchmark for future combination testing. Other preclinical trials using alternative agents to target these pathways such as talazoparib (PARP inhibitor), AZD1390 (ATM inhibitor), CX-5461 (DNA G-quadruplex stabilizer) in combination with ATR<sub>i</sub>s and other standard NB agents (cyclophosphamide and topotecan) are currently underway and data will be available for presentation at the annual meeting.

	<b>SJNBL046_X</b> <b>(MYCN amplified)</b>	<b>SJNBL047443_X</b> <b>(ATRX mutation)</b>
<b>Treatment Group</b>	<b>Avg. days on study</b>	<b>Avg. days on study</b>
Control	25.3	34.3
nal-IRI	51.2	39.4
VX-970	30.7	32.1
AZD6738	30.8	27.8
VX-970 + nal-IRI	48	41.8
AZD6738 + nal-IRI	64	44.4

## **P331 Flow Cytometry for Neuroblastoma**

Kimpo, M.K.<sup>2</sup>

National University Hospital Singapore, Paediatrics, Singapore, Singapore <sup>2</sup>

### Background/Introduction:

Flow cytometry is a technique that can be used to detect neuroblastoma cells. There is a need to be able to monitor minimal residual disease in neuroblastoma and this can be used as adjunct to other tests

### Aims:

We review our experience with the use of flow cytometry in diagnosis and residual disease monitoring. We looked at the relationship with the use of histomorphology .

### Methods/Materials:

Specimens collected from patients with high risk neuroblastoma from bone marrow, skull lesion and lymph node. Bone marrow samples were collected in sodium heparin tubes. Other lesions were placed in normal saline following biopsy and sent to the flow cytometry laboratory within an hour. Flow cytometry was performed upon harvesting in most samples. Some were banked and subsequently thawed and checked for viability. Markers used to detect neuroblastoma were GD2, CD56 and CD81. CD45, CD33 were also included to distinguish neuroblastoma cells from those of hematopoietic cells. Bone marrow aspirates and bone marrow biopsies were obtained at the same time. For bone marrow biopsies, neuroblastoma cells were identified based on morphology and immunohistochemical stains for NSE, chromogranin and synaptophysin.

### Results:

Twenty bone marrow specimens were tested from eight patients. Two patients had diagnostic bone marrow tests due to initial diagnosis of leukemia. The rest of the patients has received treatment previously, two with both chemotherapy and Dinutuximab beta. Five bone marrow specimens were read as negative on flow cytometry but were positive on histology. However the number of cells noted were low levels at two percent or less ( rare cells). Three were positive with flow cytometry at low levels ( 0.03%, 0.19%, 0.12%). One bone marrow specimen from a patient whose disease progressed after Dinutuximab beta showed tumor cells with partial dim expression of GD2. However we did not have the diagnostic sample for this patient.

Two samples were obtained from patients who had relapsed disease post chemotherapy, with one having had five cycles of Dinutuximab beta.

### Summary/Conclusions:

Flow cytometry adds value to measuring disease level in the bone marrow. This can be used as an adjunct with histology. However, the sensitivity of the test depends on the number of viable cells obtained. Neuroblastoma is known to cause patchy involvement of the bone marrow which could explain some of the inconsistencies between the tests.

Detection of expression of the GD2 antigen by tumor cells may guide in the decision to treat with anti GD2 antibody.

## **P332 MODULATION OF THE UNFOLDED PROTEIN RESPONSE IN NEUROBLASTOMA INDUCES APOPTOSIS ESPECIALLY IN *MYCN*-AMPLIFIED, CHEMORESISTANT CELLS**

*Rodríguez-Hernández, C.J.*<sup>2</sup>, *García, M.*<sup>3</sup>, *Lemos, I.*<sup>4</sup>, *Lavarino, C.*<sup>5</sup>, *Mora, J.*<sup>6</sup>

Fundació Sant Joan de Déu, Pediatric Cancer, Barcelona, Spain <sup>2</sup>, Fundació Sant Joan de Déu, Pediatric Cancer, Barcelona, Spain<sup>3</sup>, Fundació Sant Joan de Déu, Pediatric Cancer, Barcelona, Spain<sup>4</sup>, Fundació Sant Joan de Déu, Pediatric Cancer, Barcelona, Fundació Sant Joan de Déu<sup>5</sup>, Fundació Sant Joan de Déu, Pediatric Cancer, Barcelona, Spain<sup>6</sup>

### Background/Introduction:

We have previously reported that activation of the calcium-sensing receptor (CaSR) inhibits neuroblastoma (NB) tumor growth by promoting Endoplasmic Reticulum (ER) stress-mediated apoptosis and differentiation. The ER is a major regulator of multiple intracellular processes, such as the proper folding of the newly synthesized proteins. The tumor cell is exposed to several extra and intracellular stresses that can impair this folding efficiency, thus inducing the so called Unfolded Protein Response (UPR). The UPR can promote survival by hindering translation and inducing autophagy, but if the stress becomes unbearable cells undergo apoptosis. UPR is also involved in the acquisition of chemoresistance, the major responsible for the progression or relapse in many cancer patients, including NB, after the first line of treatment.

### Aims:

To characterize the ER stress/UPR modulation in NB as a putative therapeutic strategy, especially for aggressive, chemoresistant tumors.

### Methods/Materials:

Six NB cell lines with different *MYCN* and *TP53* status were used to assess toxicity promoted by *in vitro* exposure to several ER stress inducers (bortezomib, thapsigargin and tunicamycin) and one autophagy inhibitor (chloroquine). Synergism between these ER stress inducers and the autophagy inhibitor was also evaluated according to the Chou-Talalay method. Synergism was also evaluated *in vivo* in two xenograft models generated in immunocompromised mice with *MYCN*-Amplified IMR5 and *MYCN*-Non Amplified SH-SY5Y cell lines. Cell lines resistant to chemotherapeutic agents commonly used for NB treatment (cisplatin, doxorubicin and vincristine) were generated. Rescue of this chemoresistance by the modulators of the ER stress response bortezomib and chloroquine was evaluated in the resistant cell lines and their wild type counterparts.

### Results:

All the cell lines tested showed similar sensitivity to bortezomib, thapsigargin and tunicamycin. Apoptosis occurred due to induction of ER stress and the subsequent UPR as determined by RT-qPCR and western blot. The combination of these stimuli with the inhibition by chloroquine of the pro-survival autophagic branch of UPR resulted in an increased cell death but, interestingly, a synergistic collaboration was only significant in some of the cell lines (IMR5, SK-N-JD, SK-N-LP and LA-N-1). A similar scenario was found when evaluating the rescue of chemoresistance by the ER stress/UPR modulators. *in vivo* studies confirmed these results. Although the *MYCN*-Amplified cell lines were clearly the most responsive to the interactions tested, we also explored other features as putative contributors for this differential response. p53 and mTOR pathways and stemness status exhibited relevance in this phenotype.

### Summary/Conclusions:

These data would provide proof-of-principle indicating that modulation of the ER stress and the UPR might be a new therapeutic approach for malignant neuroblastomas, particularly the aggressive, *MYCN*-amplified ones with acquired or constitutive chemoresistance.





## **P333 XIAP inhibition through ARTS mimetics as an anticancer therapeutic in Neuroblastoma**

*Choo, Z.<sup>2</sup>, Loh, A. H. P.<sup>3</sup>, Larisch, S.<sup>4</sup>, Chen, Z. X.<sup>5</sup>, ,<sup>6</sup>*

National University of Singapore, Physiology, Singapore, Singapore<sup>2</sup>, KK Women's and Children's Hospital, Paediatric Surgery/VIVA-KKH Paediatric Brain and Solid Tumour Programme, Singapore, Singapore<sup>3</sup>, University of Haifa, Biology/ Cell Death and Cancer Research Laboratory, Haifa, Israel<sup>4</sup>, National University of Singapore/ National University Cancer Institute, Physiology, Singapore, National University of Singapore/ National University Cancer Institute<sup>5</sup>, , ,<sup>6</sup>

### **Background/Introduction:**

XIAP (X-linked Inhibitor of Apoptosis), the most prominent member of mammalian inhibitors of apoptosis proteins (IAPs), is a critical regulator of cell death and survival that suppresses apoptosis and enhances survival signaling. Despite being targeted in multiple adult cancers, XIAP inhibition is an underexplored therapeutic strategy in pediatric solid tumors. Neuroblastoma is one of the several neural crest-derived cancers that share a common developmental biology requiring XIAP. XIAP inhibition by XAF1 has previously been demonstrated to play a critical role in mediating KIF1B $\beta$ -driven tumor suppression in neuroblastoma. KIF1B $\beta$ -XAF1-XIAP pathway is required for normal sympathetic nervous system development. Removal of XIAP is required for targeted sympathetic neuronal progenitors to gain apoptotic competence prior to developmental culling. In high-risk, poor prognosis neuroblastoma patients, KIF1B $\beta$  is often deleted or inactivated. This leads to low XAF1 and correspondingly, high XIAP expression. Therefore, we hypothesized that high-risk, poor prognosis neuroblastoma or other neural crest-derived cancers may be addicted to XIAP for aggressive growth and survival and that patients may benefit from a treatment approach targeting XIAP.

### **Aims:**

To evaluate the utility, efficacy and mechanism of XIAP inhibition as an anticancer strategy for neuroblastoma stratified according to XIAP.

### **Methods/Materials:**

Small molecule compounds targeting pan-IAPs (SMAC mimetics) and XIAP alone (ARTS mimetics) were screened and tested on a panel of commercial and patient-derived neuroblastoma cell lines. Dose response curves established from cell viability assay were used to determine and rank the efficacy of each compound. The expression of IAPs proteins and cell death were assessed via Western Blot, Crystal Violet and biochemical apoptotic assays.

### **Results:**

Among the compounds tested, ARTS mimetic A4 demonstrated the best efficacy across the neuroblastoma panel. It is most effective against high XIAP-expressing cells (BE(2)-C and KELLY), which are considered to be aggressive and resistant to conventional chemotherapeutics. ARTS (Sept4\_i2) induces apoptosis through direct binding and specifically degrading XIAP through the ubiquitin proteasome system. ARTS mimetics, are distinct from all other IAP antagonist as they can both bind and degrade XIAP. Moreover, its efficacy appears to be stratified according to XIAP and MYCN expression status (a hallmark of high-risk, poor prognosis neuroblastomas). Encouragingly, A4 was best tolerated by immortalized non-cancer cell lines representing liver and bone marrow, two common metastatic sites for neuroblastoma. Preliminary pharmacokinetic testing of A4 showed good stability and availability in plasma. Furthermore, A4 acts synergistically when used in combination with cytotoxic agents such as vincristine and topotecan. Silencing of XIAP induces apoptosis in neuroblastoma cells while forced expression of XIAP protects cells from drug-induced cell death, indicating XIAP inhibition is necessary and sufficient to kill neuroblastoma cells.

### **Summary/Conclusions:**

ARTS mimetic A4 with XIAP-specific inhibition, showed the highest efficacy against neuroblastoma cells lines and exhibited the lowest toxicity to non-cancer cells, while pan-IAP inhibitors showed the

converse with the lowest anti-tumor efficacy and the highest toxicity. Taken together, inhibiting XIAP in a subset of aggressive neuroblastoma cells characterized by high XIAP may be a potential breakthrough treatment or sensitizer for these cells that are currently resistant to existing chemotherapeutics.

### **P334 DFMO can reduce anti-GD2 induced allodynia and increase anti-GD2 efficacy in animal models**

*Diccianni, M.<sup>2</sup>, Huang, J-R.<sup>3</sup>, Hung, F.<sup>4</sup>, Kempinska, K.<sup>5</sup>, Gangotti, J.<sup>6</sup>, Sorkin, L.<sup>7</sup>, Yu, A.<sup>8</sup>*

University of California San Diego, Pediatrics, San Diego, USA <sup>2</sup>, Chang Gung University, Institute of Stem Cell and Translational Cancer Research, Taoyuan, Taiwan<sup>3</sup>, Chang Gung University, Institute of Stem Cell and Translational Cancer Research, Taoyuan, Taiwan<sup>4</sup>, University of California San Diego, Pediatrics, San Diego, University of California San Diego<sup>5</sup>, University of California San Diego, Pediatrics, San Diego, USA<sup>6</sup>, University of California San Diego, Anesthesiology, San Diego, USA<sup>7</sup>, University of California San Diego, Pediatrics, San Diego, USA<sup>8</sup>

#### Background/Introduction:

High levels of polyamines are found in many human cancers including neuroblastoma. Elevated polyamines contribute significantly to tumor immunosuppression and polyamine depletion can exert antitumor effects and promote immunity. In addition, polyamines can enhance neuronal sensitization, including development of allodynia and other forms of pathological pain. Anti-GD2 therapy with dinutuximab is effective in improving the survival of high-risk neuroblastoma patients in remission and after relapse. However, only slightly over half of the patients with advanced disease have a favorable outcome, with allodynia hindering its usage at higher doses.

#### Aims:

The aims of this study were to determine whether polyamine depletion might be an effective anti-tumor, anti-allodynia and pro-immune system strategy when used in conjunction with immunotherapy.

#### Methods/Materials:

Allodynia experiments were conducted in male Sprague-Dawley rats where tail vein injection of anti-GD2 (14G2a) causes a paw-touch withdrawal reflex that strongly mimics allodynia seen in children. Tumor formation experiments utilized A/J mice injected intravenously with NXS2 neuroblastoma cells and C57BL/6 mice injected subcutaneously with EL4 lymphoma cells. Animals were also allowed to drink regular water or water containing various concentrations of DFMO (0.25%, 0.5% or 1.0%) for 7 days prior to any injections. DFMO and polyamine levels were determined by HPLC and mass spectrometry. Splenocytes were analyzed by flow cytometry.

#### Results:

Rats injected with both 1 and 2 mg/kg 14G2a developed significant allodynia with similar onsets of development (latency) of about 1 hour post-injection, with maximum allodynia occurring about 3-5h's post-injection ( $p < 0.001$ , repeated measures ANOVA). In contrast, animals allowed to drink water-containing 1% DFMO displayed withdrawal thresholds that were not significantly different from pre-injection levels for the 1 mg/kg injected animals ( $p > 0.05$ ). In animals injected with 2 mg/kg 14G2a, 1% DFMO significantly attenuated but did not completely prevent pain behavior. Consistent with the known actions of DFMO, serum putrescine and spermidine levels were significantly reduced in the DFMO-treated animals.

In both mouse models, survival was significantly improved for animals drinking water containing 1% DFMO (Log rank test,  $p < 0.01$ ), but suboptimal 14G2a injections (100  $\mu$ g i.p. once weekly,  $p > 0.05$ ) did not yield additional survival benefit. In contrast, when the suboptimal growth suppressor concentrations of 0.25% to 0.5% DFMO were used in combination with 14G2a (100  $\mu$ g i.p.), a significant prolongation of survival was observed ( $p < 0.01$ ). A significant reduction in the percent of myeloid-derived suppressor cells (MDSCs), which can inhibit anti-tumor immune reactions and stimulate tumor growth and metastasis, was observed in splenocytes of the 0.25% DFMO-treated C57BL/6 mice ( $p < 0.05$ ), with an even more significant reduction in the DFMO + 14G2a treated animals ( $p < 0.01$ ).

#### Summary/Conclusions:

Given its favorable toxicity profile and ability to reduce anti-GD2 -induced allodynia, increase survival and modulate the tumor microenvironment, DFMO may be an important adjunct to anti-GD2 immunotherapy in addition to a role as a potential anti-cancer therapeutic.

## **P335 Modeling and Deciphering COJEC Resistance Using Neuroblastoma PDX Models**

*Mañas, A.<sup>2</sup>, Aaltonen, K.<sup>3</sup>, Radke, K.<sup>4</sup>, Hansson, K.<sup>5</sup>, Burgos, R.<sup>6</sup>, Esfandyari, J.<sup>7</sup>, Yasui, H.<sup>8</sup>, Gisselsson, D.<sup>9</sup>, Bexell, D.<sup>10</sup>*

Lund University, Department of Laboratory Medicine, Lund, Sweden <sup>2</sup>, Lund University, Department of Laboratory Medicine, Lund, Sweden<sup>3</sup>, Lund University, Department of Laboratory Medicine, Lund, Sweden<sup>4</sup>, Lund University, Department of Laboratory Medicine, Lund, Lund University<sup>5</sup>, University of Valencia-INCLIVA, Pathology Department, Valencia, Spain<sup>6</sup>, Lund University, Department of Laboratory Medicine, Lund, Sweden<sup>7</sup>, Lund University, Department of Laboratory Medicine, Lund, Sweden<sup>8</sup>, Lund University, Department of Pathology; Department of Laboratory Medicine, Lund, Sweden<sup>9</sup>, Lund University, Department of Laboratory Medicine, Lund, Sweden<sup>10</sup>

### **Background/Introduction:**

Current intense multi-modal treatment against high-risk neuroblastoma has significantly increased overall survival, but resistance and relapses are common, and side effects are severe. One critical step is rapid COJEC induction therapy, consisting of cycled administration of Cisplatin, Vincristine, Etoposide, Cyclophosphamide and Carboplatin. COJEC has been used in the clinic for many years, but a comprehensive preclinical study investigating the mechanisms of resistance to COJEC has not been performed.

### **Aims:**

The aims of this project are: 1) to establish a clinically relevant COJEC protocol with neuroblastoma patient-derived xenograft (PDX) models; 2) to analyze the mechanisms of COJEC treatment resistance; and 3) to establish tumor organoids from COJEC-resistant PDX tumors.

### **Methods/Materials:**

High-risk *MYCN*-amplified neuroblastoma PDX models were established in nude and NSG mice. Mice were treated with a COJEC-like protocol for 6 weeks. Several dose protocols were tested, and treatment was personalized depending on tolerance. Mice that responded well were also subjected to surgical resection and observed for relapse. All tumors were analyzed using chromosomal copy number analysis, RNA sequencing and immunohistochemistry. Fresh PDX tumors were dissociated to establish COJEC-resistant tumor organoids.

### **Results:**

Neuroblastoma PDXs treated with an optimized COJEC schedule present responses comparable to those seen in humans, with high rates of resistance or relapse after surgery. Multidrug induction has variable results in PDXs derived from different patients, ranging from significant increased survival to poor or no response. Considerable tumor volume reduction was observed for most responding tumors, allowing surgical removal with a 50% relapse rate.

Data from a responsive PDX model showed that treated tumors displayed a more differentiated histological profile, with higher levels of key extracellular matrix components and increased cell death. Surprisingly, substantial tumor cell proliferation was still observed during treatment, pointing to a possible population of resistant cells. Moreover, treated tumors presented an accumulation of chromosomal aberrations, with sub-clonal dynamics enhanced in relapsed tumors. Additionally, preliminary data from tumors that did not relapse showed a downregulation of genes associated with cell cycle and DNA replication/repair at the moment of surgical removal. This significant downregulation can also be observed in patients that did not relapse.

Finally, PDX tumors that were resistant or relapsed after surgery were dissociated and successfully cultured in stem cell medium as organoids, providing a useful tool to study resistance mechanisms to COJEC drugs.

### **Summary/Conclusions:**

We have established and optimized a highly clinically relevant COJEC-induction protocol using high-risk neuroblastoma PDX models. This protocol will be helpful for studying COJEC resistance and identify biomarkers that could be indicative of resistance and relapse. Additionally, our COJEC-resistant organoids represent a useful tool for testing novel therapeutics that can overcome drug resistance in high-risk neuroblastoma.

### **P336 Estimation of life expectancy and the expected years of life lost with Neuroblastoma in children of Taiwan**

*Chi-Wei, T.<sup>2</sup>, Meng-Yao, L.<sup>3</sup>, Wen-Ming, H.<sup>4</sup>, Jung-Der, W.<sup>5</sup>, ,<sup>6</sup>*

College of Medicine, National Cheng Kung University, Department of Public Health, Tainan, Taiwan<sup>2</sup>, National Taiwan University Hospital, Department of Pediatrics, Taipei, Taiwan<sup>3</sup>, National Taiwan University Hospital and Taiwan University College of Medicine, Department of Surgery, Taipei, Taiwan<sup>4</sup>, National Cheng Kung University Hospital, College of Medicine, Department of Occupational and Environmental Medicine, Tainan, National Cheng Kung University Hospital, College of Medicine<sup>5</sup>, , ,<sup>6</sup>

#### **Background/Introduction:**

Neuroblastoma is a childhood cancer of the peripheral sympathetic nervous system and it ranks as the second most common extracranial solid tumor of children and adolescents in Taiwan. Most patients present with disseminated disease, which confers a poor prognosis and is associated with a high mortality rate.

#### **Aims:**

This study aimed to quantify the lifetime risks, life expectancy (LE) and the expected years of life lost (EYLL) after the diagnosis of neuroblastoma using a semiparametric method.

#### **Methods/Materials:**

A total of 218 patients with neuroblastoma were registered with the Taiwan Cancer Registry during 2007 to 2015. They were linked to the National Mortality Registry through the end of 2017. We estimated the cumulative incidences rate for 0-19 years (CIR<sub>0-19</sub>). The survival is estimated by Kaplan-Meier method up to the end of follow-up and extrapolated the survival function to lifetime by assuming a "constant excess hazard" for these cancer patients compared with age-, sex-, and calendar year matched referents generated from vital statistics. In this way, the LE of the neuroblastoma in childhood cancer cohort (with extrapolation up to 240 months or 960 months) after diagnosis was estimated. The EYLL was calculated by subtracting the LE from that of age- and sex-matched referents.

#### **Results:**

Most patients with neuroblastoma were males (62.8%), and 98% were less than 10 years old, with incidence rates of 3.4, 1.4, 0.3, <0.1, and <0.1 per 100,000 person-year, respectively, for age strata of 0-1.4, 1.5-4, 5-9, 10-14, and 15-19. The average EYLL was 29.6 years after extrapolation up to 80 years. Diagnosis at younger than 1.5 years old seemed to improve survival with a LE of 66.1 years in contrast to those diagnosed during 1.5-19 years with an LE of 27.80 years. EYLL can adjust for different age, sex, and calendar year of diagnosis, which also showed a smaller loss for those diagnosed before 1.5 years of age.

#### **Summary/Conclusions:**

We concluded that early diagnosis of patients with neuroblastoma before age of 1.5 years appeared to be the key for a better outcome and deserve attention.



### **P337 Response Evaluation of I-131-mIBG therapy in high risk neuroblastoma**

*Plum, G.<sup>2</sup>, Decarolis, B.<sup>3</sup>, Lieberz, D.<sup>4</sup>, Van Eijkelenburg, N. K. A.<sup>5</sup>, Simon, T.<sup>6</sup>, Berthold, F.<sup>7</sup>, Hero, B.<sup>8</sup>, Schmidt, M.<sup>9</sup>*

University Hospital of Cologne, Pediatric Oncology and Hematology, Cologne, Germany <sup>2</sup>, University Hospital of Cologne, Pediatric Oncology and Hematology, Cologne, Germany<sup>3</sup>, University Hospital of Cologne, Pediatric Oncology and Hematology, Cologne, Germany<sup>4</sup>, Princess Máxima Center for Pediatric Oncology, Pediatric Oncology, Utrecht, Princess Máxima Center for Pediatric Oncology<sup>5</sup>, University Hospital of Cologne, Pediatric Oncology and Hematology, Cologne, Germany<sup>6</sup>, University Hospital of Cologne, Pediatric Oncology and Hematology, Cologne, Germany<sup>7</sup>, University Hospital of Cologne, Pediatric Oncology and Hematology, Cologne, Germany<sup>8</sup>, University Hospital of Cologne, Nuclearmedicine, Cologne, Germany<sup>9</sup>, ,

#### **Background/Introduction:**

I-131-mIBG-therapy is an established treatment of high-risk neuroblastoma used in initial and relapsed multimodal therapy concepts. Diagnostic I-123-mIBG-scintigraphy is a valuable tool at initial diagnosis and for response evaluation. Semiquantitative extensions scores allow estimation of the extent of mIBG-positive osteomedullary metastasis.

#### **Aims:**

We were interested in the response evaluation of mIBG-therapy using semiquantitative score methods.

#### **Methods/Materials:**

MIBG scans of patients treated with I-131-mIBG-therapy at our center between 2004 and 2018 were analysed. Diagnostic I-123-mIBG scans acquired before mIBG-therapy were scored and compared to I-123-mIBG scans acquired 3-8 months after mIBG-therapy for response assessment. In addition, post-therapeutic I-131-mIBG scans acquired 4h to 48hrs after I-131-mIBG injection were scored.

The SIOPEN scoring method was used for semi-quantitative estimation of osteomedullary involvement. For each pair of scans, the ratio between the score after mIBG-therapy and prior to mIBG-therapy was calculated. Response was defined as score ratio <0.8, stable disease as score ratio from 0.8-1.2 and progression as score ratio >1.2. Response was correlated to treatment episode (initial vs. relapse treatment), score prior to mIBG-therapy (<8 vs="="">8), and *MYCN* status.

#### **Results:**

A total of 179 scans of 50 patients undergoing 63 I-131-mIBG therapies were analysed. Median age at diagnosis was 43 months (range 2-255 months) and at mIBG-therapy 74 months (range 21-260 months). *MYCN* amplification was found in 6 out 48 evaluable patients.

Twenty-four mIBG-therapies were given during the first line treatment, 39 for relapse treatment. MIBG-therapies were applied in combination with high-dose chemotherapy either with autologous stem cell rescue (n=42) or with haplo-identical stem cell rescue (n=3), in combination with other systemic treatment (n=10) and without additional treatments (n=8). Three patients received immunotherapy with ch14.18 between mIBG-therapy and response assessment.

The SIOPEN score of 63 pre-therapeutic I-123-mIBG scans ranged from 0 to 54 (median 1) with 27 scans revealing only uptake at the primary site (Score 0). The post-therapeutic I-131-mIBG scans identified more metastatic lesions (median 2, range 0-56, p<0.001). At the response assessment after mIBG-therapy, the median score score was 0 (range 0-61; p=0.03).

Because no metastatic osteomedullary lesion was seen in 27 pre-therapeutic I-123-mIBG scans, response could only be assessed for 36 pairs of scans revealing a median score ratio of 0.61 (range 0-3.2). A response as defined above (score ratio <0.8) was observed after 20, stable disease after 12

and progression after four out of 36 evaluable mIBG-therapies. In 10 patients, all osteomedullary lesions detected prior to mIBG-therapy (median 6.5, range 1-52) were cleared after mIBG-therapy.

Response was seen after 12 out of 17 mIBG-therapies in first-line treatment and in eight out of 19 mIBG-therapies in relapse treatment ( $p=0.106$ ). The median score ratio of patients treated in first line treatment was 0.53 (range 0-1.9) and 1.0 (range 0-3.2) for patients treated in relapse ( $p=0.22$ ). Similarly, neither for SIOPEL score prior to mIBG-therapy ( $p=0.95$ ) nor for *MYCN* status ( $p=0.26$ ) a correlation was found.

#### Summary/Conclusions:

Semiquantitative mIBG scoring showed response to mIBG-therapy applied in combination with other systemic treatment in part of the patients. Further evaluation in larger cohorts treated with I-131-mIBG is needed.

## **P338 Neurologic Complications from Central Nervous System Neuroblastoma Metastases**

*Kramer, K.<sup>2</sup>, Khakoo, Y.<sup>3</sup>*

Memorial Sloan Kettering Cancer Center, Pediatrics, New York, USA <sup>2</sup>, Memorial Sloan Kettering Cancer Center, Pediatrics, New York, USA<sup>3</sup>

### **Background/Introduction:**

Neuroblastoma metastatic to the central nervous system (CNS NB) is associated with significant mortality (median survival <6 months, <10% survival at 36 months). Significant neurologic compromise can occur at the onset of CNS disease or during the course of treatment.

### **Aims:**

To analyze the incidence and type of neurologic complications that occur in patients with CNS NB treated at Memorial Sloan Kettering Cancer Center (MSK).

### **Methods/Materials:**

We reviewed the medical records of all patients with radiographically and/or pathologically confirmed diagnosis of CNS NB treated at MSK over a 30 year period. For this cohort, disease evaluation generally every 3 month MIBG, CT of the primary disease, bone marrow evaluation and brain CT +/- MR brain and spine. Patients were classified as having CNS NB detected while being asymptomatic and during routine extent of disease evaluations, or having symptoms that promoted further evaluation and imaging. The type of symptoms were noted.

### **Results:**

171 patients with CNS NB were evaluated and treated for CNS NB. 51 patients (30%) were asymptomatic and had disease detected by routine radiographic evaluation. The most common symptom prompting imaging was headache (N=52, 30%) +/- vomiting (N=37, 22%). New onset seizures occurred in 22 patients (13%). Altered mental status was observed in 21 patients (13%) who "felt funny" (N=1), had slurred speech (N=1), lethargy (N=4) or became acutely obtunded (N=8). Motor weakness occurred in 12 patients (7%), ataxia in 9 (5%), and cranial neuropathies (anisocoria, hearing deficit, facial palsy) in 13 (8%). Other uncommon symptoms included eye pain (N=1), aphasia (N=1) and opsoclonus myoclonus syndrome (N=1).

### **Summary/Conclusions:**

With the current radiographic evaluation guidelines, 30% of patients will have CNS metastases detected by routine neurologic imaging. 70% of patients are symptomatic at disease presentation with headache and vomiting being the most common sign. The most serious complication, acute obtundation, occurs in 5% of patients. Clinicians should be aware that even subtle neurologic changes can be a harbinger of CNS metastases.

### **P339 Application of a sustained release carrier containing the Okinawan bioresource having anticancer activity**

*Ikematsu, S.*<sup>2</sup>, *Yabe, Y.*<sup>3</sup>, *Kono, H.*<sup>4</sup>, *Fujita, S.*<sup>5</sup>, *Tsubota, S.*<sup>6</sup>, *Kadomatsu, K.*<sup>7</sup>

National Institute of Technology, Okinawa College, Bioresources Engineering, Nago, Japan<sup>2</sup>, National Institute of Technology, Okinawa College, Bioresources Engineering, Nago, Japan<sup>3</sup>, National Institute of Technology, Tomakomai College, Applied Chemistry and Biochemistry, Tomakomai, Japan<sup>4</sup>, National Institute of Technology, Tomakomai College, Applied Chemistry and Biochemistry, Tomakomai, National Institute of Technology, Tomakomai College<sup>5</sup>, Nagoya University Graduate School of Medicine, Biochemistry, Nagoya, Japan<sup>6</sup>, Nagoya University Graduate School of Medicine, Biochemistry, Nagoya, Japan<sup>7</sup>

#### Background/Introduction:

Midkine is a growth factor expressed in various tumors including neuroblastoma, and its expression positively correlates with poor prognosis of the patients. We previously investigated antitumor activities of natural products obtained in Okinawa, a subtropical region in Japan, using midkine as an indicator. We obtained a candidate material which suppressed midkine secretion by human neuroblastoma cells. Further we found the hydrogel prepared from carboxymethyl cellulose (CMC) (Carbohydrate Polymers 106 (2014) 84-93) could be useful as a drug delivery system (DDS) carrier matrix for these bioresources, which gradually releases materials over a long period of time, and therefore could be a potential a DDS.

#### Aims:

The purpose of this study is to evaluate and apply a DDS for controlled release of anticancer bioresource products. This substance already has been confirmed to show sustained release of lysozyme and BSA. In the current study, we investigated the efficacy of the newly-developed DDS on neuroblastoma.

#### Methods/Materials:

We prepared the powder-like CMC based hydrogel as a DDS matrix. To assess the DDS, we used a neuroblastoma cell line;SK-N-SH. We assessed the effects of the CMC based hydrogel conjugated with Jabuticaba(*Myricaria cauliflora*) leaf ethanol extract and with carboplatin on neuroblastoma cell growth.

#### Results:

The CMC based hydrogel conjugated with Jabuticaba significantly suppressed neuroblastoma cell growth, and its effect is comparable with the CMC based hydrogel conjugated with carboplatin. Our DDS using electrospinning for carboplatin efficiently suppressed in situ tumor growth without any serious side effects of the anticancer drug.

#### Summary/Conclusions:

At ANR2018, we have shown that our DDS using electrospinning for carboplatin efficiently suppressed in situ tumor growth without any serious side effects of the anticancer drug. But, electrospinning type DDS had to be manufactured at high heat. Therefore, it was not possible to use a substance that is weak to heat. On the other hand, the new DDS is not affected by heat. This DDS may be useful for cancer treatment including neuroblastoma.

#### Key words

Jabuticaba(*Myricaria cauliflora*), drug delivery system (DDS), CMC based hydrogel



## **P340 A phase I/II proof-of-concept study using novel hybrid imaging technique [<sup>124</sup>I]mIBG PET/CT and PET/MRI in improving imaging of neuroblastoma**

*Chua, S.<sup>2</sup>, Patel, S.<sup>3</sup>, Flux, G.<sup>4</sup>, Wan, S.<sup>5</sup>, Bomanji, J.<sup>6</sup>, Papathanasiou, N.<sup>7</sup>, Biassoni, L.<sup>8</sup>, Humphries, P.<sup>9</sup>, Peckitt, C.<sup>10</sup>, Burns, L.<sup>11</sup>, Murray, I.<sup>12</sup>, Aldridge, M.<sup>13</sup>, Mellor, S.<sup>14</sup>, Moreno, L.<sup>15</sup>, Pearson, A.<sup>16</sup>, Brown, G.<sup>17</sup>, Vaidya, S.<sup>18</sup>, Gaze, M.<sup>19</sup>*

The Royal Marsden NHS Foundation Trust, Nuclear Medicine and PET Department, London, United Kingdom<sup>2</sup>, The Royal Marsden NHS Foundation Trust, Nuclear Medicine & PET/CT, London, United Kingdom<sup>3</sup>, Institute of Cancer Research, Head of Radioisotope Physics, Sutton, United Kingdom<sup>4</sup>, University College London Hospital, Nuclear Medicine & Radiology, London, University College London Hospital<sup>5</sup>, University College London Hospital, Institute of Nuclear Medicine, London, United Kingdom<sup>6</sup>, University Hospital of Patras, Nuclear Medicine Nuclear Medicine & PET/CT Department, Patras, Greece<sup>7</sup>, Great Ormond Street Hospital for Children NHS Foundation Trust, Nuclear Medicine, London, United Kingdom<sup>8</sup>, Great Ormond Street Hospital for Children NHS Foundation Trust, Radiology, London, United Kingdom<sup>9</sup>, Royal Marsden Hospital, Statistics, Sutton, United Kingdom<sup>10</sup>, Cancer Research UK, Drug Development- Research and Innovation, London, United Kingdom<sup>11</sup>, Royal Marsden Hospital, Physics, Sutton, United Kingdom<sup>12</sup>, University College London Hospital, Physics, London, United Kingdom<sup>13</sup>, Cancer Research UK, Drug Development- Research and Innovation, London, United Kingdom<sup>14</sup>, Hospital University Vall d'Hebron, Paediatric Haematology & Oncology, Barcelona, Spain<sup>15</sup>, The Royal Marsden NHS Foundation Trust, Pediatric Oncology, London, United Kingdom<sup>16</sup>, Beatson Institute of Cancer Research, R&D Radiochemistry, Glasgow, United Kingdom<sup>17</sup>, Institute of Cancer Research, Pediatric Oncology, Sutton, United Kingdom<sup>18</sup>, University College London Hospital, Clinical Oncology, London, United Kingdom<sup>19</sup>

### Background/Introduction:

Functional imaging plays a critical role in prognostic stratification, response assessment and treatment decisions makings for patients with high-risk neuroblastoma.

Meta-iodobenzylguanidine (*mIBG*) is a well-established imaging and molecular radiotherapy agent in neuroblastoma, for which 95% of cases are *mIBG*-avid at diagnosis. Planar scintigraphy using *mIBG* labelled with Iodine-123 ([<sup>123</sup>I]-*mIBG*) is the current imaging gold standard for staging and response assessment, but the technique has relatively poor resolution and is not easily standardised/quantified despite the use of semi-quantitative scores. Recently, newer hybrid functional/anatomical imaging technologies have become available whose potential in neuroblastoma imaging requires evaluation.

[<sup>124</sup>I]*mIBG* as a novel PET tracer is hypothesised to improve diagnostic accuracy, prognostic stratification and treatment decisions and potentially minimise the risk of overtreatment in neuroblastoma patients, and additionally will simplify their staging/ restaging work-up by decreasing the number of investigations and the need for sedation/general anaesthesia in children.

### Aims:

The primary objective of this study is to compare the diagnostic performance of [<sup>124</sup>I]-*mIBG* PET/CT, with conventional [<sup>123</sup>I]*mIBG* imaging, in detecting an equal or greater number of neuroblastoma lesions.

Secondary and tertiary objectives include the tolerability and safety of administration of [<sup>124</sup>I]*mIBG*, and comparison of the number of lesions detected as positive by [<sup>124</sup>I]*mIBG* hybrid PET/MRI with [<sup>124</sup>I]*mIBG* PET/CT and conventional imaging with [<sup>123</sup>I]*mIBG* planar scintigraphy, SPECT/CT, routine CT and MRI.

### Methods/Materials:

A multi-centre, non-therapeutic Phase I/II clinical trial (NCT02043899) directly comparing [<sup>124</sup>I]-*mIBG* PET/CT and conventional [<sup>123</sup>I]*mIBG* imaging in patients with newly diagnosed, relapsed or refractory metastatic neuroblastoma was conducted over a period of 5 years. [<sup>124</sup>I]-*mIBG* PET/CT scans were

acquired 24 hours post injection. Results were assessed by four independent reviewers. Parameters include lesion number, site, Standardized Uptake Value-SUV(mean, max and peak), Metabolic Tumour Volume(MTV). PET/MR data includes maximum axial diameter(MAD) and mean apparent diffusion coefficient(ADC). Inter-observer agreement in scoring the number of positive lesions, confidence scores, SIOPEX and CURIE scores between observers using kappa statistics and percentage agreement were measured.

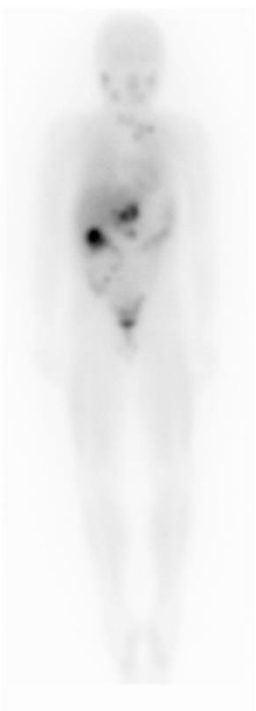
#### Results:

Nine evaluable patients (4 to 41yrs) with metastatic neuroblastoma were scanned with  $[^{123}\text{I}]\text{mIBG}$  planar scintigraphy and SPECT/CT, and  $[^{124}\text{I}]\text{mIBG}$  PET/CT. Five patients also underwent hybrid PET/MRI scans after the PET/CT scan allowing the direct comparison of the two imaging modalities. The mean injected activity was 283 MBq (92.3 to 450 MBq) for  $[^{123}\text{I}]\text{-mIBG}$  and 35 MBq (17.8 to 52 MBq) for  $[^{124}\text{I}]\text{-mIBG}$ . No adverse effects related to the imaging agents were observed. Qualitative and semi-quantitative imaging analysis results will be available in May 2020.

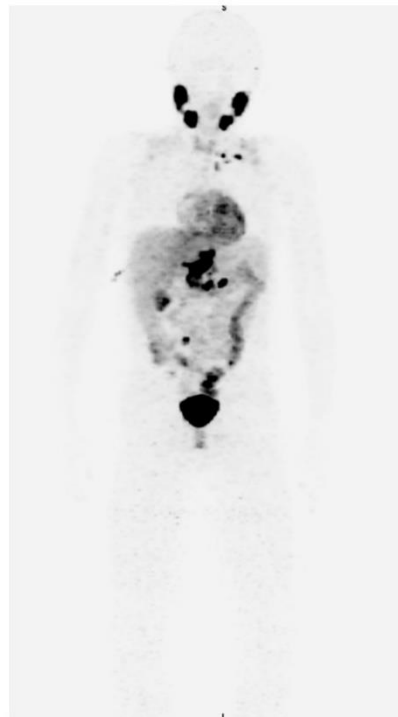
#### Summary/Conclusions:

$[^{124}\text{I}]\text{mIBG}$  PET/CT and PET/MRI are feasible and safe imaging modalities for children with high risk neuroblastoma that could potentially improve diagnostic accuracy relative to currently employed conventional imaging techniques. They could improve the accuracy of response assessment to existing and novel therapies, and reduce both the number of imaging investigations and sedation/general anaesthetics required in young children. Logistically, hybrid imaging offers a “one-stop shop” assessing morphological and functional information simultaneously and can also provide additional information on tumour cellularity. Both  $[^{124}\text{I}]\text{mIBG}$  PET/CT and PET/MRI are promising techniques worthy of further evaluation in neuroblastoma, in particular in the context of  $[^{131}\text{I}]\text{-mIBG}$  radioisotope therapy.

$[^{123}\text{I}]\text{mIBG}$  standard of care image



$[^{124}\text{I}]\text{mIBG}$  PET



$[^{124}\text{I}]\text{mIBG}$  PET/CT 4D image



Caption 1:  $[^{124}\text{I}]\text{mIBG}$  vs  $[^{123}\text{I}]\text{mIBG}$  images





## **P341 124I-MIBG PET/CT to monitor metastatic disease in children with relapsed neuroblastoma**

*Seo, Y.<sup>2</sup>, Aboian, M.<sup>3</sup>, Huang, S.<sup>4</sup>, Hernandez-Pampaloni, M.<sup>5</sup>, Huh, Y.<sup>6</sup>, Vo, K.<sup>7</sup>, Gustafson, C.<sup>8</sup>, Matthay, K.<sup>9</sup>*

University of California, San Francisco, Radiology and Biomedical Imaging, San Francisco, USA <sup>2</sup>, Yale University, Radiology, New Haven, USA<sup>3</sup>, University of California, San Francisco, Radiology and Biomedical Imaging, San Francisco, USA<sup>4</sup>, University of California, San Francisco, Radiology and Biomedical Imaging, San Francisco, University of California, San Francisco<sup>5</sup>, University of California, San Francisco, Radiology and Biomedical Imaging, San Francisco, USA<sup>6</sup>, University of California, San Francisco, Pediatrics, San Francisco, USA<sup>7</sup>, University of California, San Francisco, Pediatrics, San Francisco, USA<sup>8</sup>, University of California, San Francisco, Pediatrics, San Francisco, USA<sup>9</sup>

### **Background/Introduction:**

Iodine-123 metaiodobenzylguanidine (MIBG) scan is one of the most sensitive noninvasive lesion detection modalities for neuroblastoma. Instead of I-123, I-124 for MIBG scan offers high-resolution positron emission tomography (PET).

### **Aims:**

We have compiled the cumulative data of <sup>124</sup>I-MIBG PET/CT we have acquired at our institution to evaluate its diagnostic value over that of <sup>123</sup>I-MIBG planar and single photon emission computer tomography (SPECT)/CT as well as to estimate radiation dose of <sup>124</sup>I-MIBG.

### **Methods/Materials:**

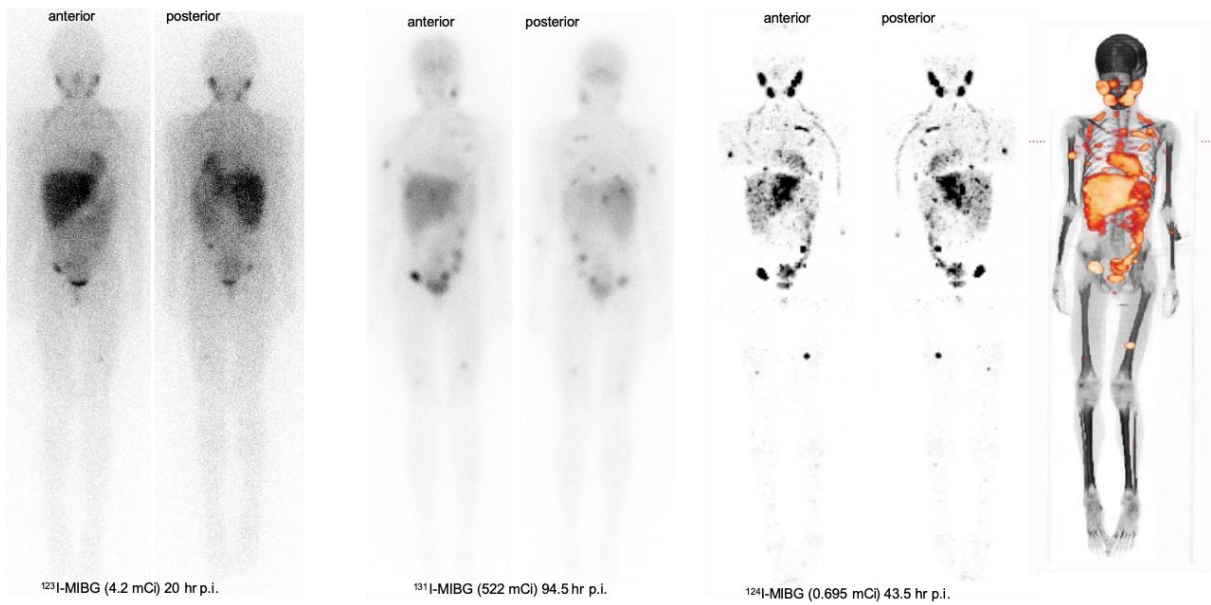
Conventional <sup>123</sup>I-MIBG scans (5.2 MBq/kg) that included whole-body anterior-posterior planar scans and a single field-of-view (FOV) SPECT/CT as well as whole-body <sup>124</sup>I-MIBG PET/CT (1.05 MBq/kg) were performed in 8 patients before <sup>131</sup>I-MIBG therapy. After therapy, 2 out of 8 patients also completed <sup>124</sup>I-MIBG PET/CT as well as paired <sup>123</sup>I-MIBG planar and SPECT/CT scans. For direct comparisons, locations of lesions identified, the number of total lesions detected, and the Curie scores were recorded for all paired imaging datasets by two highly experienced nuclear medicine physicians. In five patients, <sup>124</sup>I-MIBG PET/CT scans were performed at multiple time points for radiation dose estimation. Using patient-specific CT as voxelized phantoms for Monte Carlo simulation and ICRP103 tissue weighting factors, effective doses were calculated for <sup>124</sup>I-MIBG.

### **Results:**

In all paired datasets we reviewed, <sup>123</sup>I-MIBG whole-body planar scans, single FOV SPECT/CT, and whole-body <sup>124</sup>I-MIBG PET/CT detected 25, 32, and 87 lesions, respectively. There were 63 lesions clearly visualized by <sup>124</sup>I-MIBG PET/CT, but not visualized by <sup>123</sup>I-MIBG planar scans, and 59 lesions clearly visualized by <sup>124</sup>I-MIBG PET/CT, but not visualized by <sup>123</sup>I-MIBG SPECT/CT. However, there were 1 lesion seen by <sup>123</sup>I-MIBG planar scan and 4 lesions seen by <sup>123</sup>I-MIBG SPECT/CT, which were not clearly visualized on <sup>124</sup>I-MIBG PET/CT. These observations indicated that there were significantly more lesions identified on <sup>124</sup>I-MIBG PET/CT than those on <sup>123</sup>I-MIBG whole-body planar scans ( $p < 0.0001$ ) and <sup>123</sup>I-MIBG SPECT/CT ( $p < 0.0001$ ). Lesion identification assessment also resulted in changes in the Curie scores. The Curie scores by <sup>124</sup>I-MIBG PET/CT were higher than those by <sup>123</sup>I-MIBG planar and SPECT/CT scans in 7 out of 8 patients. Finally, the estimated effective doses were, 0.161 mSv/MBq for 122-kg female, 0.235 mSv/MBq for 63-kg male, 0.339 mSv/MBq for 40-kg male, 0.706 mSv/MBq for 29-kg female, and 0.795 mSv/MBq for 23-kg female patients. These values are approximately 10 times higher than those of <sup>123</sup>I-MIBG (0.019 mSv/MBq) for 73.7-kg male). Hence, the effective dose for <sup>124</sup>I-MIBG scan was only twice the dose for <sup>123</sup>I-MIBG scan (5.2 MBq/kg) because of the low-dose protocol (1.05 MBq/kg).

### **Summary/Conclusions:**

The first in human use of low dose <sup>124</sup>I-MIBG PET for monitoring response demonstrated superior tumor detection capability as compared to <sup>123</sup>I-MIBG planar and SPECT/CT scans.



Caption 1: Representative  $^{123}\text{I}$ -MIBG,  $^{131}\text{I}$ -MIBG, and  $^{124}\text{I}$ -MIBG images

## **P342 3D *in vitro* scaffold-based cell model of neuroblastoma for evaluating genotoxic and miRNA-targeted therapeutics.**

*Piskareva, O.*<sup>2</sup>, *Curtin, C.C.*<sup>3</sup>, *Nolan, J.N.*<sup>4</sup>, *Gallagher, C.G.*<sup>5</sup>, *O'Brien, F.*<sup>6</sup>

Royal College of Surgeons in Ireland, Department of Anatomy and Regenerative Medicine, Dublin, Ireland<sup>2</sup>, Royal College of Surgeons in Ireland, Anatomy and Regenerative Medicine, Dublin, Ireland<sup>3</sup>, Royal College of Surgeons in Ireland, Anatomy and Regenerative Medicine, Dublin, Ireland<sup>4</sup>, Royal College of Surgeons in Ireland, Department of Anatomy and Regenerative Medicine, Dublin, Royal College of Surgeons in Ireland<sup>5</sup>, Royal College of Surgeons in Ireland, Department of Anatomy and Regenerative Medicine, Dublin, Ireland<sup>6</sup>

### Background/Introduction:

Neuroblastoma is an aggressive paediatric cancer of the sympathetic nervous system. Nearly ten new cases are reported annually in Ireland, 100 in the UK and 800 in the US. Current therapies are not effective in the long term for almost 80% of patients with clinically aggressive high-risk disease due to the development of metastatic loci resistant to multiple chemotherapeutic drugs.

The accurate representation of the tumour architecture and patient diversity are two primary challenges in the identification of new agents for paediatric drug development particularly with the limited number of patients eligible for clinical trials.

### Aims:

We aimed to bridge the gap between conventional 2D culture and *in vivo* tumours for neuroblastoma by developing a 3D neuroblastoma tissue-engineered platform and exploring its therapeutic relevance to genotoxic and targeted drugs.

### Methods/Materials:

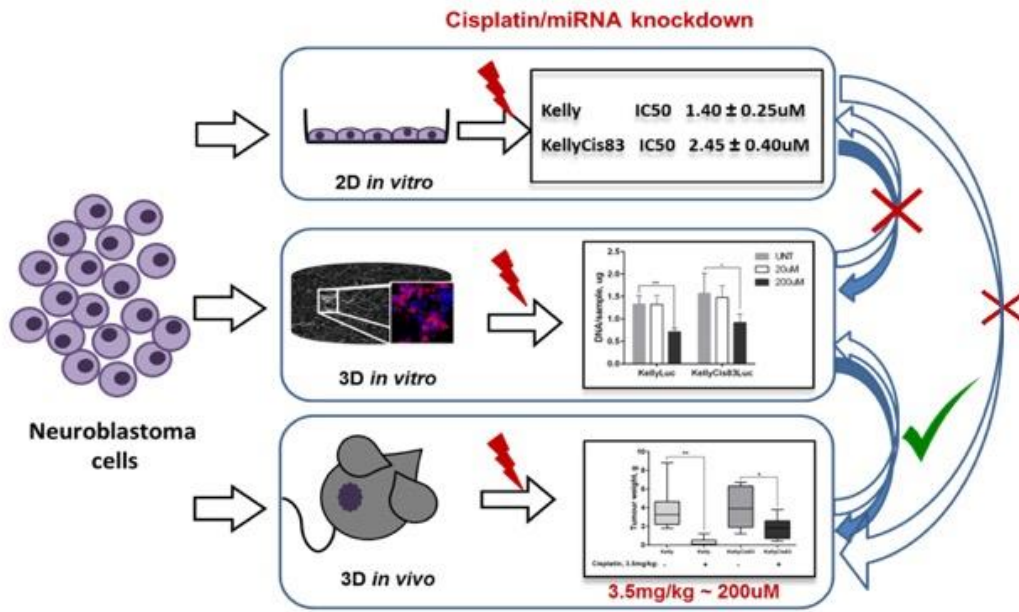
Chemotherapeutic sensitive Kelly and resistant KellyCis83 neuroblastoma cell lines were cultured in a 3D *in vitro* model on collagen-based scaffolds containing either glycosaminoglycan (Coll-GAG) or nanohydroxyapatite (Coll-nHA) and compared to 2D cell culture and an orthotopic murine model. The model was characterised by cell proliferation (DNA content) and viability, secretion of Chromogranin A (CgA) and fluorescent microscopy (DAPI). Chemosensitivity to cisplatin treatment was assessed in all tested models.

### Results:

Neuroblastoma cell lines actively infiltrated and proliferated over the 21-day timeframe and exhibited physiological activity by secreting CgA demonstrating the correlation between cell numbers and concentration of CgA. Both cell lines responded to cisplatin, a cytotoxic drug commonly used in neuroblastoma treatment, displaying > 100-fold increased resistance to cisplatin treatment when compared to 2D cultures, exhibiting chemosensitivity similar to orthotopic xenograft *in vivo* models. The data is in agreement with previous studies reporting a significant increase in cancer cell resistance to chemotherapy when grown in 3D when compared to their 2D monolayer counterparts in various cancer types. The efficacy of cellular uptake and gene knockdown by liposomes bearing miR-324-5p was similar in both 2D and 3D *in vitro* culturing models highlighting the proof-of-principle for the applicability of this model for validation of miRNA function.

### Summary/Conclusions:

We successfully established and characterised a physiologically relevant, scaffold-based 3D neuroblastoma model, strongly supporting its potential value in the evaluation of chemotherapeutic and miRNA-based drugs and investigation of neuroblastoma biology.



Caption 1: Translational relevance of 3D *in vitro* neuroblastoma models

## P343 CDK12 AS A TARGET FOR SYNTHETIC LETHAL INTERACTION WITH THE HOMOLOGOUS DNA REPAIR MACHINERY IN NEUROBLASTOMA

*De Wyn, J.*<sup>2</sup>, *Dolman, E.*<sup>3</sup>, *De Smet, E.*<sup>4</sup>, *Eggermont, A.*<sup>5</sup>, *Nunes, C.*<sup>6</sup>, *Keller, K.*<sup>7</sup>, *Olexiouk, V.*<sup>8</sup>, *De Wilde, B.*<sup>9</sup>, *Molenaar, J.*<sup>10</sup>, *Speleman, F.*<sup>11</sup>, *Durinck, K.*<sup>12</sup>

Ghent University, Department of Biomolecular Medicine, Ghent, Belgium <sup>2</sup>, Princess Máxima Center, Princess Máxima Center, Utrecht, Netherlands<sup>3</sup>, Ghent University, Department of Biomolecular Medicine, Ghent, Belgium<sup>4</sup>, Ghent University, Department of Biomolecular Medicine, Ghent, Ghent University<sup>5</sup>, Ghent University, Department of Biomolecular Medicine, Ghent, Belgium<sup>6</sup>, Princess Máxima Center, Princess Máxima Center, Utrecht, Netherlands<sup>7</sup>, Ghent University, Department of Biomolecular Medicine, Ghent, Belgium<sup>8</sup>, Ghent University, Department of Pediatric Hematology-Oncology and Stem Cell Transplantation, Ghent, Belgium<sup>9</sup>, Princess Máxima Center, Princess Máxima Center, Utrecht, Netherlands<sup>10</sup>, Ghent University, Department of Biomolecular Medicine, Ghent, Belgium<sup>11</sup>, Ghent University, Department of Biomolecular Medicine, Ghent, Belgium, <sup>12</sup>

### Background/Introduction:

Neuroblastoma (NB) is a deadly pediatric tumor arising from sympathetic neuronal progenitors. Current options for precision medicine are limited and overall survival of high-risk NB cases remains disappointingly low. In order to identify novel drug targets, we studied dynamic regulation of gene expression during *MYCN* driven NB formation in mice. We identified upregulation of key components of the homologous DNA repair machinery, including *BRCA1* and *RAD51*. In addition, both genes were identified as hits of a genome wide siRNA screen in NB cells. Of further interest, we also noted elevated CDK12 expression levels over time. In view of the impact of CDK12 on *BRCA1* and *RAD51* expression levels through global suppression of intronic polyadenylation, we hypothesized that targeted pharmacological inhibition of CDK12 using THZ531, could mediate a BRCAness phenotype rendering these cells vulnerable to drugging of synthetic lethal targets.

### Aims:

Our aim was to evaluate drug synergism of the CDK12 inhibitor THZ531 in combination with compounds affecting DNA repair and replication fork stability.

### Methods/Materials:

We evaluated the half-maximum inhibitory concentration (IC<sub>50</sub>) of THZ531 in a panel of 10 NB cell lines and determined the impact on the NB transcriptome by RNA-seq 6h post-treatment of four cell lines. In addition, we scrutinized the effects of pharmacological CDK12 inhibition on DNA damage levels. Combination treatment was tested for a small in-house panel of compounds including 3-AP (RRM2i), gemcitabine (RRM2i), talazoparib (PARPi), AZD1775 (WEE1i), LY2606368 (CHK1i), BAY1895344 (ATRi), nutlin-3 and idasanutlin (MDM2i). In addition, THZ531 was tested against a drug library of > 190 FDA approved compounds. Synergism was evaluated using the Bliss independence score.

### Results:

IC<sub>50</sub> values for THZ531 after 48h exposure range between 40nM and 3000nM. RNA-sequencing upon treatment with THZ531 confirmed significant downregulation of amongst others *RAD51D*, *RAD50*, *BRCA1*, *EME1*, *MUS81* and *XRCC2* expression levels in at least 3 out of 4 cell lines. These findings were validated at RNA and protein level by RT-qPCR and immunoblotting, respectively. Compounds with known effect on replicative stress and DNA repair (3-AP, gemcitabine, talazoparib, and checkpoint inhibitors) yielded no synergistic effects. Unexpectedly, HSP90 and BCL2 inhibitors were found to generate synergistic effects on cell viability and are currently under further study. We also observed synergism with nutlin-3 (not idasanutlin) but only in TP53 mutated cell lines, most probably through the previously reported inhibitory effect on drug efflux pumps. In view of the recently reported broad synthetic lethal interaction between homologous repair proteins and DNA polymerase

theta (POLQ), we will also evaluate the effects of combination treatment of THZ531 with a novel on-target POLQ inhibitor.

Summary/Conclusions:

We confirm downregulation of multiple DNA repair proteins upon CDK12 inhibition in NB. We did not find evidence for synergistic interaction with drugs affecting DNA repair and cell cycle checkpoints. Synergistic effects were noted for combinations with BCL2 and HSP90 inhibitors which is currently under further study.

## **P344 Combination of Carboplatin and Etoposide in Patients with Relapsed or Progressing Neuroblastoma**

*Butzer, S.<sup>2</sup>, Hero, B.<sup>3</sup>, Fischer, M.<sup>4</sup>, Vieth, S.<sup>5</sup>, Kratz, C.<sup>6</sup>, Berthold, F.<sup>7</sup>, Simon, T.<sup>8</sup>*

University Children's Hospital of Cologne, Department of Pediatric Oncology and Hematology, Cologne, Germany <sup>2</sup>, University Children's Hospital of Cologne, Department of Pediatric Oncology and Hematology, Cologne, Germany<sup>3</sup>, University Children's Hospital of Cologne, Department of Experimental Pediatric Oncology, Cologne, Germany<sup>4</sup>, University Hospital Schleswig-Holstein, Department of Pediatric Oncology and Hematology, Kiel, University Hospital Schleswig-Holstein<sup>5</sup>, Hannover Medical School, Department of Paediatric Haematology and Oncology, Hannover, Germany<sup>6</sup>, University Children's Hospital of Cologne, Department of Pediatric Oncology and Hematology, Cologne, Germany<sup>7</sup>, University Children's Hospital of Cologne, Department of Pediatric Oncology and Hematology, Cologne, Germany<sup>8</sup>

### Background/Introduction:

Despite therapeutic advances, outcome following relapse/progression of patients with neuroblastoma remains poor. We analyzed patients with relapsed/progressing neuroblastoma treated with a combination of carboplatin and etoposide (CE).

### Aims:

### Methods/Materials:

In this analysis we included patients registered in the prospective national neuroblastoma trials between 1997 and 2019 who were treated with CE (standard dose carboplatin 300 mg/m<sup>2</sup>/d and etoposide 300 mg/m<sup>2</sup>/d for two to three days) for relapse/progression. Patients receiving additional other cytotoxic agents were excluded. We correlated first and best response evaluations with clinical features, *MYCN*, and calculated time-to-progression and post-relapse overall-survival.

### Results:

A total of 38 patients with 43 relapses have been identified. Twenty-seven patients (71.1%) were diagnosed with INSS stage 4 neuroblastoma at >18 months of age or were *MYCN* amplified at first diagnosis and therefore stratified as INRG high-risk (HR)-patients. The remaining eleven patients (28.9%) either presented with metastatic relapse/progression after first-line treatment (n=4, 10.5%) or localized relapse/progression after previous intermediate risk therapy (n=7, 18.4%). Twelve of 43 (27.9%) relapses were locoregional, n=14 (32.6%) were metastatic only, n=17 (39.5%) were combined. 37/38 patients had previously been treated with etoposide/platinum containing regimens.

Among 43 relapses, CE was given for the patient's first relapse in 19 (44.2%), for the second in 10 (23.3%), and for the third or more in 14 (32.6%). A median of 2 CE cycles was applied (range 1-10 cycles), 1 cycle in 8 (18.6%), 2 cycles in 17 (39.5%) and ≥3 cycles in 18 treatment episodes (41.9%). Dose reduction for toxicity of CE was necessary in 7/43 (16.3%), delayed bone marrow recovery (>28 days between cycles) was observed in 12/43 (27.9%).

First response evaluation was done after 1 or 2 CE cycles in 29 patients and demonstrated very good partial response (VGPR) in 1 (3.4%), partial response (PR) in 6 (20.7%), stable disease (SD) in 7 (24.1%) and progressive disease (PD) in 14 (48.3%). One patient was not evaluable. Best response evaluation was performed in 36 patients after a median of 2 CE cycles (range 1-5 cycles) and revealed complete remission in 1 (2.8%), VGPR in 3 (8.3%), SD in 5 (13.9%), PR in 11 (30.6%) and PD in 16 (44.4%). In patients with PD, median time-to-progression was 2.6 months (range 0.2 - 9.2 months).

Neither for early nor for best response evaluation, response (SD or better vs. PD) correlated with number of previous events, HR at first presentation or age at relapse. Patients without *MYCN* amplification were by trend more likely to respond (SD or better in 12/16 non-amplified patients vs. PD in 7/17 patients with *MYCN* amplification, *p*=0.08).

Twelve of 20 patients received other treatments after objective response to CE, five patients were without response evaluation. Of those, six patients are alive at last follow-up, five of these presented with an early response. For the whole cohort 3-year-post-relapse overall-survival was  $0.19 \pm 0.06$ .

Summary/Conclusions:

Despite previous treatment with etoposide and platinum containing regimens, treatment with CE was effective leading to stabilization or response in a substantial part of patients with relapsed/progressing neuroblastoma. This combination therapy warrants further evaluation.



## **P345 Response to Post Induction Therapy prior to Autologous Stem Cell Transplant is Prognostic of Outcome in Patients with Refractory High Risk Neuroblastoma**

*Siddiqui, A.,<sup>1</sup> Desai, A.V.<sup>2</sup>, Oppong, A.<sup>3</sup>, Yuan, C.<sup>4</sup>, Gao, G.<sup>5</sup>, Bagatell, R.<sup>6</sup>, Berg, K.<sup>7</sup>, Sokol, E.<sup>8</sup>, MacQuarrie, K.<sup>9</sup>, Pinto, N.<sup>10</sup>, Gollapudi, A.<sup>11</sup>, Mody, R.<sup>12</sup>, Wolfe, I.<sup>13</sup>, Shusterman, S.<sup>14</sup>, Clinton, C.<sup>15</sup>, Foster, J.<sup>16</sup>, Smith, V.<sup>17</sup>, Cohn, S.<sup>18</sup>, Desai, A.V.<sup>19</sup>*

Comer Children's Hospital, University of Chicago, Department of Pediatrics, Section of Pediatric Hematology/Oncology, Chicago, USA<sup>1</sup>, Comer Children's Hospital, University of Chicago, Department of Pediatrics, Section of Pediatric Hematology/Oncology, Chicago, USA<sup>2</sup>, University of Chicago, Department of Pediatrics, Section of Pediatric Hematology/Oncology, Chicago, USA<sup>3</sup>, University of Chicago, Department of Radiology, Chicago, USA<sup>4</sup>, University of Chicago, Department of Public Health Sciences, Chicago, University of Chicago<sup>5</sup>, Children's Hospital of Philadelphia, University of Pennsylvania, Division of Pediatric Hematology/Oncology, Philadelphia, USA<sup>6</sup>, Children's Hospital of Philadelphia, University of Pennsylvania, Division of Pediatric Hematology/Oncology, Philadelphia, USA<sup>7</sup>, Ann & Robert H. Lurie Children's Hospital of Chicago, Division of Hematology, Oncology, Neuro-Oncology and Stem Cell Transplant, Chicago, USA<sup>8</sup>, Ann & Robert H. Lurie Children's Hospital of Chicago, Division of Hematology, Oncology, Neuro-Oncology and Stem Cell Transplant, Chicago, USA<sup>9</sup>, Seattle Children's Hospital, University of Washington, Division of Pediatric Hematology/Oncology, Seattle, USA<sup>10</sup>, Seattle Children's Hospital, University of Washington, Division of Pediatric Hematology/Oncology, Seattle, USA<sup>11</sup>, C.S. Mott Children's Hospital, University of Michigan, Division of Pediatric Hematology/Oncology, Ann Arbor, USA<sup>12</sup>, C.S. Mott Children's Hospital, University of Michigan, Division of Pediatric Hematology/Oncology, Ann Arbor, USA<sup>13</sup>, Boston Children's Hospital, Dana-Farber Cancer Institute, Division of Pediatric Hematology/Oncology, Boston, USA<sup>14</sup>, Boston Children's Hospital, Dana-Farber Cancer Institute, Division of Pediatric Hematology/Oncology, Boston, USA<sup>15</sup>, Texas Children's Hospital, Baylor College of Medicine, Department of Pediatrics, Section of Hematology-Oncology, Houston, USA<sup>16</sup>, Texas Children's Hospital, Baylor College of Medicine, Department of Pediatrics, Section of Hematology-Oncology, Houston, USA<sup>17</sup>, Comer Children's Hospital, University of Chicago, Department of Pediatrics, Section of Pediatric Hematology/Oncology, Chicago, USA<sup>18</sup>, Comer Children's Hospital, University of Chicago, Department of Pediatrics, Section of Pediatric Hematology/Oncology, Chicago, USA<sup>19</sup>

### **Background/Introduction:**

Outcome for high-risk neuroblastoma (HRNBL) patients with refractory disease at end of induction (EOI) is poor. It is unknown if additional therapies such as I-131-Metaiodobenzylguanidine (MIBG) or combination therapy with irinotecan, temozolomide, and dinutuximab (I/T/DIN) prior to autologous stem cell transplant (ASCT) will improve outcome.

### **Aims:**

The primary study aim is to determine the 3-year event-free survival (EFS) and overall survival (OS) of patients with HRNBL who had refractory disease at EOI treated with versus without post-induction therapy prior to ASCT.

### **Methods/Materials:**

A retrospective chart review of HRNBL patients diagnosed between 2008 and 2018 and found to have refractory disease at EOI was conducted at seven centers. Demographics, tumor biology, treatment response, and outcomes were abstracted. Response was assessed using the 2017 International Neuroblastoma Response Criteria (INRC). The Kaplan-Meier method was used to assess 3-year EFS and OS from the time of diagnosis.

### **Results:**

Three-year EFS and OS were 54% and 79% for the cohort (n=136). Ninety-one patients received no additional therapy prior to ASCT (Cohort 1); 32 patients received post-induction therapy prior to ASCT (Cohort 2); and 13 patients did not undergo ASCT (Cohort 3). Three-year EFS and OS were not

statistically significantly different between Cohort 1 (3-year EFS and OS; 62% and 81%) and Cohort 2 [3-year EFS and OS; 49% (p=0.48) and 82% (p=0.19)]. Outcome for Cohort 3 patients was significantly worse than Cohort 1 [3-year EFS: 15% vs. 62% (p<.001) and 3-year OS: 48% vs. 81% (p=0.003)] and Cohort 2 [3-year EFS: 15% vs. 49% (p<.001) and 3-year OS 48% vs. 82% (p=0.035)]. For Cohort 2 patients, post-induction therapy for the 31 patients with metastatic disease at EOI included I/T/DIN (n=12), MIBG (n=16), MIBG plus I/T/DIN (n=1), and other (n=2). Improved response in metastatic sites was observed in 10 of the 12 (83%) patients who received I/T/DIN and 9 of the 16 (56%) who received MIBG. Two additional patients responded to MIBG plus I/T/DIN (n=1) or MIBG with chemotherapy (n=1). Among the 21 patients with metastatic disease response to post-induction therapy, 3-year EFS and OS were 69% and 94%; significantly better than Cohort 2 patients who did not respond to post induction therapy [3-year EFS and OS: 11% (p=0.016) and 66% (p=0.2)]. Six patients achieved a complete response (CR) in metastatic sites following I/T/DIN (n=5) or MIBG (n=1) prior to ASCT, and all are alive without relapse with median follow-up of 3.4 years.

#### Summary/Conclusions:

No difference in EFS or OS was detected in the overall cohort of HRNBL patients with refractory disease at EOI treated with versus without post-induction therapy prior to ASCT. However, outcomes were significantly better for patients whose metastatic disease responded to post-induction therapy compared to those whose disease did not respond. Excellent survival was observed in the small cohort of patients who achieved a CR in metastatic sites prior to ASCT. Additional prospective studies testing the efficacy to I/T/DIN prior to ASCT in patients with refractory metastatic disease at EOI are warranted.

### **P346 Spinal canal invasion in peripheral neuroblastic tumors: a change in the front-line treatment approach according to the interim results of a prospective study**

*Ash, S.<sup>2</sup>, Riccardo Haupt, R.<sup>3</sup>, De Bernardi, B.<sup>4</sup>, Kraal, K.C.J.<sup>5</sup>, Plantaz, D.<sup>6</sup>, Wiecezorek, A.<sup>7</sup>, Shamanskaya, T.<sup>8</sup>, Owens, C.<sup>9</sup>, Trager, C.<sup>10</sup>, Beck-Popovic, M.<sup>11</sup>, Hero, B.<sup>12</sup>, Laureys, G.<sup>13</sup>, Canete, A.<sup>14</sup>, Gunnes, M.W.<sup>15</sup>, Constantino, C.<sup>16</sup>, Gandolfo, C.<sup>17</sup>, Erminio, G.<sup>18</sup>, Simon, T.<sup>19</sup>, Trahair, T.<sup>20</sup>, Sorrentino, S.<sup>21</sup>*

Schneider Children's Medical Center of Israel, The Rina Zaizov Pediatric Hematology Oncology Division, Petach Tikva, Israel <sup>2</sup>, IRCCS IstitutoGiannina Gaslini, Epidemiology and Biostatistics Unit, Genova, Italy<sup>3</sup>, Istituto Giannina Gaslini, Department of Haematology-Oncology, Genova, Italy<sup>4</sup>, Princess Maxima Center (PMC) for paediatric oncology, Pediatric Oncology, Utrecht, Princess Maxima Center (PMC) for paediatric oncology<sup>5</sup>, CHU de Grenoble, Hôpital Albert Michallon, Département de Pédiatrie, Grenoble, France<sup>6</sup>, University Children's Hospital of Krakow, Pediatric Oncology, Krakow, Poland<sup>7</sup>, Dmitry Rogachev National Research Center of Pediatric Hematology, Oncology and I, Department of Clinical Oncology, Moscow, Russia<sup>8</sup>, Our Lady Children's Hospital, Pediatric Oncology, Dublin, Ireland<sup>9</sup>, Karolinska Institute,, Paediatric Oncology,, Stockholm, Sweden<sup>10</sup>, University Hospital (CHUV), Dept. of Paediatrics, Lausanne, Switzerland<sup>11</sup>, Children's Hospital, University of Cologne, Department of Pediatric Hematology and Oncology, Cologne, Germany, <sup>12</sup>, Prinses Elisabeth Kinderziekenhuis Universitair Ziekenhuis, Department of Paediatric Haematology/Oncology, Gent, Belgium <sup>13</sup>, Hospital Universitari I Politecnic La Fe, Unidad de Oncología Pediátrica, Valencia, Spain <sup>14</sup>, Haukeland University Hospital, Paediatric Department, Bergen, Norway <sup>15</sup>, Instituto Português de Oncologia de Lisboa, Serviço de Pediatria, Lisboa, Portugal <sup>16</sup>, Istituto Giannina Gaslini, Neuroradiology unit, Genova, Italy <sup>17</sup>, IRCCS IstitutoGiannina Gaslini, Epidemiology and Biostatistics Unit, Genova, Italy <sup>18</sup>, Children's Hospital, University of Cologne, Department of Pediatric Hematology and Oncology, Cologne, Germany <sup>19</sup>, Sydney Children's Hospital-Centre for Children's Cancer & Blood Disorders, Pediatric Oncology, Sydney, Australia <sup>20</sup>, Istituto Giannina Gaslini, Department of Haematology-Oncology, Genova, Italy <sup>21</sup>

#### **Background/Introduction:**

Patients with Peripheral Neuroblastic Tumors (PNTs) and spinal canal invasion (SCI) have an excellent survival probability but are prone to develop late neurologic and orthopedic sequelae. The therapeutic approach may include neurosurgical decompression, chemotherapy, and radiation therapy. During the last 2 decades, studies have focused more on primary chemotherapy management of patients with spinal cord compression although there is a bias of treatment for patients with severe symptoms favoring surgical approach. The optimal treatment for these patients has not been determined yet.

#### **Aims:**

The aim of the study was to describe the natural history of PNTs presenting with SCI and the applied treatments, to evaluate the combined effects of risk factors and to develop treatment guidelines.

#### **Methods/Materials:**

We set up a prospective registry collecting clinical, pathological, biological, therapeutic and follow-up data on symptomatic and asymptomatic patients with SCI.

#### **Results:**

135 patients have been registered from 18 countries. 60% had localized disease. The intraspinal extension occupied more than two third of the spinal canal in 66% of the patients. 64% were symptomatic, with motor deficit (41%) and pain (26%) being the most common symptoms. Patients were treated by SIOPEN/institutional protocols. Chemotherapy at diagnosis was performed in 78% of cases; early neurosurgery in 16% and correlated with the presence of symptoms; other approaches at diagnosis were extraspinal tumor resection (3%) and wait and see (3%). The treatment decision was significantly influenced by the presence of symptoms, but not by the degree of symptoms or spinal

canal invasion. Improvement of symptoms (motor deficit), if any, was possible within the first 6-12 months since diagnosis. Neurosurgery is more likely to be used in symptomatic patients, although the medium-term outcome is comparable to that of chemotherapy only.

Summary/Conclusions:

This is the first prospective study collecting data on patient characteristics, treatment approaches and outcome of PNT's presenting with SCI. The study allows the correlation between clinical features at diagnosis and type of treatment with clinical response and late effects. Chemotherapy is the most frequent front-line approach with comparable outcome to neurosurgery. Logistic regression analysis is planned when more data will be available.

### **P347 A multicentric retrospective study on local control in children with high risk neuroblastoma treated with radiotherapy in Belgium**

*Boterberg, T.<sup>2</sup>, Ledoux, B.<sup>3</sup>*

Ghent University Hospital, Department of Radiation Oncology, Ghent, Belgium <sup>2</sup>, Cliniques Universitaires Saint-Luc, Department of Radiation Oncology, Brussels, Belgium<sup>3</sup>

#### Background/Introduction:

External radiotherapy is part of the multi-modality treatment of high risk neuroblastoma. Although most patients with high-risk neuroblastoma die from uncontrolled metastatic disease, improvement of local control may also improve overall survival.

#### Aims:

The aim of this retrospective study is to analyse the local control and overall survival obtained in the Belgian cohort of patient treated for a high risk neuroblastoma.

#### Methods/Materials:

We identified 109 patients treated in Belgium between May 2002 and September 2018, of whom 92 could be analysed. 75 did not progress during the preceding treatment with induction chemotherapy, surgery, high dose chemotherapy, and autologous stem cell infusion and were treated with radiotherapy to a dose of 21 Gy on the tumour bed. We retrospectively analysed the medical records and treatments of all these patients, in collaboration with the 5 Belgian paediatric radiotherapy centres.

#### Results:

With a median follow-up of 3,6 years [0,0-16,3 years] of the global cohort of 92 patients, the 5-year overall survival (OS) was 52,6% ± 5,4% and the 5-year event free survival (EFS) was 50,8% ± 5,3%. Amongst the 75 irradiated patients, the 5-year local recurrence (LR) was 12,3% ± 3,8%, with a median follow up of 4,4 years [0,8-16,3 years]. LR was significantly influenced by the resection status with a LR of 3,5% and 32,7%, respectively for patients with or without macroscopic complete resection ( $p=0,001$ ). This translates into a significant difference in the 5-year OS of 59,2% and 27,3% for completely and incompletely resected patients respectively. Equally, OS was significantly influenced by the LR with a 5y-OS and mean OS of 62,2% and 10,08 years for patient without LR and 0% and 2,17 years for patient with LR ( $p<0,0001$ ).

#### Summary/Conclusions:

In our cohort of Belgian patients, overall survival in high-risk neuroblastoma is influenced by local control. Since radiotherapy can improve local control, there is a rationale to use it to improve the overall survival, potentially even more in patients with residual disease after surgery. Efforts should be made to enrol patients in prospective randomised trials in order to improve our knowledge on the benefit of radiotherapy in high-risk neuroblastoma and the best regimen to use. Of note, the future SIOPEN HR-NBL2 trial has a randomisation for patients with residual disease after surgery to a tumour bed dose of 21.6 Gy followed by a boost dose up to 36 Gy, compared to tumour bed irradiation only of 21.6 Gy.

## **P348 The INRG visualization and analytics platform**

*Volchenbourn, S.*<sup>2</sup>, *Cohn, S.*<sup>3</sup>, *Furner, B.*<sup>4</sup>, *Graglia, L.*<sup>5</sup>, *Tian, J.*<sup>6</sup>, *Applebaum, M.*<sup>7</sup>, *Kang, W.*<sup>8</sup>, *Birz, S.*<sup>9</sup>

University of Chicago, Pediatrics, Chicago, USA <sup>2</sup>, University of Chicago, Pediatrics, Chicago, USA<sup>3</sup>, University of Chicago, Center for Research Informatics, Chicago, USA<sup>4</sup>, University of Chicago, Pediatrics, Chicago, University of Chicago<sup>5</sup>, University of Chicago, Center for Research Informatics, Chicago, USA<sup>6</sup>, University of Chicago, Pediatrics, Chicago, USA<sup>7</sup>, University of Chicago, Center for Research Informatics, Chicago, USA<sup>8</sup>, University of Chicago, HiQ Analytics, Chicago, USA<sup>9</sup>

### Background/Introduction:

The INRG Data Commons houses data on over 21,000 children diagnosed with neuroblastoma over the past 25 years. With data on over 30 clinical and phenotypic variables from subjects in North America (COG), Europe (SIOPEN, GPOH) and Japan. Updated outcomes data are periodically added. Current use is restricted to cohort discovery, data requests, and linkage to other data (TARGET, BPC). The purpose of this study is to add visualization and analysis tools to the INRG data commons to provide researchers with a platform for discovery and experimental planning.

### Aims:

Aim 1 - Allow researchers to select a cohort of interest based selection from the 30+ variables

Aim 2 - Give researchers the ability to build survival curves to examine the event-free (EFS) and overall survival (OS)

Aim 3 - Enable the platform to ingest variables for survival curve analysis from other platforms (e.g., Gabriella Kids First Data Resource).

### Methods/Materials:

The INRG data commons platform went live in 2015 and is publicly-available at <http://www.inrgdb.org> for cohort discovery over 21,000 international research subjects and 30+ clinical and phenotypic variables. To create a visualization, the search criteria for a given cohort are passed to the visualization page on which the user provides additional parameters for analysis, including the survival endpoint (OS / EFS), the time window (e.g., 0-10 years), and the variable against which to plot survival (e.g., *MYCN* status).

The visualization platform makes a call to the API to retrieve a data set consisting of survival time, survival status, and the variable of interest (e.g., *MYCN* status). These data are then packaged in an R data frame and sent in a call to an R server process running in a web service wrapper. R generates a temporary image file that gets stored on the file system and then displayed in the browser. All of the processing happens server side to prevent the transfer of line-level data to users.

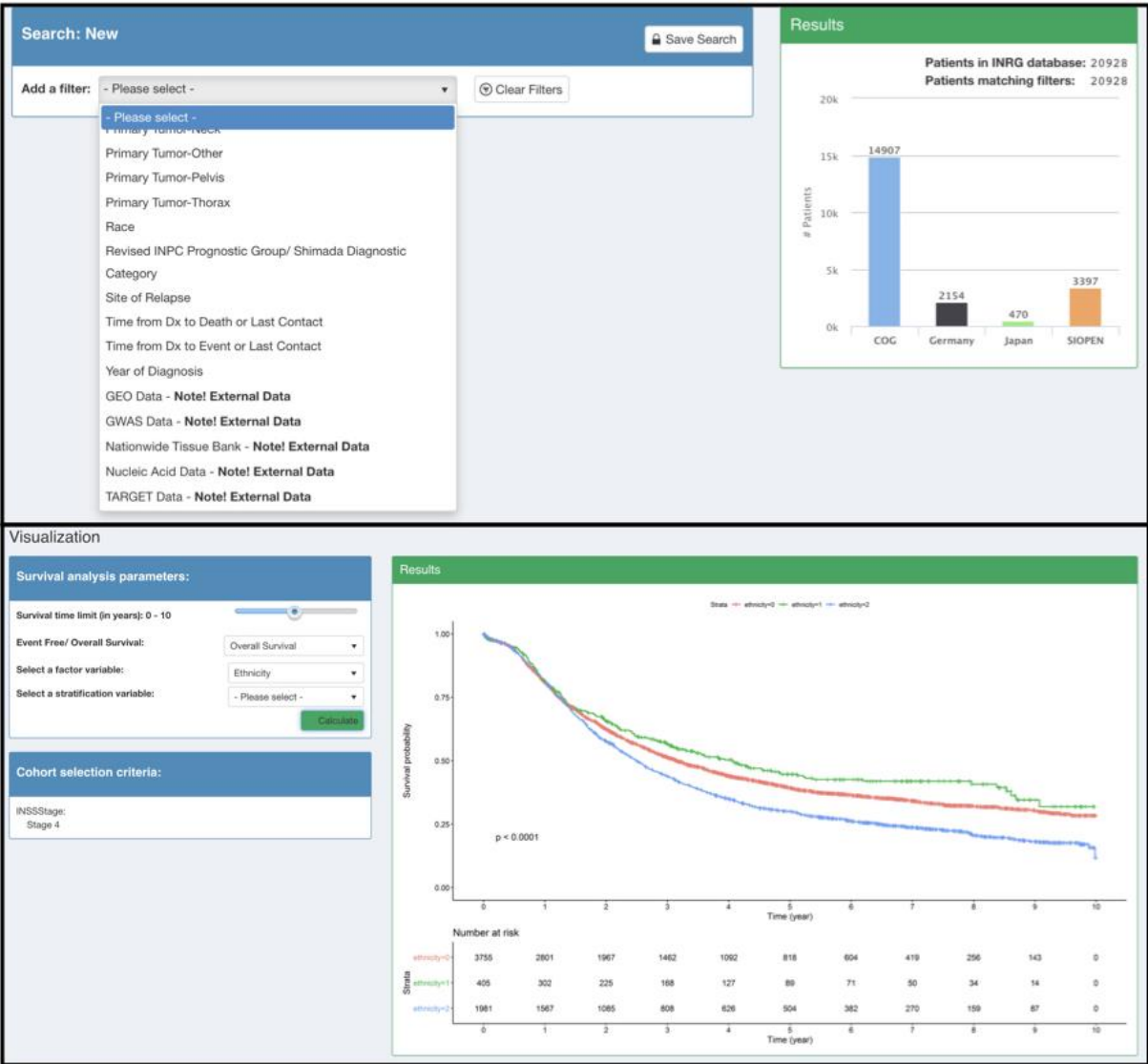
### Results:

A beta version of the platform was released on January 2020 for testing. Initial results demonstrate a highly-effective platform for generating survival curves for cohorts of patients. Further enhancements will be added to increase functionality and add features. Users will be able to save and download their visualizations as well as use them for formal data requests. Finally, connections will be made to other data sources to allow the consumption of genetic data on the INRG subjects, keyed on the publicly-available Universal Specimen Identifier (USI).

### Summary/Conclusions:

This represents the first time that a visualization and analysis platform will be available to the research community that facilitates searching over harmonized data from an international cohort of patients. This powerful platform will allow researchers to develop and test hypotheses for further examination. The platform should be useful for a wide variety of researchers, from clinicians with little informatics experience up to bioinformaticians that want an easy cloud-based platform for data visualization and

analysis. The platform will be demonstrated during the presentation and a variety of different kinds of analyses will be shown.



Caption 1: Figure 1 - INRG cohort selection and visualization. Top: Cohort discovery. Bottom: Survival curve generation. Details in the text.

## **P349 In vivo identification of active drugs and drug combinations for high-risk neuroblastoma using zebrafish models**

*He, S.<sup>2</sup>, Martel, M.W.<sup>3</sup>, Aves, M.<sup>4</sup>, Debiasi, D.<sup>5</sup>, Zimmerman, M.W.<sup>6</sup>, Look, A.T.<sup>7</sup>*

Dana-Farber Cancer Institute, Pediatric Oncology, Boston, USA <sup>2</sup>, Dana-Farber Cancer Institute, Pediatric Oncology, Boston, USA<sup>3</sup>, Dana-Farber Cancer Institute, Pediatric Oncology, Boston, USA<sup>4</sup>, Dana-Farber Cancer Institute, Pediatric Oncology, Boston, Dana-Farber Cancer Institute<sup>5</sup>, Dana-Farber Cancer Institute, Pediatric Oncology, Boston, USA<sup>6</sup>, Dana-Farber Cancer Institute, Pediatric Oncology, Boston, USA<sup>7</sup>

### Background/Introduction:

Neuroblastoma is a high-risk pediatric solid tumor that accounts for 15% of childhood cancer deaths. Most children with high-risk neuroblastoma respond initially to chemotherapy, but a large proportion will experience therapy-resistant relapse. In addition, due to intensified therapies, survivors of high-risk neuroblastoma often face long-term toxicities such as skeletal dysplasia, cardiac dysfunction and premature mortality.

### Aims:

We aim to identify drugs and drug combinations with selectively activity against high-risk neuroblastoma cells in vivo, without undue toxicity to normal tissues.

### Methods/Materials:

We capitalize on the advantages of zebrafish as a model organism to address pressing questions relevant to the generation of effective and selective therapies for high-risk neuroblastoma. We have optimized zebrafish growth and feeding conditions to obtain consistent and reproducible neuroblastoma tumor onset, tumor penetrance, drug response and fish survival across multiple fish lines and batches. The zebrafish neuroblastoma tumor cells, expressing a dbh:GFP fluorescent reporter, are visualized in living fish using fluorescent stereomicroscope, and the drug effects are quantified by comparing the fluorescent tumors before and after the treatment.

### Results:

We have developed zebrafish models of high-risk neuroblastoma driven by overexpression of *MYCN* or *MYC* oncogene, as well as zebrafish models of refractory neuroblastoma harboring either hyperactivation of RAS-MAPK pathway or inactivation of the p53 pathway. These zebrafish develop aggressive neuroblastoma with up to 80% tumor penetrance by 3 weeks of age, ideal for the rapid analysis of small-molecule drugs by adding the drugs to fish water in 12-well plates. Using these faithful preclinical models of refractory neuroblastoma, we are analyzing FDA-approved drugs and drugs currently in clinical trials by simultaneously assessing drug toxicity to normal tissues and activity against primary neuroblastoma tumors in juvenile zebrafish. Post-treatment fish can be followed for tumor relapse, normal development and long-term toxicities. In addition, we also developed assays to analyze drug effects on the progression of very early tumor lesions after tumor initiation in vivo, as well as the awakening of dormant disseminated neuroblastoma cells. Using these zebrafish models, we screened a panel of kinase inhibitors, and identified entrectinib (a RTK inhibitor that is active against NTRK1/2/3, ALK and ROS1) as the most active candidate drug. We validated its anti-neuroblastoma activity in human neuroblastoma cell lines. Our data showed that entrectinib is more active than the ALK inhibitor lorlatinib or the NTRK inhibitor larotrectinib, and less toxic than the multi-RTK inhibitor crizotinib. Importantly, we found that the activity of entrectinib is independent of *MYC/MYCN*, ALK, NF1, P53 mutation status, suggesting entrectinib as a potential anti-neuroblastoma drug.

### Summary/Conclusions:

We have established zebrafish neuroblastoma models for preclinical basic science studies, as multiple dose levels of each drug alone and in combination with other drugs can be readily tested at low cost and in high-throughput. The effective and safe drugs and drug combinations identified in our zebrafish



platforms will inform follow-up testing in murine neuroblastoma primary patient-derived xenografts, before moving forward in clinical trials for neuroblastoma patients.

## **P350 Repurposing a psychoactive drug for children with cancer: p27<sup>Kip1</sup>-dependent inhibition of metastatic neuroblastomas by Prozac.**

*Sala, A.*<sup>2</sup>, *Tsakaneli, A.*<sup>3</sup>, *Bibbo, S.*<sup>4</sup>, *Capone, E.*<sup>5</sup>, *Purgato, S.*<sup>6</sup>, *Sallese, M.*<sup>7</sup>, *Nieddu, V.*<sup>8</sup>, *De Laurenzi, V.*<sup>9</sup>, *Iezzi, M.*<sup>10</sup>, *Perini, G.*<sup>11</sup>, *Sala, G.*<sup>12</sup>, *Rossi, C.*<sup>13</sup>, *Ciuffici, P.*<sup>14</sup>

Brunel University London, Life Sciences, London, United Kingdom <sup>2</sup>, Brunel University London, Department of Life Sciences, London, United Kingdom<sup>3</sup>, Università' di Chieti-Pescara, Centro di Studi e Tecnologie Avanzate (CAST), Chieti, Italy<sup>4</sup>, Università' di Chieti-Pescara, Centro di Studi e Tecnologie Avanzate (CAST), Chieti, Università' di Chieti-Pescara<sup>5</sup>, Università' di Bologna, Dipartimento di Farmacia e Biotecnologia, Bologna, Italy<sup>6</sup>, Università' di Chieti-Pescara, Centro di Studi e Tecnologie Avanzate (CAST), Chieti, Italy<sup>7</sup>, Università' di Chieti-Pescara, Centro di Studi e Tecnologie Avanzate (CAST), Chieti, Italy<sup>8</sup>, Università' di Chieti-Pescara, Centro di Studi e Tecnologie Avanzate (CAST), Chieti, Italy<sup>9</sup>, Università' di Chieti-Pescara, Centro di Studi e Tecnologie Avanzate (CAST), Chieti, Italy<sup>10</sup>, Università' di Bologna, Dipartimento di Farmacia e Biotecnologia, Bologna, Italy<sup>11</sup>, Università' di Chieti-Pescara, Centro di Studi e Tecnologie Avanzate (CAST), Chieti, Italy, <sup>12</sup>, Università' di Chieti-Pescara, Centro di Studi e Tecnologie Avanzate (CAST), Chieti, Italy <sup>13</sup>, Università' di Chieti-Pescara, Centro di Studi e Tecnologie Avanzate (CAST), Chieti, Italy <sup>14</sup>

### Background/Introduction:

The MYC family of transcription factors are major drivers of human cancer and potential therapeutic targets. However, no clinically viable drugs have been yet developed that are able to directly tackle MYC oncoproteins. In our laboratory, we are exploring alternative approaches aiming to disturb signalling downstream of MYC. *MYCN* is frequently activated in neuroblastoma, a paediatric solid malignancy that, in its metastatic form, has a very poor prognosis. An important pathway regulated by MYC is the *CKS1/SKP2/p27<sup>Kip1</sup>* axis. In this study, we have repurposed the anti-psychotic drug Prozac to disrupt *CKS1/SKP2/p27<sup>Kip1</sup>* signalling and assess its potential as an anti-neuroblastoma agent *in vitro* and *in vivo*

### Aims:

Validate Prozac as an anticancer drug for MYC+ CKS1b+ neuroblastomas

### Methods/Materials:

crispr cas9 DNA editing; MTT and colony assays; cell cycle profile and apoptosis assays using flow cytometry; NSG metastatic mouse models.

### Results:

Using DNA editing technology, we show that stabilisation of p27<sup>Kip1</sup> operated by Prozac in MYC-activated cells is essential for the anti-neuroblastoma activity of the drug.

Furthermore, dosing mice with a concentration of Prozac equivalent to that used in long-term clinical trials in children with psychiatric disorders caused a significant reduction of metastatic disease in two models of high-risk neuroblastoma. The anti-proliferative effect of Prozac was strictly dependent on *MYCN* levels, suggesting that its expression could be used as a bio-marker to guide therapeutic interventions.

### Summary/Conclusions:

The favourable toxicity profile of Prozac suggests that long term treatments might be implemented in children with MYC/*CKS1<sup>high</sup>* neuroblastomas.

## **P351 Discovery of novel thiazoles (TT-42) as potent inhibitor of Phosphatidylinositol 3-kinase (PI3K) against neuroblastoma**

Singh, U.P.<sup>2</sup>

Sam Higginbottom University of Agriculture, Technology and Sciences, Department of Pharmaceutical Sciences, Allahabad, India <sup>2</sup>

### Background/Introduction:

Neuroblastoma is a cancer often found in the small glands on top of the kidneys (adrenal glands). It can develop in the stomach, chest, neck, pelvis and bones. The existing modalities to treat neuroblastoma are associated with a low survival rate. Phosphatidylinositol 3-kinase (PI3K) is a cellular proto-oncogene and an essential lipid kinase, that plays an important role in the regulation of cell proliferation, survival and motility. Several preclinical studies have shown that this pathway is hyper activated in neuroblastoma.

### Aims:

The present study showed the development of novel PI3K inhibitors against neuroblastoma.

### Methods/Materials:

The compounds were designed and developed on the basis of pharmacophoric requirements to interact with the active site of PI3K. The molecule was allowed to dock with 3D crystal structure of PI3K protein to assess the structural requirement for binding. The molecules were tested for their activities against PI3K $\alpha$  enzyme via Kinase-Glo Luminescent Kinase Assay kit (Promega, Madison, WI, USA).

### Results:

The molecules were developed in considerable yields. The docking results showed excellent shape complementarity between ligand and the binding pocket. The thiazole moiety occupied the ATP binding pocket, where N1 nitrogen forms a critical hydrogen bond to Val882, and Lys833 in the protein hinge region. The morpholine group fragment lies over Trp812, apparently adding a hydrophilic shield to the hydrophobic heterocycle. The  $\pi$ - stacking interaction was also observed with Asp964. The compound showed IC<sub>50</sub> of 2.31  $\mu$ M against PI3K $\alpha$ .

### Summary/Conclusions:

In summary, using structure-based design approach, we have successfully designed and synthesized a novel series of thiazoles as PI3K inhibitor against neuroblastoma.

## **P352 MYCN Amplification Predicts Sensitivity to PC-002 in Neuroblastoma**

Li, X.,<sup>1</sup>Jiang, C.<sup>2</sup>, He, N.<sup>3</sup>, Zhang, M.<sup>4</sup>, Lv, Y.<sup>5</sup>, Yu, Y.<sup>6</sup>, Hou, X.F.<sup>7</sup>, Hao, Y.B.<sup>8</sup>, An, Z.D.<sup>9</sup>, Zhang, H.W.<sup>10</sup>, Yang, Z.<sup>11</sup>, Zhai, H.Y.<sup>12</sup>, Cao, Z.X.<sup>13</sup>, Jiang, C.<sup>14</sup>, Chen, Y.Y.<sup>15</sup>

COTHERA BIOPHARMA, INC., Medical Research and Development, Wilmington, USA<sup>1</sup>, COTHERA BIOPHARMA, INC., Medical Research and Development, Wilmington, USA<sup>2</sup>, COTHERA BIOPHARMA, INC., Medical Research and Development, Wilmington, USA<sup>3</sup>, COTHERA BIOPHARMA, INC., Medical Research and Development, Wilmington, USA<sup>4</sup>, COTHERA BIOPHARMA, INC., Medical Research and Development, Wilmington, USA<sup>5</sup>, COTHERA BIOPHARMA, INC., Medical Research and Development, Wilmington, USA<sup>6</sup>, COTHERA BIOPHARMA, INC., Medical Research and Development, Wilmington, USA<sup>7</sup>, COTHERA BIOPHARMA, INC., Medical Research and Development, Wilmington, USA<sup>8</sup>, COTHERA BIOPHARMA, INC., Medical Research and Development, Wilmington, USA<sup>9</sup>, COTHERA BIOPHARMA, INC., Medical Research and Development, Wilmington, USA<sup>10</sup>, COTHERA BIOPHARMA, INC., Medical Research and Development, Wilmington, USA<sup>11</sup>, COTHERA BIOPHARMA, INC., Medical Research and Development, Wilmington, USA<sup>12</sup>, COTHERA BIOPHARMA, INC., Medical Research and Development, Wilmington, USA<sup>13</sup>, COTHERA BIOPHARMA, INC., Medical Research and Development, Wilmington, USA<sup>14</sup>, COTHERA BIOPHARMA, INC., Medical Research and Development, Wilmington, USA<sup>15</sup>

### Background/Introduction:

Oncogene *MYCN* amplification is the most important negative prognostic factor in neuroblastoma (NB). Survival for high-risk patients who are resistant to conventional chemotherapy remains below 40%. Therefore novel therapeutic strategies are urgently needed. PC-002 is a novel small-molecule agent with broad antitumor activity *in vitro* and *in vivo*. In this study we investigated the efficacy and the mechanism of PC-002 as an antitumor drug in *MYCN*-driven neuroblastoma.

### Aims:

### Methods/Materials:

### Results:

We performed EdU incorporation assay to assess cell proliferation 72h after PC-002 treatment. Four cell lines with *MYCN* amplification (MNA) were highly sensitive to PC-002 with IC50 ranging from 0.62nM to 11.7nM, whereas two cell lines without *MYCN* amplification (non-MNA) showed much higher IC50 values (59.06nM and >220nM). Apoptosis was triggered in MNA cell line with PC-002 treatment, while non-MNA cells did not show a specific response. PC-002 also caused cell regrowth inhibition and DNA damage in MNA cells. We further demonstrated that PC-002 significantly downregulated *MYCN* protein through reducing protein half-life without causing transcriptional repression in MNA cells. USP7 (ubiquitin-specific protease) interacts with *MYCN* and induces deubiquitination and stabilization of *MYCN*. PC-002 inhibited USP7 deubiquitinase activity *in vitro* and disrupted *MYCN*-USP7 binding. In contrast, extremely low *MYCN* protein expression was detected in non-MNA cells, and *MYCN* protein level was not significantly affected by PC-002 treatment. The *in vivo* anticancer activity of PC-002 was assessed in neuroblastoma xenograft models. In MNA NB xenograft models, PC-002 significantly inhibited the tumor growth. In contrast, no tumor growth inhibition effect was observed in non-MNA models.

### Summary/Conclusions:

Taken together, our data suggested that PC-002 selectively inhibited tumor growth in MNA neuroblastoma via *MYCN* protein degradation. Therefore, future clinical evaluation of PC-002 in MNA neuroblastoma is warranted.

## **P353 A novel therapeutic strategy against *MYCN*-amplified neuroblastoma by chemical-induced copy number reduction combined with PARP inhibitors**

*Takatori, A.*<sup>2</sup>, *Yoda, H.*<sup>3</sup>, *Watanabe, T.*<sup>4</sup>, *Shinozaki, Y.*<sup>5</sup>, *Nagase, H.*<sup>6</sup>

Chiba Cancer Center Research Institute, Division of Innovative Cancer Therapeutics, Chiba, Japan <sup>2</sup>,  
Chiba Cancer Center Research Institute, Division of Innovative Cancer Therapeutics, Chiba, Japan<sup>3</sup>,  
Chiba Cancer Center Research Institute, Division of Innovative Cancer Therapeutics, Chiba, Japan<sup>4</sup>,  
Chiba Cancer Center Research Institute, Division of Cancer Genetics, Chiba, Chiba Cancer Center  
Research Institute<sup>5</sup>, Chiba Cancer Center Research Institute, Division of Cancer Genetics, Chiba,  
Japan<sup>6</sup>

### Background/Introduction:

*MYCN* amplification is a predictor for clinical outcome of patients with neuroblastoma and correlates with tumor aggressiveness. Inhibition of *MYCN* is a promising therapeutic strategy for *MYCN*-driven neuroblastoma although direct targeting of *MYCN* has been challenging. We have recently reported a DNA-alkylating pyrrole-imidazole polyamide, *MYCN*-A3 (CCC-002), that sequence-specifically binds to the *MYCN* gene and demonstrates anti-tumor activity (Yoda, et al., *Cancer Res.*, 2019). CCC-002 induced site-specific DNA damage, copy number reduction of amplified *MYCN* and cell death in a *similar* manner to CRISPR/Cas9.

### Aims:

In this study, we examined the efficacy of PARP inhibitors, olaparib and rucaparib, as a potential add-on therapy to CCC-002.

### Methods/Materials:

CCC-002 was designed to bind to the minor groove of genomic DNA in the coding region of *MYCN* and synthesized for site-specific alkylation of *MYCN*.

### Results:

Olaparib and rucaparib treatment sensitized *MYCN*-amplified CHP-134 cells to CCC-002 but not in *MYCN*-non-amplified SK-N-AS cells. We also used IMR5-75-sh*MYCN* cells expressing tetracycline-inducible shRNA targeting *MYCN*. Knockdown of *MYCN* expression showed no synergistic effect with olaparib, suggesting that the above combinatorial effect of CCC-002 and PARP inhibitors may occur by DNA damage accumulation rather than the suppression of *MYCN* expression. Indeed, western blot and immunofluorescence analyses confirmed the elevated level of gamma-H2AX and phosphorylated RPA32 after the combination treatment with CCC-002 and olaparib.

### Summary/Conclusions:

Site-specific DNA damage induced by CCC-002 leads to suppression of the *MYCN* gene and potent anti-tumor activity against *MYCN*-amplified neuroblastoma. The present data suggest that DNA repair inhibition may provide an effective therapeutic strategy to accumulate DNA damage by CCC-002 in *MYCN*-amplified neuroblastoma cells.

## **P354 GSK-3 $\beta$ inhibition by 9-ING-41: A Potential Therapy for High-Risk Neuroblastoma.**

*Moreno-Smith, M.*<sup>2</sup>, *Tao, L.*<sup>3</sup>, *Zhang, C.*<sup>4</sup>, *Rajapakshe, K.*<sup>5</sup>, *Grimm, S.L.*<sup>6</sup>, *Foster, J.H.*<sup>7</sup>, *Coarfa, C.*<sup>8</sup>, *Giles, F.*<sup>9</sup>, *Barbieri, E.*<sup>10</sup>

Baylor College of Medicine, Department of Pediatrics, Dan L Duncan Comprehensive Cancer Center, Houston, USA <sup>2</sup>, Baylor College of Medicine, Department of Pediatrics, Dan L Duncan Comprehensive Cancer Center, Houston, USA<sup>3</sup>, Baylor College of Medicine, Department of Pediatrics, Dan L Duncan Comprehensive Cancer Center, Houston, USA<sup>4</sup>, Baylor College of Medicine, Department of Molecular and Cellular Biology, Houston, Baylor College of Medicine<sup>5</sup>, Baylor College of Medicine, Department of Molecular and Cellular Biology, Houston, USA<sup>6</sup>, Baylor College of Medicine, Department of Pediatrics, Dan L Duncan Comprehensive Cancer Center, Houston, USA<sup>7</sup>, Baylor College of Medicine, Department of Molecular and Cellular Biology, Houston, USA<sup>8</sup>, Actuate Therapeutics, Inc., Actuate Therapeutics, Fort Worth, Tx, USA<sup>9</sup>, Baylor College of Medicine, Department of Pediatrics, Dan L Duncan Comprehensive Cancer Center, Houston, USA<sup>10</sup>

### Background/Introduction:

High-risk neuroblastoma (NB) is characterized by poor differentiation and chemoresistance. Thus, there is an urgent need for new effective therapeutic agents. Glycogen synthase kinase 3 $\beta$  (GSK-3 $\beta$ ), a serine/threonine kinase with roles in neuronal progenitor homeostasis and cell metabolism, has been identified as a potential target in NB. 9-ING-41 is a small-molecule inhibitor of GSK-3 $\beta$  which has exhibited anti-cancer activity and no significant attributable toxicity in an ongoing phase I/II study in adults with refractory hematological malignancies and solid tumors (NCT03678883).

### Aims:

The specific goals of our study were to: 1) determine the anti-tumor activity of 9-ING-41 in multiple pre-clinical models of NB, and 2) identify the transcriptional programs activated by 9-ING-41.

### Methods/Materials:

The effect of 9-ING-41 on NB cell proliferation, apoptosis, and tumorigenic activity was determined by MTT, caspase 3, and clonogenic assays in multiple *MYCN*-amplified (MNA) and no-MNA cell lines. Protein expression of pro- and anti-apoptotic proteins upon 9-ING-41 treatment was assessed by western blotting. Genetic depletion of p53 in p53 wild-type lines was obtained via lentiviral-mediated shRNA. To elucidate the mechanism of action of 9-ING-41, transcriptomic analysis (RNA-seq) was performed in MNA cells upon 9-ING-41 treatment at different time points (8 and 24h). Lastly, the anti-tumor activity of 9-ING-41 was determined in MNA orthotopic cell-derived xenografts.

### Results:

9-ING-41 blocks cell proliferation and promotes apoptosis to a greater extent in MNA cells (IC<sub>50</sub>: 0.2  $\mu$ M, 5 fold-caspase activation) compared to no-MNA cells (IC<sub>50</sub>: 0.98 $\mu$ M, < 2 fold-caspase activation). 9-ING-41 also remarkably suppresses colony formation independently of *MYCN* status. Because we observed high apoptosis in MNA cells which retain wild-type p53, we asked whether 9-ING-41 could stimulate p53 signaling. Western blotting analysis of MNA cell lines upon treatment with 9-ING-41 showed a clear stabilization of p53 and p53-responsive proteins (p21 and Bax), suggesting that 9-ING-41 effectively activates p53 downstream signaling. Supporting this finding, genetic depletion of p53 in MNA cells completely abrogates the effects of 9-ING-41 on cell proliferation and apoptosis, confirming that these effects are indeed p53-dependent. RNA-seq analysis identified a total of 452 genes differentially expressed upon 9-ING-41 at 24h (FDR < 0.05, FC  $\geq$  1.5). Hallmark pathway enrichment analysis determined the top enriched pathways as the ones associated with p53 (-log<sub>10</sub> P=29.3), TNF $\alpha$  signaling, hypoxia, apoptosis, and cell cycle regulation. Notably, 9-ING-41 as single agent (40mg/Kg i.p. 5 days/week for three weeks) effectively blocked the tumor growth of MNA xenografts (p=0.03). Lastly, 9-ING-41 was able to sensitize NB cells to conventional therapies, such as irinotecan (IC<sub>50</sub> irinotecan: 0.94 $\mu$ M; combo: 0.16 $\mu$ M) and temozolomide (IC<sub>50</sub> temozolamide: 74 $\mu$ M; combo: 12 $\mu$ M).

Summary/Conclusions:

9-ING-41 has significant pre-clinical activity in NB. Mechanistically, 9-ING-41 activates p53-driven apoptosis and upregulates gene expression programs promoting cytokine signaling, hypoxia, apoptosis, and cell cycle regulation. These data confirm that GSK-3 $\beta$  inhibition is a potentially effective therapeutic approach for high-risk NB, and provide a strong rationale for the inclusion of patients with advanced NB in clinical studies of 9-ING-41. These data also support the conduct of clinical studies of 9-ING-41 combined with irinotecan or temozolamide.

## **P355 Fatty acid metabolism is essential for neuroblastoma**

*Ruiz Perez, M.V.*<sup>2</sup>, *Sainero Alcolado, L.*<sup>3</sup>, *Eyre Sanchez, E.*<sup>4</sup>, *Arsenian Henriksson, M.*<sup>5</sup>

Karolinska Institutet, Microbiology, Tumor and Cell Biology, Stockholm, Sweden <sup>2</sup>, Karolinska Institutet, Microbiology, Tumor and Cell Biology, Stockholm, Sweden<sup>3</sup>, Karolinska Institutet, Microbiology, Tumor and Cell Biology, Stockholm, Sweden<sup>4</sup>, Karolinska Institutet, Microbiology, Tumor and Cell Biology, Stockholm, Karolinska Institutet<sup>5</sup>

### Background/Introduction:

High-risk neuroblastoma frequently acquire therapy resistance and metastatic spread, with fatal clinical outcome. Moreover, these patients may suffer long-term side effects after treatment, including slowed mental and physical development. High MYC signaling or *MYCN* amplification are strongly correlated to poor prognosis and treatment failure in neuroblastoma patients. Alternative approaches, as differentiation-inducing therapy, are especially interesting for high-risk patients in which resistance to conventional chemotherapy arises. Lipids perform critical roles not only as energy fuels and cell membrane components, but also as signaling molecules regulating many cellular processes. We have previously shown that *MYCN* inhibition induces accumulation of neutral lipids in the cytoplasm of neuroblastoma cells, together with induction of neural differentiation (Zirath et al., PNAS 2013). We recently published that fatty acid  $\beta$ -oxidation is a major source of energy production in neuroblastoma cells, and the inhibition of fatty acid oxidation *in vivo* with the small molecule etomoxir reduces tumor growth in a *MYCN*-amplified neuroblastoma xenograft model in mice (Oliynyk, Ruiz-Pérez et al., iScience 2019).

### Aims:

Here our objective is to analyze the crosstalk between lipid metabolism and cell differentiation regulated by MYC(N), to identify new therapeutic targets with potential use for induction of differentiation.

### Methods/Materials:

We have performed bioinformatics analysis of patient gene expression datasets, as well as metabolomics, Seahorse extracellular flux metabolic assays, cell and molecular biology techniques, and xenografts using *MYCN*-amplified and non-amplified cells in mice.

### Results:

The analysis of functional processes in a neuroblastoma patient gene expression dataset highlights lipid metabolism as one of the most relevant functions for this type of tumor. Proteomics of neuroblastoma cells shows that lipid metabolism and related signaling elements, as well as proteins involved in cell differentiation, are consistently affected by *MYCN* downregulation. Furthermore, high expression of acetyl-CoA carboxylase (*ACACA*) and fatty acid synthase (*FASN*), the enzymes involved in fatty acid synthesis, correlates to poor survival in neuroblastoma patients, suggesting fatty acid synthesis and degradation as promising targets for treatment. Metabolomic analysis shows that *MYCN* inhibition has an impact on the relative contribution of glucose- and glutamine-derived carbons to fatty acid synthesis, and to reduced fatty acid content in neuroblastoma cells. The inhibition of *de novo* fatty acid synthesis (targeting either ACC or FASN) triggers MYC(N) protein downregulation and differentiation of both *MYCN*-amplified and non-amplified neuroblastoma cells. Moreover, fatty acid synthesis inhibition has an impact in mitochondrial structure and function. The withdrawal of major nutrients as glucose and glutamine does not induce neuroblastoma differentiation, while the incubation in delipidized cell culture medium induces neurite outgrowth. Consequently, cell membrane-permeable fatty acids prevent the phenotypes observed, suggesting a very specific relationship of lipid metabolism with induction of differentiation. Inhibition of *de novo* fatty acid *in vivo* prevents the growth of *MYCN*-amplified and non-amplified neuroblastoma xenografts and induces expression of differentiation markers in the tumor cells.



#### Summary/Conclusions:

Our work aims to elucidate the impact of lipid metabolism on neuroblastoma biology, with potential implications for metabolism-targeted and differentiation-based therapies. Here we show that targeting fatty acid metabolism could be an interesting therapeutic strategy for neuroblastoma treatment, with a special potential for induction of differentiation.

## **P356 4SC-205-mediated targeting of KIF11, a new prognostic marker and therapeutic target in neuroblastoma.**

Segura, M.F.<sup>2</sup>

Vall Hebron Research Institute, Pediatric Oncology, Barcelona, Spain <sup>2</sup>

### Background/Introduction:

High-risk neuroblastoma tumors are highly proliferative and, therefore, need a robust cell division machinery. Furthermore, targeting cell division has been demonstrated to be effective in various preclinical studies in pediatric malignancies. Early phase studies of pharmacological inhibition of cell-cycle regulators in the treatment of cancer in children showed appreciable tolerability, and support the incorporation of combination approaches.

Preliminary results of our laboratory have shown a clear association of motor proteins expression (i.e. kinesins) with poor patient survival. In particular, we observed that tumors from patients in advanced stages of the disease or relapsed tumors, express high levels of KIF11 (kinesin family member 11), a mitotic spindle-specific kinesin which is essential for bipolar spindle formation and mitotic progression in human cells.

Loss of function experiments and pharmacological inhibition of KIF11 showed efficacy in patient-derived orthotopic xenografts (PDOX), suggesting that KIF11 is essential for the proliferation and viability of neuroblastoma cells, thus positioning KIF11 as a possible new therapeutic target for high-risk, refractory and/or relapsed neuroblastoma.

### Aims:

This project aims to define the contribution of KIF11 to the aggressiveness of high-risk neuroblastoma and evaluate its potential as a prognostic tool and therapeutic target. We will test the therapeutic potential of the new KIF11 inhibitor, 4SC-205 (currently in clinical trials for adult tumors), orally administered and highly specific for KIF11.

### Methods/Materials:

To analyze KIF11 mRNA expression levels and correlation with clinical parameters, we used the GSE49710 (SEQC, Revet et al, 2010, n=498) dataset. SiRNA, shRNA targeting KIF11 and a small-molecule inhibitor (i.e. 4SC-205, 40mg/kg three times/week) were used to investigate the effects of KIF11 inhibition in proliferation, apoptosis and cell cycle in multiple neuroblastoma cell lines *in vitro* and *in vivo* using PDOX.

### Results:

The expression analysis of KIF11 mRNA in NB patients revealed that expression levels above the median correlated with poor event-free and overall survival. Multivariate Cox regression analysis showed that KIF11 mRNA expression can be an independent prognostic factor for lower overall survival. Furthermore, genetic and pharmacologic KIF11 inhibition strikingly reduced cell proliferation and viability in multiple chemoresistant neuroblastoma cell lines. Specifically, KIF11 inhibition arrested neuroblastoma cells in G2/M-phase and triggered distinct molecular events compatible with mitotic catastrophe and apoptotic cell death. The oral administration of the KIF11 inhibitor 4SC-205 in mice bearing neuroblastoma PDOX significantly impaired tumor growth and triggered the expected molecular response in the tumors (e.g. increase in the phosphorylation of Histone 3). Moreover, 4SC-205 reduced metastatic burden and expanded the overall survival of mice with neuroblastoma liver metastasis.

### Summary/Conclusions:

Our findings suggest that the expression of KIF11 could be an independent prognostic factor in neuroblastoma and its pharmacological inhibition a potential new therapeutic strategy to treat high-risk neuroblastoma.

## **P357 A Novel Chimeric Antigen Receptor Targeting Anaplastic Lymphoma Kinase (ALK) for Childhood Cancer Immunotherapy**

*Halliwel, E.<sup>2</sup>, Anderson, J.<sup>3</sup>, Pataillot-Meakin, T.<sup>4</sup>, Chester, K.<sup>5</sup>, Straathof, K.<sup>6</sup>*

UCL ICH, DBC Cancer, London, United Kingdom <sup>2</sup>, UCL ICH, DBC Cancer, London, United Kingdom<sup>3</sup>, Imperial, Imperial, London, United Kingdom<sup>4</sup>, UCL CI, CI, London, UCL CI<sup>5</sup>, UCL ICH, DBC Cancer, London, United Kingdom<sup>6</sup>

### Background/Introduction:

Manipulating patient T cells through the insertion of chimeric antigen receptors (CARs) has transformed the treatment of haematological malignancies by enabling T cell redirection. Success rates in solid tumours have been less efficacious. High risk neuroblastoma (NB) is the most common extracranial solid tumour of childhood accounting for ~15% of childhood cancer related deaths. Anaplastic lymphoma kinase (ALK), a tyrosine kinase receptor, is speculated to be involved in healthy neural development but also in NB progression. Both germline and somatic ALK mutations are implicated in constitutively active oncogenic signalling. Previous CAR T cells targeted against NB, such as GD2 and L1-CAM do not yet yield desired results, demonstrating the demand for novel targets.

### Aims:

To evaluate an ALK CAR as potential novel therapy based on *in vitro* cytotoxic activity, cytokine production and proliferative capabilities.

### Methods/Materials:

A panel of second generation ALK-specific CARs was initially investigated; consisting of five unique ALK single chain variable fragments (scFv), two stalks regions of different lengths and a CD28 costimulatory domain with CD3 $\zeta$ , in order to determine a lead combination. Human primary  $\alpha\beta$  T cells were virally transduced to express the CAR on their surface and co-cultured with cell lines of varying ALK surface expression to determine *in vitro* cytotoxicity, cytokine production, short- and long-term proliferative capacity with and without antigen re-stimulation.

### Results:

All ALK CARs created were successfully expressed on the surface of  $\alpha\beta$  T cells and demonstrated robust cytotoxicity and antigen-dependant cytokine generation when co-cultured with ALK positive cell lines but not with ALK negative cell lines. When NB cell lines were co-cultured, with lower ALK surface expression, cytotoxicity and the ability to produce cytokines by the ALK CARs was lowered in a correlative manner. Furthermore, all CARs demonstrated the ability to proliferate at short time points and produce IL-2 and IFN $\gamma$  after re-stimulation. Despite this, their perseverance at longer time points appeared to be limited. Interestingly, differences in stalk regions appeared to influence whether CAR proliferation was antigen-dependant or antigen-independent.

### Summary/Conclusions:

We have successfully demonstrated that CAR T cells directed against ALK show clear antigen dependant cytotoxicity and cytokine production when ALK target expression is high. There was a clear correlative relationship between ALK surface density and the level of cytotoxic activity and cytokine production, shown when NB cell lines were utilised, suggesting ALK may be expressed below the threshold antigen density required to trigger responses. There is hope to combine to targeting of ALK with another NB associated antigen in a co-CAR system also.

## **P358 Metabolic switch between serine/glycine pathway and TCA cycle for neuroblastoma therapy**

*Hsieh, C.*<sup>2</sup>, *Huang, C.*<sup>3</sup>, *Chung, Y.*<sup>4</sup>, *Liu, Y.*<sup>5</sup>, *Hsu, C.*<sup>6</sup>, *Hsu, W.*<sup>7</sup>, *Huang, H.*<sup>8</sup>, *Juan, H.*<sup>9</sup>

National Taiwan University, Institute of Molecular and Cellular Biology, Taipei, Taiwan<sup>2</sup>, National Taiwan University, Graduate Institute of Biomedical Electronics and Bioinformatics, Taipei, Taiwan<sup>3</sup>, National Taiwan University, Department of Life Science, Taipei, Taiwan<sup>4</sup>, Taipei Medical University Hospital, Department of Pediatrics, Taipei, Taipei Medical University Hospital<sup>5</sup>, National Taiwan University, Department of Agricultural Chemistry, Taipei, Taiwan<sup>6</sup>, National Taiwan University Hospital, Department of Surgery, Taipei, Taiwan<sup>7</sup>, National Yang-Ming University, Institute of Biomedical Informatics, Taipei, Taiwan<sup>8</sup>, National Taiwan University, Department of Life Science, Taipei, Taiwan<sup>9</sup>

### Background/Introduction:

Neuroblastoma is the most common extracranial childhood tumor of the sympathetic nervous system. Clinical outcome remains poor in patients with high-risk neuroblastoma, therefore, novel treatment approaches are required. Repositioning existing drugs for new indications could deliver the productivity increases and reduce development risk because they have well-known safety and pharmacokinetic profiles.

### Aims:

In this study, we aim to discover targets and repositioning drugs for neuroblastoma therapy.

### Methods/Materials:

We performed an integrative transcriptome analysis of high-risk neuroblastoma to find out potential drug targets. We further systematically investigated small-molecule gene-expression signatures across multiple cell lines from the L1000 data of the Library of Integrated Network-based Cellular Signatures (LINCS) project. These perturbational signatures were indeed linked to cancer hallmark and drug sensitivity. The drug-protein interaction was predicted by docking simulation, and the isothermal titration calorimetry (ITC) assay was further used to verify the prediction. Additionally, we utilized MS/MS spectrometry to measure the metabolites in the serine/glycine pathway and tricarboxylic acid (TCA) cycle in drug-treated neuroblastoma cells. The anti-tumor impact of the drug is also dissected by *in vitro* and *in vivo* study.

### Results:

We uncovered phosphoglycerate dehydrogenase (PHGDH) as a disease-relevant gene. High expression of PHGDH was found to positively correlate with poor survival in patients with neuroblastoma. Consistent with this finding, PHGDH knockdown significantly reduced cell viability and clonogenic growth in SK-N-DZ cells expressing high levels of endogenous PHGDH, whereas PHGDH overexpression conferred selective growth advantage to SK-N-AS cells harboring low endogenous PHGDH. This suggests a key role of PHGDH in cellular programming and tumor aggressiveness in neuroblastoma. From the LINCS analysis, we predicted that PHGDH expression can be strongly inhibited by homoharringtonine (HHT), a natural alkaloid used as a traditional Chinese medicine for treating acute myeloid leukemia (AML) and chronic myeloid leukemia (CML). Consistent with the prediction, treatment of neuroblastoma cells with HHT decreased PHGDH expression, enhanced cytotoxicity, and triggered apoptosis at nanomolar concentrations. Docking simulation and ITC assay further verified that HHT can bind to PHGDH and inhibit its enzyme activity by competing with the substrate NAD<sup>+</sup>. Overexpression of PHGDH in SK-N-AS and SK-N-SH neuroblastoma cells conferred resistance to HHT, further supporting the drug-target relationship. Given that PHGDH catalyzes the first step in the *de novo* serine synthesis pathway (SSP) and is essential for turnover of amino acids including glycine, we evaluated the changes of some selected metabolites in neuroblastoma cells treated with HHT. We found that HHT treatment decreased SSP while activating TCA cycle, accompanied with an increase in reactive oxygen species (ROS). Lastly, we examined the *in vivo* efficacy of HHT in a mouse neuroblastoma xenograft model by subcutaneously injecting SK-N-BE(2)C

cells. Consistent with *in vitro* results, HHT treatment significantly reduced tumor volume and increased mouse survival, without observable changes in body weight or damages to organs such as liver, spleen, and kidney.

Summary/Conclusions:

Taken together, these findings suggest that metabolic switch between serine/glycine pathway and TCA cycle with a new PHGDH inhibitor is a powerful therapeutic strategy for neuroblastoma.

## **P359 MRI Native T1 mapping of neuroblastoma pathology - Non-invasive early detection of response to *MYCN*-targeted therapy in the Th-*MYCN* model**

*Jamin, Y.*<sup>2</sup>, *Zormpas-Petridis, K.*<sup>3</sup>, *Poon, E.*<sup>4</sup>, *Yuan, Y.*<sup>5</sup>, *Chesler, L.*<sup>6</sup>, *Robinson, S.P.*<sup>7</sup>

The Institute of Cancer, London, Division of Radiotherapy and Imaging, Sutton, United Kingdom<sup>2</sup>, The Institute of Cancer, London, Radiotherapy and Imaging, Sutton, United Kingdom<sup>3</sup>, The Institute of Cancer, London, Division of Clinical studies, Sutton, United Kingdom<sup>4</sup>, The Institute of Cancer, London, Division of Molecular Pathology, Sutton, The Institute of Cancer, London<sup>5</sup>, The Institute of Cancer, London, Division of Clinical Studies, Sutton, United Kingdom<sup>6</sup>, The Institute of Cancer, London, Division of Radiotherapy and Imaging, Sutton, United Kingdom<sup>7</sup>

### Background/Introduction:

The integration of advanced mouse modelling to accelerate the evaluation of novel therapies against high-risk neuroblastoma provides a unique opportunity to evaluate non-invasive functional imaging biomarkers to characterize *MYCN*-driven tumour micro-environment and its response to therapy. The Th-*MYCN* mouse which spontaneously develops tumours which are stroma-poor, undifferentiated or poorly-differentiated with a high mitosis-karyorrhexis index, mirroring the histology of childhood high-risk tumours. Small molecule inhibitors targeting the stability of *MYCN* protein via the selective inhibition of Aurora A kinase or mTOR activity have shown promising results in genetically-engineered murine (GEM) models of neuroblastoma and are currently being evaluated in early-phase paediatric clinical trials.

### Aims:

This study aimed to demonstrate that native T<sub>1</sub>-mapping, a functional MRI technique, is sensitive to neuroblastoma rich histopathological heterogeneity and how a reduction in native tumour spin-lattice relaxation time T<sub>1</sub> is a sensitive biomarker of response to treatment with Aurora A kinase inhibitor alisertib (MLN8237) and mTOR inhibitor vistusertib (AZD2014) in the Th-*MYCN* model.

### Methods/Materials:

MRI was performed before (*Day 0*) and 24 hours after treatment started (*Day 2*). Th-*MYCN* mice were treated p.o. on *Day 1* with 30 mg/kg Alisertib, 25 mg/kg Vistusertib or vehicle. Tumour T<sub>1</sub> maps were compared with spatially-registered computed maps of undifferentiated, differentiated and apoptotic neuroblasts, generated from digitized H-E sections using a machine learning-based algorithm for the automatic detection and classification of neuroblasts.

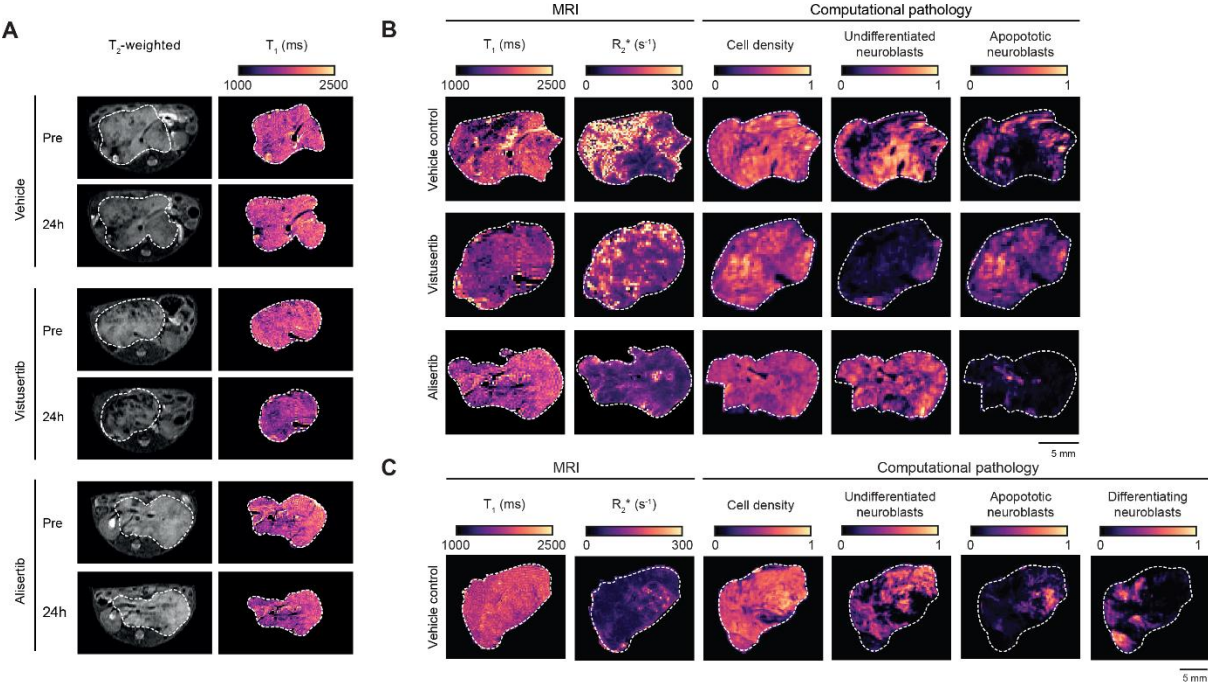
### Results:

A significant reduction in tumour median T<sub>1</sub> value was observed 24h following treatment with Vistusertib (1712±25ms to 1553±25ms, p<0.0001) or Alisertib (1776±26ms to 1679±25ms, p<0.0001) (Fig.1A). Regions of high T<sub>1</sub> values spatially corresponded to regions of undifferentiated neuroblasts in treatment-naïve tumours, while lower T<sub>1</sub> values reflected erythrocyte content or extensive tissue damage (Fig.1B). The reduction in tumour T<sub>1</sub> observed upon treatment with Vistusertib was associated with a significant increase in apoptotic cells, i.e. a reduction of the fraction of undifferentiated neuroblasts, but not with Alisertib. Combining the MRI data from vistusertib- and alisertib- treated mice with matched histopathology revealed a significant negative correlation between the treatment-induced reduction in T<sub>1</sub> over 24h and the proportion of apoptotic neuroblasts present in the tumour at the study endpoint ( $r = -0.55$ ,  $P = 0.04$ ). Overall combining the MRI data from vehicle control-, vistusertib- and alisertib-treated tumours with matched histopathology showed a positive correlation between median native T<sub>1</sub> and the ratio of undifferentiated neuroblasts ( $r = 0.70$ ,  $P < 0.0001$ ) and a negative correlation with the fraction of apoptotic cells ( $r = -0.63$ ,  $P = 0.006$ ). Interestingly, in three tumours exhibiting a significant amount of differentiating neuroblasts, regional differences in native tumour T<sub>1</sub> visually and spatially

corresponded to differences in undifferentiated neuroblast density, with regions of lower  $T_1$  values corresponding to hotspots of differentiating neuroblasts (Fig.1C).

Summary/Conclusions:

This study demonstrates the sensitivity of MRI  $T_1$ -mapping for the rich pathology of neuroblastoma and its therapeutic modulation, which warrants further evaluation of its utility in the clinic, for diagnostic, guiding both surgical biopsies and resection and in clinical trials of novel therapies against neuroblastoma.



Caption 1: The spin lattice T1 is sensitive to neuroblastoma rich pathology and its modulation by MYCN-targeted therapeutics in the Th-MYCN model



## **P360 Nucleolin is a prognostic tumorigenic factor and a novel cell surface protein for targeted therapy in neuroblastoma**

*Brignole, C.,<sup>1</sup> Ponzoni, M.<sup>2</sup>, Cangelosi, D.<sup>3</sup>, Bensa, V.<sup>4</sup>, Corrias, M.V.<sup>5</sup>, Del Zotto, G.<sup>6</sup>, Bruno, S.<sup>7</sup>, Fonseca, N.A.<sup>8</sup>, Cruz, A.F.<sup>9</sup>, Emionite, L.<sup>10</sup>, Malaguti, F.<sup>11</sup>, Carlini, B.<sup>12</sup>, Morandi, F.<sup>13</sup>, Tamma, R.<sup>14</sup>, Perri, P.<sup>15</sup>, Moura, V.<sup>16</sup>, Ribatti, D.<sup>17</sup>, Cilli, M.<sup>18</sup>, Eva, A.<sup>19</sup>, Sementa, A.R.<sup>20</sup>, Moreira, J.N.<sup>21</sup>, Ponzoni, M.<sup>22</sup>, Pastorino, F.<sup>23</sup>*

IRCCS Istituto G. Gaslini, Laboratory of Experimental Therapies in Oncology, Genoa, Italy<sup>1</sup>, IRCCS Istituto G. Gaslini, Laboratory of Experimental Therapies in Oncology, Genoa, Italy<sup>2</sup>, IRCCS Istituto G. Gaslini, Laboratory of Molecular Biology, Genoa, Italy<sup>3</sup>, IRCCS Istituto G. Gaslini, Laboratory of Experimental Therapies in Oncology, Genoa, Italy<sup>4</sup>, IRCCS Istituto G. Gaslini, Laboratory of Experimental Therapies in Oncology, Genoa, IRCCS Istituto G. Gaslini<sup>5</sup>, IRCCS Istituto G. Gaslini, Department of Research and Diagnostics, Genoa, Italy<sup>6</sup>, University of Genoa, Department of Experimental Medicine, Genoa, Italy<sup>7</sup>, University of Coimbra, Center for Neurosciences and Cell Biology, Coimbra, Portugal<sup>8</sup>, University of Coimbra, Center for Neurosciences and Cell Biology, Coimbra, Portugal<sup>9</sup>, IRCCS Ospedale Policlinico San Martino, Animal Facility, Genoa, Italy<sup>10</sup>, IRCCS Istituto G. Gaslini, Department of Pathology, Genoa, Italy<sup>11</sup>, IRCCS Istituto G. Gaslini, Department of Pathology, Genoa, Italy<sup>12</sup>, IRCCS Istituto G. Gaslini, Laboratory of Stem Cell and Cell Therapy, Genoa, Italy<sup>13</sup>, University of Bari, Department of Basic Medical Sciences, Neurosciences, and Sensory Organs, Bari, Italy<sup>14</sup>, IRCCS Istituto G. Gaslini, Laboratory of Experimental Therapies in Oncology, Genoa, Italy<sup>15</sup>, Treat U, Treat U, Coimbra, Portugal<sup>16</sup>, University of Bari, Department of Basic Medical Sciences, Neurosciences, and Sensory Organs, Bari, Italy<sup>17</sup>, IRCCS Ospedale Policlinico San Martino, Animal Facility, Genoa, Italy<sup>18</sup>, IRCCS Istituto G. Gaslini, Laboratory of Molecular Biology, Genoa, Italy<sup>19</sup>, IRCCS Istituto G. Gaslini, Department of Pathology, Genoa, Italy<sup>20</sup>, University of Coimbra, Center for Neurosciences and Cell Biology, Coimbra, Portugal<sup>21</sup>, IRCCS Istituto G. Gaslini, Laboratory of Experimental Therapies in Oncology, Genoa, Italy<sup>22</sup>, IRCCS Istituto G. Gaslini, Laboratory of Experimental Therapies in Oncology, Genoa, Italy<sup>23</sup>

### **Background/Introduction:**

The use of targeted anti-cancer therapies is severely limited by the lack of specific receptors. Nucleolin (NCL), a multifunctional nucleolar protein involved in many biological processes, is overexpressed in adult tumors, where is also shuttled to the cell surface of both tumor cells and tumor endothelial cells. Little is reported about pediatric tumors, including neuroblastoma (NB).

### **Aims:**

To investigate NCL expression and function in NB.

### **Methods/Materials:**

The prognostic value of NCL was assessed in a cohort of 709 NB patients (whose gene expression of the primary tumor was profiled by Agilent customized 4x44k oligonucleotide microarray; ArrayExpress: E-MTAB-1781) and on 20 stage 4, NB patients, alive or dead, at 10 years follow-up. NCL expression was evaluated by immunohistochemistry (IHC) analysis in Schwannian-stroma poor NB patients, and by Flow Cytometry (FC) analysis in tumor fragments derived from relapsed NB patients and in bone marrow-infiltrating NB cells from patients, at either onset or relapse. In a translational setting, NCL expression was evaluated on human NB cell lines in culture and after injection in mice, by Imaging FC (IFC) and IHC analyses, respectively. The NCL-targeting peptide F3 was used to decorate liposomes (L) to test NB cell association and internalization. The cytotoxic potential of doxorubicin (DXR)-loaded, F3-decorated L (NCL-L[DXR]) was evaluated in terms of inhibition of NB cell proliferation and cell death induction *in vitro* and by increasing life span in orthotopic and metastatic animal models of NB, mimicking the growth and spread of NB in patients, respectively.

### **Results:**

E-MTAB-1781 assay demonstrates that NCL mRNA expression is significantly higher in amplified versus (vs) not-amplified *MYCN* tumors ( $p < 0.0001$ ), and in INSS stage 4 vs INSS stage 1, 2, 3, and 4s tumors ( $p < 0.002$ ). Kaplan-Meier and multivariate analyses of overall survival show a significant worse survival for patients with high NCL expression. This result was confirmed in stage 4 patients with a 10 years of follow-up, where a significantly higher expression of NCL corresponds to death events ( $p = 0.015$ , death vs alive) and worst survival ( $p = 0.0215$ , high vs low NCL expression). A strong NCL protein expression in NB tumors, also shuttled to the cell surface of almost all the examined patients, was observed. Moreover, NCL is expressed at different extent on the surface of all NB cell lines analyzed. Notably, NCL staining is evident on both tumor and endothelial tumor cells in well-established NB xenografts. Moreover, F3-decorated rhodamine-labeled liposomes strongly associate to NB cells compared to untargeted formulations, exhibiting a co-localization with cell surface NCL. Finally, NCL-L[DXR] result significantly more effective, in terms of inhibition of cell proliferation and reduction of cell viability *in vitro*, and of delay of tumor growth *in vivo*, compared to untargeted L[DXR].

#### Summary/Conclusions:

Our findings indicate that NCL is an unfavorable independent prognostic factor for NB patients and demonstrate that cell surface NCL might represent a novel protein to be targeted by drug-loaded nanoparticles as innovative therapeutic strategy against NB.

## **P361 Exploiting DNA damage response defects with ATR and PARP inhibitors for the treatment of high risk neuroblastoma**

*Southgate, H.E.D.<sup>2</sup>, Watson, J.<sup>3</sup>, Chen, L.<sup>4</sup>, Curtin, N.J.<sup>5</sup>, Tweddle, D. A.<sup>6</sup>*

Faculty of Medical Sciences, Newcastle University, Wolfson Childhood Cancer Research Centre, Newcastle Centre for Cancer, Newcastle Upon Tyne, United Kingdom <sup>2</sup>, Faculty of Medical Sciences, Newcastle University, Wolfson Childhood Cancer Research Centre, Newcastle Centre for Cancer, Newcastle Upon Tyne, United Kingdom<sup>3</sup>, Faculty of Medical Sciences, Newcastle University, 1Wolfson Childhood Cancer Research Centre, Newcastle Centre for Cancer, Newcastle Upon Tyne, United Kingdom<sup>4</sup>, Faculty of Medical Sciences, Newcastle University, Newcastle Centre for Cancer, Newcastle Upon Tyne, Faculty of Medical Sciences, Newcastle University<sup>5</sup>, Faculty of Medical Sciences, Newcastle University, Wolfson Childhood Cancer Research Centre, Newcastle Centre for Cancer, Newcastle Upon Tyne, United Kingdom<sup>6</sup>

### Background/Introduction:

DNA damage response (DDR) defects are frequently observed in high-risk neuroblastoma (HR-NB) including allelic loss and loss of function mutations in key DDR genes on chromosome 11q, oncogene induced replication stress (RS) and cell cycle checkpoint dysfunction. Around 50% of HR-NB tumours have amplification of the *MYCN* oncogene that promotes rapid DNA replication and cell cycle dysfunction, leading to errors and RS. Defects in the p53 pathway, a key tumour suppressor pathway, are frequently observed at NB relapse, including mutation in *TP53* (gene coding for p53). Cancer cells with defective cell cycle checkpoint signalling and/or increased oncogene-driven RS are acutely dependent on the DNA damage sensor kinase ATR, which orchestrates DNA repair by homologous recombination (HRR). PARP inhibition results in unrepaired single strand DNA breaks progressing to replication, further increasing RS. We hypothesise that combining PARP and ATR inhibition will lead to greater cytotoxicity due to increased RS.

### Aims:

To determine the molecular features conferring sensitivity to ATR inhibition in cell lines derived from HR-NB tumours.

To assess synergy of PARP and ATR inhibition including RS and cytotoxicity in HR-NB cell lines.

### Methods/Materials:

The effects of the ATR inhibitors, VE-821 and AZD6738, and the PARP inhibitor, olaparib were investigated in a panel of 10 NB cell lines with varying *MYCN*, 11q and *TP53* status. All inhibitors were purchased from Stratech (Cambridge, UK). Growth inhibition following exposure to increasing concentrations of VE-821 or olaparib for 72 hours was assessed by XTT assay (Roche) in all 10 NB cell lines. The effect of ATR inhibition on olaparib-induced growth inhibition in 4 cell lines: SHSY5Y (non-*MYCN* amplified (MNA), *TP53* wt), SKNAS (non-MNA, *TP53* mut), NGP (MNA, *TP53* wt), and N20\_R1 (MNA, *TP53* mut). Basal expression of DDR proteins including ATM and ATR was assessed using Western blotting. *CHK1*<sup>S345</sup> and *H2AX*<sup>S129</sup> phosphorylation was assessed using Western blotting to determine ATR activity and RS respectively. RS and HRR activity was also measured by gH2AX and Rad51 foci formation using immunofluorescent microscopy.

### Results:

Sensitivity to VE-821 was associated with *MYCN* amplification and/or low protein ATM expression ( $p < 0.05$ ) but not p53 status or 11q status. ATR inhibition by VE-821 or AZD6738 was synergistic with olaparib (CI value 0.04-0.89) independent of *MYCN* or ATM status. Olaparib increased *H2AX*<sup>S129</sup> phosphorylation which was further increased by VE-821. Rad51 foci formation in response to olaparib was reduced by VE-821 suggesting inhibition of HRR.

### Summary/Conclusions:

*MYCN* amplification and low ATM protein expression are determinants of ATRi sensitivity in NB cell lines and could be useful as predictive biomarkers. ATR inhibition is synergistically cytotoxic with PARP inhibition. This synergy is likely a result of an increase in PARPi-induced RS and inhibition of HRR by ATR inhibition. These findings suggest a potential novel therapeutic strategy for the treatment of HR-NB.

## **P362 Establishment of a preclinical 3D *in vitro* model simulating the neuroblastoma microenvironment enabling investigation of tumor-stroma drug targeting**

Kock, A.,<sup>1</sup> Larsson, K.<sup>2</sup>, Bergqvist, F.<sup>3</sup>, Steinmetz, J.<sup>4</sup>, Elfman, L.<sup>5</sup>, Korotkova, M.<sup>6</sup>, Johnsen, J.<sup>7</sup>, Jakobsson, P.-J.<sup>8</sup>, Kogner, P.<sup>9</sup>, Larsson, K.<sup>10</sup>

Karolinska institutet, Dep. of Children's and Women's Health, Solna, Sweden<sup>1</sup>, Karolinska institutet, Department of Medicine, Stockholm, Sweden<sup>2</sup>, Karolinska institutet, Department of Medicine, Stockholm, Sweden<sup>3</sup>, Karolinska institutet, Department of Medicine, Stockholm, Sweden<sup>4</sup>, Karolinska institutet, Dep. of Children's and Women's Health, Stockholm, Karolinska institutet<sup>5</sup>, Karolinska institutet, Department of Medicine, Stockholm, Sweden<sup>6</sup>, Karolinska institutet, Dep. of Children's and Women's Health, Stockholm, Sweden<sup>7</sup>, Karolinska institutet, Department of Medicine, Stockholm, Sweden<sup>8</sup>, Karolinska institutet, Dep. of Children's and Women's Health, Stockholm, Sweden<sup>9</sup>, Karolinska institutet, Department of Medicine, Stockholm, Sweden<sup>10</sup>

### Background/Introduction:

Cancer related inflammation is a complex tumor-promoting interaction between malignant cancer cells and benign stromal cells. We previously reported that the neuroblastoma microenvironment contains infiltrating cancer-associated fibroblasts (CAFs) expressing microsomal prostaglandin E synthase-1 (mPGES-1), responsible for prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) synthesis. The bioactive lipid PGE<sub>2</sub> promotes immunosuppression, angiogenesis, tumor growth and therapy resistance. Conventional therapies used in the clinic to treat cancer, i.e. chemotherapy and irradiation, induce apoptosis and cell death, processes that leads to increased inflammation and PGE<sub>2</sub> production creating a niche for tumor cell repopulation.

### Aims:

To investigate the impact of mPGES-1/PGE<sub>2</sub> inhibition on cells in the tumor microenvironment in neuroblastoma with the purpose to increase efficiency of treatment and host immunity. We propose that targeting the CAF derived PGE<sub>2</sub> production in combination with cytotoxic drugs will abrogate tumor recovery and improve treatment outcome.

### Methods/Materials:

To evaluate mPGES-1 inhibition in combination with conventional cancer therapies *in vitro* we established a multicellular tumor spheroid (MCTS) model, simulating the neuroblastoma microenvironment by co-culturing neuroblastoma cells and fibroblasts. MCTS were treated with doxorubicin or vincristine in combination with small molecule mPGES-1 inhibitors.

### Results:

The MCTS model successfully recapitulated the neuroblastoma microenvironment where fibroblasts express mPGES-1 and several markers for CAFs. Combination treatment of MCTS with mPGES-1 inhibitors CIII and 934 revealed an enhanced cytotoxic effect of the chemotherapeutic drugs doxorubicin and vincristine. The combination treatment also reduced post treatment MCTS recovery. The *in vitro* findings were translational to an *in vivo* neuroblastoma mouse model where mPGES-1 inhibition with CIII enhanced the effect of vincristine in reducing tumor growth.

### Summary/Conclusions:

The conventional therapies used in the clinic today mainly target the cancer cells overlooking the contribution of tumor-promoting inflammation to tumor progression, and therapy resistance. In the present work, we established an *in vitro* 3D neuroblastoma model, enabling screening for stroma-targeting therapies in combination with established tumor cells targeting therapies. MCTS were treated with a combination of mPGES-1 inhibitor and vincristine, and inhibition of mPGES-1 was found to enhance the cytotoxic effect of vincristine, also translated to an enhanced effect *in vivo*.



### **P363 Identification of novel vulnerabilities of therapy resistant Neuroblastomas**

*Rösch, L.<sup>2</sup>, Ridinger, J.<sup>3</sup>, Hummel, M.<sup>4</sup>, Kopp-Schneider, A.<sup>5</sup>, Michaelis, M.<sup>6</sup>, Cinatl, J.<sup>7</sup>, Jones, D.T.W.<sup>8</sup>, Witt, O.<sup>9</sup>, Oehme, I.<sup>10</sup>*

KiTZ Heidelberg / DKFZ / DKTK, CCU Pediatric Oncology, Heidelberg, Germany <sup>2</sup>, KiTZ Heidelberg / DKFZ / DKTK, CCU Pediatric Oncology, Heidelberg, Germany<sup>3</sup>, DKFZ, Division of Biostatistics, Heidelberg, Germany<sup>4</sup>, DKFZ, Division of Biostatistics, Heidelberg, DKFZ<sup>5</sup>, University of Kent, School of Biosciences, Canterbury, United Kingdom<sup>6</sup>, Goethe University Hospital, Institute for Medical Virology, Frankfurt Am Main, Germany<sup>7</sup>, KiTZ Heidelberg / DKFZ / DKTK, Pediatric Glioma Research, Heidelberg, Germany<sup>8</sup>, KiTZ Heidelberg / DKFZ / DKTK / University Hospital Heidelberg, CCU Pediatric Oncology, Heidelberg, Germany<sup>9</sup>, KiTZ Heidelberg / DKFZ / DKTK, CCU Pediatric Oncology, Heidelberg, Germany<sup>10</sup>

#### **Background/Introduction:**

Relapse, often accompanied by treatment resistance, is a common clinical problem in high-risk childhood malignancies. The INFORM registry “INdividualized Therapy FO r R elapsed M alignancies in Childhood” addresses this by molecular-biologically characterizing tumor samples from pediatric patients with relapsed or refractory high-risk disease. The ERBB family of receptor tyrosine kinases is well known for its role in cancer development and progression. However, the role of ERBB4 in relapsed neuroblastoma has not been studied widely. Aberrant activation of ERBB4 can lead to the activation of various cancer-relevant pathways, including the PI3K/AKT-, SRC- and MAPK-pathways.

#### **Aims:**

We aim to identify and target novel vulnerabilities in relapsed neuroblastoma to induce efficient cell death of the resistant cells. Furthermore, we elucidate which subpopulation of relapsed neuroblastomas can be targeted by a certain vulnerability. As there are many different mechanisms of programmed cell death we further intent to unravel which type is achieved by the novel treatment combination.

#### **Methods/Materials:**

To identify novel vulnerabilities, gene expression data of relapsed INFORM tumors was compared to publicly available gene expression datasets from primary tumors using a rank based analysis. The identified lists of differentially expressed genes between primary and relapsed tumors were then tested for enrichment of molecular pathways. We validated ERBB4 expression in susceptible and resistant neuroblastoma cell lines on RNA and protein levels. The effect of treatment combination of vincristine (VCR) and the ERBB4 inhibitor afatinib on cell viability was controlled by trypan blue exclusion. Influence on the downstream pathways was monitored by western blot. To differentiate between different types of programmed cell death, we established a toolbox to capture several characteristic features: e.g. caspase activity as marker for apoptosis or lipid peroxidation as sign of ferroptosis.

#### **Results:**

Differentially expressed genes identified between primary and relapsed tumors were enriched in pathways involving the extracellular matrix (ECM) and ERBB signaling, especially ERBB4. Combining afatinib with VCR overcame resistance in VCR resistant BE(2)-C cells. Inhibition of the individual downstream pathways (PI3K, MAPK, SRC) of ERBB4 signaling was less efficient in breaking VCR resistance. Moreover, analysis of downstream signaling revealed no effect of afatinib treatment on the PI3K/AKT- and the MAPK-pathways. SRC phosphorylation in resistant BE(2)-C was significantly decreased compared to the control cells. For the elucidation of cell death mechanism, so far, a panel of high-risk neuroblastoma cell lines has been tested for apoptosis, which shows significant differences in caspase activation between different cell lines.

#### **Summary/Conclusions:**

We identified pathways of the ECM and ERBB4 signaling to be associated with relapsed, therapy resistant neuroblastoma. Inhibition of ERBB4 with afatinib breaks resistance in VCR resistant BE(2)-C cells, which involves the SRC pathway. Thus, we identified novel vulnerabilities in relapsed pediatric tumors, which might enable the efficient elimination of treatment-resistant cells through pathway targeted therapy.



## **P364 Modelling neuroblastoma using patient-derived induced pluripotent stem cells**

*Zubillaga, V.*<sup>2</sup>, *Marin-Navarro, A.*<sup>3</sup>, *Kapdi, A.*<sup>4</sup>, *Kogner, P.*<sup>5</sup>, *Bexell, D.*<sup>6</sup>, *Falk, A.*<sup>7</sup>, *Wilhelm, M.*<sup>8</sup>

Karolinska Institutet, Microbiology, tumor and cell biology, Stockholm, Sweden <sup>2</sup>, Karolinska Institutet, Of Microbiology, Tumor Cell biology, Stockholm, Sweden<sup>3</sup>, Karolinska Institutet, Of Microbiology, Tumor Cell biology, Stockholm, Sweden<sup>4</sup>, Karolinska Institutet, Women's and Children's health, Stockholm, Karolinska Institutet<sup>5</sup>, Lund University, Translational Cancer Research, Lund, Sweden<sup>6</sup>, Karolinska Institutet, Neuroscience, Stockholm, Sweden<sup>7</sup>, Karolinska Institutet, Microbiology, tumor and cell biology, Stockholm, Sweden<sup>8</sup>

### **Background/Introduction:**

Neuroblastoma (NB) is a neuroendocrine tumor of the peripheral nervous system usually manifesting in the adrenal gland, in the abdomen and chest. The origin of NB is thought to be Neural Crest cells (NCC) and its derivative sympathoadrenal (SA) cells. Although hereditary NB is rare, germ line mutations have been found in ALK, mimicking common sporadic ALK mutations in NB. *in vitro* modeling of complex diseases is now a possibility with the use of induced pluripotent stem cells (iPS) cells.

### **Aims:**

We aim to model neuroblastoma onset and progression using reprogrammed patient cells carrying familial driver mutations in ALK.

### **Methods/Materials:**

To study the contribution of ALK mutations in NB development, we reprogrammed non-cancerous fibroblasts from NB patients carrying germline mutations in ALK (R1275Q) and somatic cells from healthy individuals into iPS cells. Then, we used a NCC differentiation protocol deriving trunk NCC from the iPS cells. For further SA differentiation, trunk NCC were cultured in neurotrophic medium which yields a high percentage of sympathoadrenal progenitors and sympathetic neurons.

### **Results:**

The patient-specific iPS cells and iPS cells from healthy individuals have been characterized by morphology, expression of pluripotency markers, as well as differentiation to all three germ layers. More importantly, we have established a feeder- and matrigel-free neural crest cell (NCC) differentiation protocol using iPS cells and are able to generate NCC that express relevant markers such as p75, SOX10, SOX9, TFAP2A and PAX3. To examine the tumorigenic ability of these cells, we have orthotopically transplanted luciferase-labeled Ctrl or patient NCC into the adrenal medulla of immunocompromised (NOD-SCID-Fcg) mice and monitored tumor growth over time using the IVIS bioluminescence *in vivo* system. Luciferase activity could be detected in mice injected with patient NCC cells eight weeks after injection and luciferase signal intensities increased over time, while no luciferase activity was observed in mice injected with Ctrl NCC.

### **Summary/Conclusions:**

By using somatic cells carrying germ line mutation as the cell source, instead of using cancer cells derived from an established tumor that carry many more mutations, we will be able to follow the stepwise tumor development resulting from the original mutation. We believe our model will help to uncover new networks important for tumor initiation, discover potential early-stage biomarkers, and give us better models system in which therapeutics can be evaluated.

## **P365 Norepinephrine transporter-targeted macromolecular prodrug of SN-38 causes shrinkage and lasting regrowth inhibition of primary and metastatic tumors in experimental models of drug-resistant neuroblastoma**

Chorny, M.,<sup>1</sup> Brodeur, G.M.<sup>2</sup>, Guerrero, D.T.<sup>3</sup>, Alferiev, I.S.<sup>4</sup>, Nguyen, F.<sup>5</sup>, Guan, P.<sup>6</sup>, Kolla, V.<sup>7</sup>, Fishbein, I.<sup>8</sup>, Brodeur, G.M.<sup>9</sup>

The Children's Hospital of Philadelphia, Pediatrics, Philadelphia, USA<sup>1</sup>, The Children's Hospital of Philadelphia, Pediatrics, Philadelphia, USA<sup>2</sup>, The Children's Hospital of Philadelphia, Pediatrics, Philadelphia, USA<sup>3</sup>, The Children's Hospital of Philadelphia, Pediatrics, Philadelphia, USA<sup>4</sup>, The Children's Hospital of Philadelphia, Pediatrics, Philadelphia, The Children's Hospital of Philadelphia<sup>5</sup>, The Children's Hospital of Philadelphia, Pediatrics, Philadelphia, USA<sup>6</sup>, The Children's Hospital of Philadelphia, Pediatrics, Philadelphia, USA<sup>7</sup>, The Children's Hospital of Philadelphia, Pediatrics, Philadelphia, USA<sup>8</sup>, The Children's Hospital of Philadelphia, Pediatrics, Philadelphia, USA<sup>9</sup>

### Background/Introduction:

*Uptake-1*, a tissue-specific mechanism of catecholamine reuptake, can be exploited for delivery of molecular cargoes to primary and metastatic tumors. Tumor-targeted delivery of radionuclides using this process has previously been evaluated as a treatment of neuroblastoma (NB). However, due to limited tumor selectivity and non-discriminatory damaging effects of the high-dose radiation on healthy adrenergic and non-adrenergic tissues, this strategy has shown suboptimal therapeutic efficacy ( $\leq 30\%$  clinical response, which is seldom curative) and serious acute and delayed toxicities, including secondary malignancies later in life.

### Aims:

In this study, we investigated NB pharmacotherapy targeted to uptake-1 using an *in situ* activatable tripartite prodrug constructed with a bioeliminable polymer as a carrier. This strategy integrates a replication-dependent mode of drug action with neuroendocrine tissue selectivity, which directs the pharmacological effect to actively dividing tumor cells and minimizes toxicity to normal tissues.

### Methods/Materials:

We used xenograft models of refractory NB established using multidrug-resistant NB cells [BE(2)C] derived at relapse after intensive chemo-radiotherapy, paired with bioluminescent imaging to investigate tumor uptake and antitumor efficacy of the experimental prodrug. Tumor uptake analysis was carried out using an HPLC assay in orthotopic BE(2)C xenografts allowed to reach  $\sim 1 \text{ cm}^3$  (n=5). The effectiveness of prodrug-mediated targeted delivery against refractory disease was evaluated in mouse models of primary (orthotopic) and metastatic, chemoresistant NB. The results were analyzed by Kruskal-Wallis one-way ANOVA and the log-rank test.

### Results:

Drug delivery in the form of a macromolecular prodrug, taking advantage of the uptake-1 process, resulted in enhanced intratumoral accumulation of SN-38, a potent anticancer drug uniquely targeted to topoisomerase I. Unlike its clinically used precursor (irinotecan), SN-38 delivered as a polymer-linked prodrug was stably present in the tumor tissue at levels  $\geq 100$  times the drug concentration required for fully suppressing growth of chemoresistant NB tumors. Consistent with these results, SN-38 administered as the prodrug (2x/week, 4 weeks) caused rapid tumor regression, fully suppressed regrowth of chemoresistant orthotopic BE(2)C xenografts and markedly extended event-free survival ( $>14$  weeks). At the same dosing regimen, the prodrug also caused rapid elimination of established multifocal tumor deposits in the model of refractory metastatic disease, with no detectable regrowth over  $>15$  weeks. In contrast, irinotecan given at equivalent doses as a control had no significant effect on the disease progression in either model.

### Summary/Conclusions:

Tumor-guided delivery using a new prodrug design translated into rapid shrinkage and sustained regrowth inhibition of aggressive primary and disseminated tumors in the experimental settings of relapsed, multidrug-resistant NB that shows no response to conventional therapy. The results of our studies confirm feasibility and effectiveness of using macromolecular prodrugs targeted to tissue-specific mechanisms for treating localized and metastatic forms of refractory disease. Thus, targeted delivery of polymer-linked prodrug agents represents a new, promising strategy for treating high-risk NB, as well as other aggressive malignancies in children and adults.

## **P366 Dual PI3K/HDAC inhibitor CUDC-907 blocks tumor growth and sensitizes *MYCN* amplified neuroblastoma to BCL-2 inhibition through the destabilization of MCL-1**

*Koach, J.*<sup>2</sup>, *Nasholm, N.*<sup>3</sup>, *Van, C.*<sup>4</sup>, *Weiss, W.*<sup>5</sup>, *Matthay, K.*<sup>6</sup>, *Dubois, S.*<sup>7</sup>, *Gustafson, C.*<sup>8</sup>

University of California, Pediatric Hematology Oncology, San Francisco, USA <sup>2</sup>, University of California, Pediatric Hematology Oncology, San Francisco, USA<sup>3</sup>, University of California, Pediatric Hematology Oncology, San Francisco, USA<sup>4</sup>, University of California, Neurology and Pediatrics, San Francisco, University of California<sup>5</sup>, University of California, Pediatric Hematology Oncology, San Francisco, USA<sup>6</sup>, Dana-Farber Cancer Institute and Harvard Medical School, Pediatrics, Boston, USA<sup>7</sup>, University of California, Pediatric Hematology Oncology, San Francisco, USA<sup>8</sup>,

### Background/Introduction:

Amplification of *MYCN* proto-oncogene occurs commonly in high-risk neuroblastoma and marks a particularly aggressive and high-risk form of the disease. Recent advances in therapy for neuroblastoma have been mostly through toxic escalations in conventional cytotoxic chemotherapy and radiation. Most high-risk neuroblastoma patients will relapse with highly refractory disease, therefore new therapies for neuroblastoma are focused on more targeted approaches, many of which involve *MYCN* regulation. Small molecule inhibitors of phosphoinositide 3-kinase (PI3K) and histone deacetylase (HDAC), which regulates *MYCN* expression, have shown pre-clinical efficacy in neuroblastoma and several of these are in clinical trials as monotherapy in children with neuroblastoma. However, resistance to targeted monotherapies is common in cancers. One means of bypassing resistance is to target two or more pathways simultaneously through combinations of novel drugs.

### Aims:

We hypothesize that inhibitors of *MYCN* and associated pathways each block distinct aspects of *MYCN* biology, and when used in combination, will both enhance efficacy and block emergent resistance. We aim to assess the efficacy and mechanism of action in the combination treatment between CUDC-907 and BH3 mimetics, venetoclax (ABT-199) and navitoclax (ABT-263), for the treatment of high-risk neuroblastomas.

### Methods/Materials:

We analyzed the effect of CUDC-907 on a panel of neuroblastoma cell lines; using Cell Titre-Glo assay to measure cell growth; immunoprecipitation and quantitative PCR to assess changes in protein and mRNA expression levels, respectively. Using protein arrays and cell-based assays, we identified synergy between CUDC-907 and BCL-2 inhibitors: venetoclax and navitoclax. Other mechanistic studies include flow cytometry, caspase activation assay, and mitochondrial membrane potential assay. We assessed the efficacy of CUDC-907 in combination with venetoclax or navitoclax, *in vivo* using PDX and TH-*MYCN* mouse models.

### Results:

We show dual PI3K/HDAC inhibitor CUDC-907 can reduce neuroblastoma cell growth and is highly effective against MYC and *MYCN* driven neuroblastoma. CUDC-907 suppresses *MYCN* by both blocking histone acetylation and downregulating *MYCN* transcription, and destabilizes *MYCN* and MCL-1 proteins by regulating the phosphorylation of GSK3 $\beta$ . We found CUDC-907 altered several apoptotic proteins, priming neuroblastoma cells for apoptosis induced by BCL-2 inhibitors venetoclax and navitoclax. We show CUDC-907 is most potent in combination with navitoclax, significantly decreasing neuroblastoma cell growth by preventing cells from entering S-phase during mitosis and thus inducing cell cycle arrest. It also induces cytochrome c release and activation of caspase-3 leading to apoptosis. Furthermore, CUDC-907 in combination with navitoclax showed greater efficacy in our *in vivo* PDX model by significantly delaying neuroblastoma tumor growth.

### Summary/Conclusions:

In this study, we demonstrate that the CUDC-907 primes neuroblastoma cells for navitoclax mediated death through down-regulation of MCL-1, thus releasing the pro-apoptotic proteins, Bim and Bax. Importantly, mitochondrial priming of the cells for death by CUDC-907 requires elevated levels of *MYCN* or *MYC*, and high levels of BCL-2 or BCL-XL for synergy between CUDC-907 and navitoclax. Our research provides strong evidence demonstrating the efficacy of CUDC-907 and navitoclax as novel targeted therapy for the treatment of neuroblastoma.

### **P367 Telomerase inhibition acts synergistically with chemotherapy in preclinical high-risk neuroblastoma models**

*Fischer, J.*<sup>2</sup>, *Otte, F.*<sup>3</sup>, *Roderwieser, A.*<sup>4</sup>, *Rosswog, C.*<sup>5</sup>, *Kahlert, Y.*<sup>6</sup>, *Acht, T.*<sup>7</sup>, *Fischer, M.*<sup>8</sup>

University Children's Hospital of Cologne, Pediatric Oncology, Cologne, Germany<sup>2</sup>, University Children's Hospital of Cologne, Pediatric Oncology, Cologne, Germany<sup>3</sup>, University Children's Hospital of Cologne, Pediatric Oncology, Cologne, Germany<sup>4</sup>, University Children's Hospital of Cologne, Pediatric Oncology, Cologne, Germany<sup>5</sup>, University Children's Hospital of Cologne, Pediatric Oncology, Cologne, Germany<sup>6</sup>, University Children's Hospital of Cologne, Pediatric Oncology, Cologne, Germany<sup>7</sup>, University Children's Hospital of Cologne, Pediatric Oncology, Cologne, Germany<sup>8</sup>

#### Background/Introduction:

Telomerase is activated in the majority of high-risk neuroblastomas. We have recently demonstrated that telomerase-interacting compounds, such as 6-thio-2'-deoxyguanosine (6-thio-dG), significantly impair growth of neuroblastoma cell lines harboring *TERT* rearrangements, *MYCN* amplification, or high *TERT* expression in the absence of these alterations both *in vitro* and *in vivo*.

#### Aims:

We here aimed to examine whether telomerase-interacting compounds in combination with established chemotherapy or *ALK* inhibitors act synergistically on neuroblastoma growth *in vitro* and *in vivo*.

#### Methods/Materials:

Growth-inhibitory effects of varying concentrations of 6-thio-dG in combination with etoposide, doxorubicin or ceritinib on neuroblastoma cell lines with *TERT* rearrangements (CLB-GA, GI-ME-N), high *TERT* expression (SH-SY5Y) or *MYCN* amplification (SK-N-BE(2)c, IMR-32, Kelly, LS, TR-14) were determined after 96 hours of substance incubation using CellTiter-Glo®. Drug synergy was calculated using the Chou-Talalay method. Tumor growth inhibition of subcutaneous xenografts was assessed upon treatment with 6-thio-dG, imetelstat, etoposide, or combinations of these compounds. Telomerase activity in imetelstat-treated and control tumors was measured using a TRAP assay.

#### Results:

Strong synergies were observed between 6-thio-dG and etoposide or doxorubicin in most telomerase-positive neuroblastoma cell lines *in vitro*. By contrast, we did not observe a synergistic effect of 6-thio-dG in combination with ceritinib in *ALK*-mutated or *ALK*-wildtype cell lines *in vitro*. Treatment of mouse xenografts with combinations of 6-thio-dG and etoposide or imetelstat and etoposide significantly attenuated growth of cell lines bearing a *TERT* rearrangement (CLB-GA;  $p < 0.001$  and  $p = 0.001$  respectively) or high *TERT* expression (SH-SY5Y;  $p = 0.001$  and  $0.011$  respectively) in comparison to xenografts treated with single compounds. Telomerase activity in xenograft tumors treated by imetelstat was decreased by roughly 50%, indicating intermediate on-target specificity in this setting.

#### Summary/Conclusions:

Telomerase inhibition by imetelstat or 6-thio-dG act synergistically with genotoxic drugs in a fraction of telomerase-positive neuroblastoma and may thus represent a promising treatment option for high-risk patients. Our data also suggest that the development of telomerase-interacting compounds with higher on-target specificity may further improve the efficacy of such treatment strategies.

### **P368 TIAL1 is a MYCN-regulated alternative splicing protein that modifies NF1 to increase RAS-MAPK signaling in neuroblastoma**

*Zheng, T.*<sup>2</sup>, *Sperring, C.*<sup>3</sup>, *Farrel, A.*<sup>4</sup>, *An, Z.*<sup>5</sup>, *Huang, M.*<sup>6</sup>, *Maris, J.*<sup>7</sup>, *Matthay, K.*<sup>8</sup>, *Weiss, W.*<sup>9</sup>

UCSF, Medical Scientist Training Program and Biomedical Sciences, San Francisco, USA<sup>2</sup>, Columbia University, College of Physicians and Surgeons, New York City, USA<sup>3</sup>, Children's Hospital of Philadelphia, Biomedical and Health Informatics, Philadelphia, USA<sup>4</sup>, UCSF, Neurology, San Francisco, UCSF<sup>5</sup>, Children's Hospital of Los Angeles, Pediatrics, Los Angeles, USA<sup>6</sup>, Children's Hospital of Philadelphia, Pediatrics, Philadelphia, USA<sup>7</sup>, UCSF, Pediatrics, San Francisco, USA<sup>8</sup>, UCSF, Neurology, Pediatrics, and Neurological Surgery, San Francisco, USA<sup>9</sup>

#### Background/Introduction:

Activation of RAS-MAPK signaling occurs commonly in relapsed neuroblastoma. Recent studies suggest that increased RAS-MAPK signaling confers aggression in primary and relapsed tumors. NF1 is a GTPase activating protein (GAP) that negatively regulates RAS-MAPK signaling, and is mutated in relapsed neuroblastoma. Inclusion of exon 23a in NF1 mRNA generates an NF1<sup>23a</sup>, a hypomorphic GAP that inefficiently blocks RAS-MAPK signaling. Inclusion of NF1 exon 23a is regulated in-part by the splicing protein TIAL1. Amplification of *MYCN* drives approximately half of high-risk neuroblastoma, and is further known to transcriptionally regulate the alternative splicing landscape. We hypothesize that TIAL1 is a central effector of primary *MYCN*-amplified neuroblastoma, driving RAS-MAPK through negatively regulating NF1.

#### Aims:

The aims of this study were to 1) evaluate TIAL1 as a transcriptional target of *MYCN*, 2) determine whether TIAL1 regulates RAS-MAPK signaling through alternative splicing of NF1, and 3) analyze functional relevance of the *MYCN*-TIAL1-NF1 axis.

#### Methods/Materials:

We developed a system implementing lentiviral doxycycline-inducible dCas9 with a secondary lentiviral sgRNA. This CRISPRi system allows us to reversibly regulate TIAL1 knockdown in human patient cell lines and patient derived xenograft lines. We have also introduced these systems into neural crest cells of the sympathoadrenal lineage derived from human iPSCs. We cloned lentiviral constructs to overexpress TIAL1 and *MYCN*. We analyzed ChIP-seq and RNA-seq results from existing neuroblastoma patient tumor sets to assess *MYCN* binding and *MYCN*, TIAL1, and NF1<sup>23a</sup> vs NF1<sup>WT</sup> expression. We performed growth and signaling studies. We injected our modified cells orthotopically into the renal capsules of NSG mice, as an *in vivo* mouse model.

#### Results:

We found that *MYCN* binds multiple sites in the TIAL1 promoter in a variety of patient derived xenograft tumor lines and cell lines. High expression of TIAL1 correlated inversely with survival. Relapsed patient tumors showed higher levels of incorporation of higher NF1 exon 23a compared to primary tumors. We found that lentiviral misexpression of *MYCN* increased expression of TIAL1, and also increased ratios of NF1<sup>23a</sup>/NF1<sup>WT</sup>. Knockdown of TIAL1 by CRISPRi decreased relative incorporation of NF1 exon 23a. Further, knockdown of TIAL1 led to decreased levels of RAS-GTP. Conversely, we also found that TIAL1 overexpression led to increased abundance of activated Erk. Knockdown of TIAL1 decreased proliferation more effectively in *MYCN*-high cell lines compared *MYCN*-low cell lines. Our preliminary results *in vivo* suggest that TIAL1 knockdown increases tumor latency.

#### Summary/Conclusions:

Our study explored if alternative splicing of NF1 could potentially modulate a *MYCN*-driven axis in aggressive neuroblastoma tumors. We have identified TIAL1 as an alternative splicing protein which is regulated by *MYCN* and which affects NF1 exon 23a inclusion to regulate overall NF1 activity and downstream RAS/MAPK signaling. Thus, TIAL1 could be a potentially novel targetable component acting in high-risk and relapsed neuroblastoma. We are continuing to delve into mechanisms of TIAL1 in neuroblastoma aggression and therapy resistance.





## **P369 Effect of CDK4 and MDM2 inhibition on cell viability in neuroblastoma**

Martinez-Monleon, A.<sup>2</sup>, Fransson, S.<sup>3</sup>, Gaarder, J.<sup>4</sup>, Djos, A.<sup>5</sup>, Javanmardi, N.<sup>6</sup>, Kogner, P.<sup>7</sup>, Martinsson, T.<sup>8</sup>

Gothenburg University, Department of Laboratory Medicine, Gothenburg, Sweden<sup>2</sup>, University of Gothenburg, Department of Laboratory Medicine, Gothenburg, Sweden<sup>3</sup>, University of Gothenburg, Department of Laboratory Medicine, Gothenburg, Sweden<sup>4</sup>, University of Gothenburg, Department of Laboratory Medicine, Gothenburg, University of Gothenburg<sup>5</sup>, University of Gothenburg, Department of Laboratory Medicine, Gothenburg, Sweden<sup>6</sup>, Karolinska Institutet, Department of Women's and Children's Health, Stockholm, Sweden<sup>7</sup>, University of Gothenburg, Department of Laboratory Medicine, Gothenburg, Sweden<sup>8</sup>

### Background/Introduction:

High-grade amplification of 12q13.3-14.1 and 12q15 were detected in small subset of tumors in our Swedish cohort of neuroblastoma patients including some with fatal outcome. Genes with an important role in cancer progression that are located in these amplified areas includes *CDK4*, which is involved in the cell cycle and *MDM2*, which is a proto-oncogene involved in p53 regulation.

### Aims:

The aim of the project is to investigate the effect of CDK4 and/or MDM2 inhibition on cell viability in relation to genetic background to determine in which cases these treatments could be beneficial for the patients. Furthermore, we wanted to study the interaction between CDK4 and MDM2 inhibitors to analyze if there is any possibility of a synergetic interaction between them.

### Methods/Materials:

For this project ten NB cell lines were used including two 12q-amplified cell lines. The cell lines were treated with Ribociclib (CDK4 inhibitor), Nutlin-3a (MDM2 inhibitor) or a combination of Ribociclib and Nutlin-3a after 24h seeding in 96 well plates. The viability assays were done with PrestoBlue at different time points (24-48-78h). IC50 analysis was calculated by Prism 8 while the synergy analysis was performed using SynergyFinder. Western blot was used to evaluate the downstream effects of the inhibitors.

### Results:

The viability analyses showed that nine out of ten cell lines response to CDK4 inhibition with Ribociclib, resulting in a 50% cell viability reduction (IC50) after 72h treatment. The resistance of the remaining cell line to Ribociclib treatment, SK-N-FI, could possibly be explained by its high expression of MDR1 (Multidrug Resistance Protein 1).

In the case of MDM2 inhibition through Nutlin-3a, five out of ten cell lines were sensitive to the treatment where 50% cell viability reduction occurred after 24h; the other five cell lines were resistant to Nutlin-3a even after 72h of treatment.

The response of Nutlin-3a was dependent of *TP53* mutation status where all cell lines resistant to the treatment are *TP53* mutated. On the other hand, the sensitive cell lines were *TP53* wild type (wt), except for NGP which is *MDM2* amplified. Further studies of NGP are needed in order to identify if the sensitivity to the treatment is related with the amplification of *MDM2*, if NGP is heterozygous for *TP53* or if the mutant retains some wt functionality.

No significant synergy could be detected with combined Ribociclib and Nutlin-3a treatment.

### Summary/Conclusions:

This study indicates that CDK4 inhibition could provide an efficient treatment for NB, possibly also in patients with *CDK4* amplified tumors and that MDM2 inhibition is a potential treatment option for *TP53*

wt NB. Interestingly, combined CDK4 and MDM2 inhibition does not provide any synergistic effects on the NB cell lines that have been used.

## **P370 Preclinical assessment of MEK inhibitors in combination with chemotherapy in neuroblastoma**

Chen, L.<sup>2</sup>, Ashley, A.L.<sup>3</sup>, O'Neill, E.<sup>4</sup>, Goodman, A.<sup>5</sup>, Bown, N.<sup>6</sup>, Rodriguez, A.<sup>7</sup>, Simko, S.<sup>8</sup>, Tweddle, D.A.<sup>9</sup>

Newcastle University, Wolfson Childhood Cancer Research Centre, Newcastle Upon Tyne, United Kingdom<sup>2</sup>, Newcastle University, Wolfson Childhood Cancer Research Centre, Newcastle Upon Tyne, United Kingdom<sup>3</sup>, Newcastle University, Wolfson Childhood Cancer Research Centre, Newcastle Upon Tyne, United Kingdom<sup>4</sup>, The Newcastle upon Tyne Hospitals NHS Foundation Trust, Northern Genetics Service, Newcastle Upon Tyne, The Newcastle upon Tyne Hospitals NHS Foundation Trust<sup>5</sup>, The Newcastle upon Tyne Hospitals NHS Foundation Trust, Northern Genetics Service, Newcastle Upon Tyne, United Kingdom<sup>6</sup>, F. Hoffmann-La Roche AG, Oncology, Basel, Switzerland<sup>7</sup>, Genentech, Oncology, South San Francisco, Ca, USA<sup>8</sup>, Newcastle University, Wolfson Childhood Cancer Research Centre, Newcastle Upon Tyne, United Kingdom<sup>9</sup>, ,

### Background/Introduction:

Treatment of patients with high-risk neuroblastoma remains clinically challenging. The use of novel small molecule inhibitors targeting oncogenic pathways perturbed in neuroblastoma offers a non-DNA damaging, potentially more effective and less toxic treatment strategy. Aberrations in the RAS/MAPK occur frequently in relapsed neuroblastoma and support the use of MEK inhibitors as a potential novel therapy in high-risk neuroblastoma.

### Aims:

To determine the sensitivity of preclinical models of neuroblastoma to MEK inhibitors alone and in combination with cytotoxic chemotherapies used in the treatment of neuroblastoma.

### Methods/Materials:

Using XTT assays, 72h GI<sub>50</sub> values of MEK inhibitors, trametinib, MEK-162 and cobimetinib were determined in a large panel of neuroblastoma cell lines of varying MAPK pathway status. Synergistic interactions between MEK inhibitors with topotecan or temozolomide were determined in a *KRAS* mutant neuroblastoma cell line using median-effect analysis together with CalcuSyn software. Induction of apoptosis was assessed using Caspase-Glo 3/7 assays. *In vivo* efficacy studies were conducted using a subcutaneous xenograft model of *NRAS* mutant neuroblastoma.

### Results:

Sensitivity to trametinib, MEK-162 and cobimetinib correlated with the presence of a MAPK pathway mutation with cell lines with *RAF/RAS* aberrations being more sensitive than those with *NF1* aberrations. Combinations of MEK inhibitors with topotecan or temozolomide were shown to be synergistic with average CI values at ED<sub>50</sub>, ED<sub>75</sub> and ED<sub>90</sub> within the synergistic range (CI value 0.3-0.7). In support of the latter, all combination treatments led to increased levels of apoptosis as evident by higher levels of caspase 3/7 activity compared to single agent treatment. Consistent with *in vitro* data, assessment *in vivo* demonstrated anti-tumour efficacy of cobimetinib alone and in combinations with cyclophosphamide and topotecan or irinotecan and temozolomide, with combination treatments leading to the greatest tumour growth inhibition. All treatments were well tolerated with no toxicities observed.

### Summary/Conclusions:

These data support the use of MEK inhibitors in combination with chemotherapy as a potential therapeutic option for neuroblastoma patients with aberrations in the MAPK pathway and could be a therapeutic arm of the forthcoming BEACON2 trial.



## **P371 Histone deacetylase inhibition suppresses telomerase activity in neuroblastoma cells harboring TERT rearrangements**

*Finkler, S.<sup>2</sup>, Spruessel, A.<sup>3</sup>, Weiner, J.<sup>4</sup>, Obermayer, B.<sup>5</sup>, Toedling, J.<sup>6</sup>, Schulte, J.H.<sup>7</sup>, Astrahantseff, K.<sup>8</sup>, Ackermann, S.<sup>9</sup>, Fischer, M.<sup>10</sup>, Beule, D.<sup>11</sup>, Henssen, A.G.<sup>12</sup>, Eggert, A.<sup>13</sup>, Lodrini, M.<sup>14</sup>, Deubzer, H.E.<sup>15</sup>*

Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin, Germany<sup>2</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin, Germany<sup>3</sup>, Berlin Institute of Health / Max-Delbrück Center for Molecular Medicine in the H, Core Unit Bioinformatics – CUBI, Berlin, Germany<sup>4</sup>, Berlin Institute of Health / Max-Delbrück Center for Molecular Medicine in the H, Core Unit Bioinformatics – CUBI, Berlin, Berlin Institute of Health / Max-Delbrück Center for Molecular Medicine in the H<sup>5</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin, Germany<sup>6</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin, Germany<sup>7</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin, Germany<sup>8</sup>, University of Cologne, Department of Pediatric Hematology and Oncology, Cologne, Germany<sup>9</sup>, University of Cologne, Department of Pediatric Hematology and Oncology, Cologne, Germany<sup>10</sup>, Berlin Institute of Health / Max-Delbrück Center for Molecular Medicine in the H, Core Unit Bioinformatics – CUBI, Berlin, Germany<sup>11</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin, Germany,<sup>12</sup> Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin, Germany<sup>13</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin, Germany<sup>14</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin, Germany<sup>15</sup>

### Background/Introduction:

Telomerase activation by genomic rearrangements inducing transcriptional upregulation of *TERT* defines a subgroup of high-risk neuroblastomas with poor outcome. Accordingly, telomerase activity presents a high-priority drug target with no currently available clinical candidates.

### Aims:

We aimed to assess the significance of histone deacetylase (HDAC) activity for *TERT* mRNA expression and telomerase activity in *TERT*-rearranged neuroblastoma cells, and estimate the effects of its inhibition by clinically approved drugs.

### Methods/Materials:

Transcriptional changes were analyzed via gene expression profiling. Telomerase activity was assessed with the TeloTAGGG™ Telomerase PCR ELISA<sup>PLUS</sup> assay. Chromatin immunoprecipitation (ChIP) sequencing was performed using the Illumina NEBNext® ChIP-Seq Library Prep Reagent Set and HiSeq 4000 in 50 single-read mode. The Illumina Infinium Human MethylationEPIC Beadchip was used for methylation studies. *De novo* RNA synthesis was blocked through addition of actinomycin D to the cell culture medium. Functional studies were performed in 2D and 3D cell cultures and subcutaneous neuroblastoma xenografts in mice.

### Results:

Changes in *TERT* transcript levels monitored in time-course in CLB-GA and GI-ME-N neuroblastoma cells following treatment with low nanomolar concentrations of panobinostat. *TERT* transcription was repressed after 2 hours of treatment, and remained strongly down-regulated throughout the 120-hour time-course. Treatment with vorinostat, mocetinostat or entinostat, which are either clinically approved or being evaluated in phase II or III clinical trials, similarly suppressed *TERT* transcript levels by 2- to 4.5-fold, supporting *TERT* repression as a common event of HDAC-inhibition in *TERT*-rearranged neuroblastoma cells. We assessed telomerase activity in CLB-GA and GI-ME-N whole-cell lysates to investigate whether *TERT* repression caused by HDAC inhibitor treatment is translated to the effector level, and detected an approximately 2-fold decrease. Transient plasmid-mediated enforced *TERT*

expression partly rescued the anti-tumor effects induced by HDAC inhibitor treatment *in vitro*. Treating NMRI-Foxn1<sup>nu/nu</sup> mice carrying subcutaneous CLB-GA- or GI-ME-N-derived neuroblastoma xenografts with panobinostat in either preventive or therapeutic settings considerably attenuated tumor growth. We assessed *TERT* transcript levels and telomerase activity in these xenograft tumors, and detected a reduction of up to 5-fold. Altogether, our data indicate that HDAC inhibitors, including those in or being tested for clinical use, suppress TERT in TERT-driven neuroblastoma cells *in vitro* and *in vivo*. To test for underlying epigenetic changes, we performed ChIP sequencing in GI-ME-N cells treated with panobinostat or solvent control using antibodies against histone modifications known to mark active or repressive sites as well as transcriptional elongation, and looked for changes in the chromatin context of the *TERT* locus. We have detected no major differences between HDAC inhibitor-treated and control cells in our on-going experiments. Likewise, HDAC inhibition did not substantially alter the methylation profile in the region of interest. Blocking *de novo* RNA synthesis with actinomycin D, however, reduced *TERT* transcript levels by 2-fold in HDAC inhibitor-treated cells compared to controls, suggesting reduced *TERT* transcript stability as the underlying molecular mechanism.

#### Summary/Conclusions:

Our results show high-level telomerase activity caused by genomic rearrangements is suppressed in neuroblastoma cells by treatment with clinically approved HDAC inhibitors, suggesting indirect druggability and a potential novel molecular rationale for therapeutic intervention.

## **P372 Clinical response from entrectinib and ALK-TRK-ROS1 inhibition in treatment-refractory metastatic neuroblastoma with tumor ALK activation and germline ALKAL2 mutation**

*Treis, D.*<sup>2</sup>, *Umapathy, G.*<sup>3</sup>, *Fransson, S.*<sup>4</sup>, *Wessman, S.*<sup>5</sup>, *Gordon, L.*<sup>6</sup>, *Stenman, J.*<sup>7</sup>, *Elfman, L.*<sup>8</sup>, *Hallberg, B.*<sup>9</sup>, *Palmer, R.H.*<sup>10</sup>, *Martinsson, T.*<sup>11</sup>, *Johnsen, J.I.*<sup>12</sup>, *Kogner, P.*<sup>13</sup>

Karolinska institute, Institution for Women's and Children's Health, Stockholm, Sweden <sup>2</sup>, Sahlgrenska Academy, University of Gothenburg, Dept. of Medical Biochemistry and Cell biology, Gothenburg, Sweden<sup>3</sup>, Sahlgrenska Academy, University of Gothenburg, Department of Pathology and Genetics, Gothenburg, Sweden<sup>4</sup>, Karolinska institute, Dept. of Oncology-Pathology, Stockholm, Karolinska institute<sup>5</sup>, Astrid Lindgren Children's Hospital, Karolinska University Hospital, Dept. of Pediatric Radiology, Stockholm, Sweden<sup>6</sup>, Karolinska institute, Dept. of Women's and Children's Health, Stockholm, Sweden<sup>7</sup>, Karolinska institute, Dept. of Women's and Children's Health, Stockholm, Sweden<sup>8</sup>, Sahlgrenska Academy, University of Gothenburg, Dept. of Medical Biochemistry and Cell Biology, Gothenburg, Sweden<sup>9</sup>, Sahlgrenska Academy, University of Gothenburg, Dept. of Medical Biochemistry and Cell Biology, Gothenburg, Sweden<sup>10</sup>, Sahlgrenska Academy, University of Gothenburg, Dept. of Pathology and Genetics, Gothenburg, Sweden<sup>11</sup>, Karolinska institute, Dept. of Women's and Children's Health, Stockholm, Sweden, <sup>12</sup>, Karolinska institute, Dept. of Women's and Children's Health, Stockholm, Sweden <sup>13</sup>

### Background/Introduction:

Activation of the receptor tyrosine kinases ALK or TRKA/B/C is indicted as an oncogenic event in a fraction of neuroblastomas. Their inhibitors are not routinely used in children with neuroblastoma, but have proven very beneficial in some.

### Aims:

We report clinical response in a patient treated with the ALK-TRK-ROS1 inhibitor entrectinib after failing five lines of conventional clinical chemotherapy.

### Methods/Materials:

A six-month-old previously healthy boy presented with vomiting, weight loss, abdominal mass and high arterial blood pressure. He was found to have a poorly differentiated neuroblastoma without *MYCN* amplification but with genetic aberrations including 17q-gain, 1p-deletion, and 2q-gain (involving *MYCN*, ALK and ALKAL2). There were extensive hepatic, pulmonary, and bone metastases. Bone marrow showed 0.1-0.3% involvement. Urine catecholamines were extremely elevated and hypertension necessitated triple pharmacological therapy.

The patient failed to respond initial intermediate-risk treatment (LINES, four courses etoposide/carboplatin followed by four courses cyclophosphamide/doxorubicin/vincristine). COJEC (HR-NBL-1/SIOPEN) did not induce remission but increasing bone marrow involvement. After primary tumor extirpation 9.5 months from diagnosis, metastases remained viable. Celecoxib (220mg/m<sup>2</sup> x2 p.o.) with four courses topotecan/vincristine/doxorubicin followed by two courses temozolamid/topotecan induced only limited incomplete response, remaining extensive metastatic disease and elevated catecholamine markers.

Tumor tissue stained positive for ALK and phosphorylated ALK demonstrating ALK activation, whereas sequencing showed wild-type ALK. However, a constitutional mutation in the ligand ALKAL2 was detected. Moreover, pronounced Trk-activation was detected. To target both RTK-activations, experimental treatment was started with the ALK-TRK-ROS1 inhibitor entrectinib at initial oral dose 200mg/day, increasing to 300mg and 400mg after 10 and 29 months, respectively.

### Results:

The child is alive and well after 39 months of continuous still ongoing oral therapy with entrectinib and celecoxib. General condition and tumor symptoms improved rapidly while extremely elevated urine catecholamines have gradually decreased to normal or near-normal levels. Despite excellent clinical

condition, core-needle biopsy of liver metastases still showed viable poorly differentiated tumor two months after treatment start. However, metastatic extent (number and size) in liver and lungs has continued decreasing.

Entrectinib is well tolerated and pathologic tibiae and fibulae fractures are attributed to previous chemotherapy. Growth, development and social life are completely normal 4.5 years from diagnosis.

Summary/Conclusions:

We report a patient with refractory metastatic neuroblastoma who, in the setting of exhausted therapeutic options, has responded favorably reaching a stable clinical situation from ALK-TRK-ROS1 inhibitor, entrectinib, treatment. To our knowledge, this is the first successful report of ALK inhibitor response in ALK activation possibly mediated by ALK-ligand, ALKAL2, mutation.



### **P373 The therapeutic potential of ERK inhibition in neuroblastoma**

Zhao, Y.,<sup>1</sup> Smith, V.<sup>2</sup>, Yu, Y.<sup>3</sup>, Smith, V.<sup>4</sup>, Yang, J.<sup>5</sup>, Foster, J.<sup>6</sup>

Baylor College of Medicine, Texas Children's Hospital, Pediatrics, Houston, USA<sup>1</sup>, Baylor College of Medicine, Texas Children's Hospital, Pediatrics, Houston, USA<sup>2</sup>, Baylor College of Medicine, Texas Children's Hospital, Pediatrics, Houston, USA<sup>3</sup>, Baylor College of Medicine, Texas Children's Hospital, Pediatrics, Houston, USA<sup>4</sup>, Baylor College of Medicine, Texas Children's Hospital, Pediatrics, Houston, Baylor College of Medicine, Texas Children's Hospital<sup>5</sup>, Baylor College of Medicine, Texas Children's Hospital, Pediatrics, Houston, USA<sup>6</sup>

#### Background/Introduction:

Neuroblastoma (NB) is the most common extracranial malignant solid tumor in children and drug resistance is a major reason for poor outcome of neuroblastoma. Dysregulation of MAPK pathway has been implicated in the pathogenesis of relapsed and refractory NB patients, which underscores the possibility of targeting MAPK signaling cascade as a novel therapeutic strategy. Ulixertinib is a small-molecule ERK1/2 inhibitor and assessment of Ulixertinib in clinical trials is underway in many malignancies.

#### Aims:

To evaluate the effect of ERK inhibition using Ulixertinib on neuroblastoma growth.

#### Methods/Materials:

We first examined the effect of Ulixertinib on NB cell proliferation using a panel of NB cell lines including three *MYCN*-amplified cell lines (NGP, IMR32, and SK-N-BE2) and four non-*MYCN*-amplified cell lines (CHLA255, SH-SY-5Y, SK-N-AS, and LAN-6).  $2.0 \times 10^4$  cells were seeded in each well of a 96-well plate and treated with serial dilutions of Ulixertinib for 48 hours, and cell viability was determined using Cell Counting Kit-8 (CCK-8). Then the soft agar assay was performed to test the inhibitory effect of Ulixertinib on NB cell anchorage-independence growth. A CHLA255 xenograft mouse model was used to determine the effect of Ulixertinib on tumor growth. CHLA255-Fluc cells at the concentration of  $5 \times 10^6$  were injected into NSG mice by tail vein. A week post-injection, the mice were given Ulixertinib 50 mg/kg by intraperitoneal injection every two days. Tumor growth was monitored weekly by bioluminescent imaging.

#### Results:

Ulixertinib inhibited ERK1/2-mediated signaling and suppressed cell proliferation by inducing cell apoptosis. Furthermore, Ulixertinib significantly inhibited NB tumor growth *in vivo*.

#### Summary/Conclusions:

Ulixertinib may be a potential therapeutic agent for NB treatment.

### **P374 The therapeutic potential of a pan-RAF Small molecule inhibitor agerafenib as a treatment option for neuroblastoma patients**

Zhao, Y.,<sup>1</sup>Foster, J.<sup>2</sup>, Li, H.<sup>3</sup>, Yu, Y.<sup>4</sup>, Lu, J.<sup>5</sup>, Chen, Z.<sup>6</sup>, Wu, D.<sup>7</sup>, Zhang, H.<sup>8</sup>, Hu, Y.<sup>9</sup>, Zhai, Y.<sup>10</sup>, Su, J.<sup>11</sup>, Ayinuer, A.<sup>12</sup>, De Las Casas, A.<sup>13</sup>, Shi, Z.<sup>14</sup>, Woodfield, S.<sup>15</sup>, Vasudevan, S.<sup>16</sup>, Agarwal, S.<sup>17</sup>, Yan, Y.<sup>18</sup>, Yang, J.<sup>19</sup>, Foster, J.<sup>20</sup>

Baylor College of Medicine, Texas Children's Hospital, Pediatrics, Houston, USA<sup>1</sup>, Baylor College of Medicine, Texas Children's Hospital, Pediatrics, Houston, USA<sup>2</sup>, Baylor College of Medicine, Texas Children's Hospital, Pediatrics, Houston, USA<sup>3</sup>, Baylor College of Medicine, Texas Children's Hospital, Pediatrics, Houston, USA<sup>4</sup>, Baylor College of Medicine, Texas Children's Hospital, Pediatrics, Houston, Baylor College of Medicine, Texas Children's Hospital<sup>5</sup>, Baylor College of Medicine, Texas Children's Hospital, Pediatrics, Houston, USA<sup>6</sup>, Baylor College of Medicine, Texas Children's Hospital, Pediatrics, Houston, USA<sup>7</sup>, Baylor College of Medicine, Texas Children's Hospital, Pediatrics, Houston, USA<sup>8</sup>, Baylor College of Medicine, Texas Children's Hospital, Pediatrics, Houston, USA<sup>9</sup>, Baylor College of Medicine, Texas Children's Hospital, Pediatrics, Houston, USA<sup>10</sup>, Baylor College of Medicine, Texas Children's Hospital, Pediatrics, Houston, USA<sup>11</sup>, Baylor College of Medicine, Texas Children's Hospital, Surgery, Houston, USA<sup>12</sup>, Baylor College of Medicine, Texas Children's Hospital, Pediatrics, Houston, USA<sup>13</sup>, Baylor College of Medicine, Texas Children's Hospital, Pathology, Houston, USA<sup>14</sup>, Baylor College of Medicine, Texas Children's Hospital, Surgery, Houston, USA<sup>15</sup>, Baylor College of Medicine, Texas Children's Hospital, Surgery, Houston, USA<sup>16</sup>, Baylor College of Medicine, Texas Children's Hospital, Pediatrics, Houston, USA<sup>17</sup>, Baylor College of Medicine, Texas Children's Hospital, Pediatrics, Houston, USA<sup>18</sup>, Baylor College of Medicine, Texas Children's Hospital, Pediatrics, Houston, USA<sup>19</sup>, Baylor College of Medicine, Texas Children's Hospital, Pediatrics, Houston, USA<sup>20</sup>

#### Background/Introduction:

The mitogen-activated protein kinase (MAPK) pathway is a key driver of oncogenicity in many cancers. This pathway transmits growth and mitogen signals to the nucleus via a cascade of specific phosphorylation events involving RAS, RAF, MEK and ERK. Up to 78% of relapsed neuroblastoma samples contain mutations predicted to hyperactivate MAPK signaling. These findings underscore the significance of modulating the MAPK signaling axis for NB treatment.

Agerafenib is a small-molecule pan-RAF inhibitor and assessment of agerafenib in clinical trials for treating cancer is underway. In this study, we examined the therapeutic effect of agerafenib as a treatment option for NB.

#### Aims:

To determine the therapeutic potential of a pan-RAF Small molecule inhibitor agerafenib as a treatment option for neuroblastoma patients

#### Methods/Materials:

We examined the effect of agerafenib on NB cell proliferation using a panel of NB cell lines including three *MYCN*-amplified cell lines (NGP, IMR32, and SK-N-BE(2)) and three non-*MYCN*-amplified cell lines (SH-SY-5Y, SK-N-AS, and LAN-6).  $2.0 \times 10^4$  cells were seeded in each well of a 96-well plate and treated with serial dilutions of agerafenib for 72 hours, and cell viability was determined using Cell Counting Kit-8 (CCK-8). Then the soft agar assay was performed to test the inhibitory effect of agerafenib on NB cell anchorage-independence growth. The NB xenograft and TH-*MYCN* transgenic mouse models were used to determine the therapeutic effect of agerafenib on tumor growth *in vivo*. For the xenograft mouse model, an inoculum of  $1 \times 10^6$  prepared NGP cells was injected into the left renal capsule. Two weeks later, mice bearing tumors with similar sizes (using bioluminescent imaging to monitor tumor growth) were randomly divided into two groups: DMSO control group and agerafenib treatment group (30 mg/kg by intraperitoneal (i.p.) injection once daily for 21 days). At the end of the treatment, all mice were sacrificed. Tumors, together with the right kidneys (control), were harvested and analyzed. For TH-*MYCN* transgenic NB mouse model, double transgenic TH-*MYCN* mice at four

weeks of age, following the development of an abdominal tumor (~1 mm in diameter), were treated daily for 3 consecutive weeks with single intraperitoneal (i.p.) injections of agerafenib at 30 mg/kg or an equal volume of DMSO (controls). Three weeks later, the treatment was stopped and mice were monitored daily for symptoms. Kaplan-Meier survival data were analyzed using a logrank test.

#### Results:

High expressions of RAF family kinases correlated with advanced tumor stage, high-risk disease, tumor progression, and poor overall survival. Targeted inhibition of RAF family kinases with agerafenib abrogated the activation of ERK MAPK pathway in NB cells. Agerafenib significantly inhibited the cell proliferation and colony formation ability of NB cells *in vitro* and its combination with traditional chemotherapy showed a synergistic pro-apoptotic effect. Agerafenib exhibited a favorable toxicity profile, potently suppressed tumor growth, and prolonged survival in orthotopic mouse models.

#### Summary/Conclusions:

Our preclinical data suggest that agerafenib might be an effective therapeutic agent for NB treatment, both as a single-agent and in combination with chemotherapy.

## **P375 Towards optimizing combination therapy in ALK-positive neuroblastoma**

*Gräßer, J.*<sup>2</sup>, *Claudino, N.*<sup>3</sup>, *Westermann, F.*<sup>4</sup>, *Höfer, T.*<sup>5</sup>

German Cancer Research Center (DKFZ), Division of Theoretical Systems Biology, Heidelberg, Germany <sup>2</sup>, German Cancer Research Center (DKFZ), Division of Theoretical Systems Biology, Heidelberg, Germany<sup>3</sup>, Hopp-Children's Cancer Center at the NCT (KITZ), Division of Neuroblastoma Genomics, Heidelberg, Germany<sup>4</sup>, German Cancer Research Center (DKFZ), Division of Theoretical Systems Biology, Heidelberg, German Cancer Research Center (DKFZ)<sup>5</sup>

### Background/Introduction:

Neuroblastoma (NB) is the most common extracranial solid tumor in early childhood with a poor clinical outcome in high-risk patients, underlining the need for novel therapeutic strategies. Recently, the receptor tyrosine kinase ALK (anaplastic lymphoma kinase) has been shown to be a promising therapeutic target in neuroblastoma therapy and small-molecule inhibitors are in clinical use as monotherapies. However, an optimal integration of these therapeutics into the standard first-line chemotherapy has yet to be established.

### Aims:

In this study, we aim at obtaining a general understanding of the inhibitory properties of the 3rd generation ALK inhibitor lorlatinib as monotherapy as well as in the combination treatment with standard chemotherapeutics.

### Methods/Materials:

We determined the response to lorlatinib of a panel of NB cell lines, including ALK amplification, mutation (F1174L and R1275Q) and wild-type, by measuring cell viability in a dose dependent manner. The effect of lorlatinib on ALK downstream targets and cell cycle were determined by western blot and flow cytometry respectively. In addition, we performed RNA-sequencing on a highly sensitive cell line to gain insights into transcriptome changes at lorlatinib treatment. Finally, we tested a combination treatment of lorlatinib with two chemotherapeutics, vincristine and cisplatin, using a selection of our initial cell line panel.

### Results:

In our drug response assay, we observed a high sensitivity to lorlatinib in the ALK amplified cell line NB-1, whereas ALK mutated cell lines showed an intermediate response, wild-type cell lines were non-responders. Thus, an increased ALK expression and activation is essential for an effective lorlatinib treatment which was confirmed by the analysis of the ALK downstream targets AKT and ERK1/2. Furthermore, cell cycle analysis of the responder cell line NB-1 revealed a cell cycle arrest at lorlatinib treatment. This finding was confirmed by our transcriptome analysis for early treatment time points. Finally, combination treatment of lorlatinib with vincristine or cisplatin showed a reduction in viability for the ALK-addicted cell line NB-1. We are currently investigating this combinatorial approach in a 3D spheroid context.

### Summary/Conclusions:

Our data suggests that ALK-addiction and the response to ALK inhibition by lorlatinib is not necessarily linked to the mutation status of ALK and that highly sensitive cells could benefit from a simultaneous combination treatment of lorlatinib with first-line chemotherapy. Further investigation will be necessary to find optimal combination schedules.

## **P376 Systematic target actionability reviews of preclinical proof-of-concept papers to match targeted drugs to paediatric cancers – A pilot study on MDM2 and TP53**

*Schubert, N.A.*<sup>2</sup>, *Lowery, C.D.*<sup>3</sup>, *Bergthold, G.*<sup>4</sup>, *Koster, J.*<sup>5</sup>, *Eleveld, T.F.*<sup>6</sup>, *Rodríguez, A.*<sup>7</sup>, *Jones, D.T.W.*<sup>8</sup>, *Vassal, G.*<sup>9</sup>, *Stancato, L.F.*<sup>10</sup>, *Pfister, S.M.*<sup>11</sup>, *Caron, H.N.*<sup>12</sup>, *Molenaar, J.J.*<sup>13</sup>

Princess Máxima Center for Pediatric Oncology, Research, Utrecht, Netherlands <sup>2</sup>, Eli Lilly and Company, -, Indianapolis, Indiana, USA<sup>3</sup>, Hoffman-La Roche, -, Basel, Switzerland<sup>4</sup>, Amsterdam University Medical Centers, Department of Oncogenomics, Amsterdam, Amsterdam University Medical Centers<sup>5</sup>, Princess Máxima Center for Pediatric Oncology, -, Utrecht, Netherlands<sup>6</sup>, Hoffman-La Roche, -, Basel, Switzerland<sup>7</sup>, Hopp Children's Cancer Center Heidelberg (KiTZ), -, Heidelberg, Germany<sup>8</sup>, Gustave Roussy, Department of Clinical Research, Villejuif, France<sup>9</sup>, Eli Lilly and Company, -, Indianapolis, Indiana, USA<sup>10</sup>, Hopp Children's Cancer Center Heidelberg (KiTZ), -, Heidelberg, Germany<sup>11</sup>, Hoffman-La Roche, -, Basel, Switzerland, <sup>12</sup>, Princess Máxima Center for Pediatric Oncology, Research, Utrecht, Netherlands <sup>13</sup>

### Background/Introduction:

Children with cancer are in urgent need of new therapies, as approximately 25% of patients experience a relapse and 20% succumb to their disease. Repurposing of targeted agents developed for adult indications could provide novel therapeutic options for paediatric cancer patients.

### Aims:

To prioritise targeted drugs for paediatric clinical development and efficiently guide clinical development for novel targeted agents, we applied systematic review methodology to develop a Target Actionability Review (TAR) strategy. These TARs assess the strength and completeness of published preclinical proof-of-concept (PoC) data by structured critical appraisal of and summarizing the available scientific literature for a specific target (or pathway) and the associated drugs in paediatric tumours.

### Methods/Materials:

The TAR strategy involves a sensitive literature search in PubMed and the selection of relevant papers. For each paper, the individual experimental findings are extracted, marked for paediatric tumour type, and categorised into nine separate PoC data modules. Each experimental finding is scored for experimental outcome and quality independently by two reviewers; discrepancies are assessed by a third reviewer and resolved by adjudication. Scores corresponding to one PoC module are merged for each tumour type and visualised in a heatmap matrix in the publicly available R2 data portal [r2.amc.nl].

### Results:

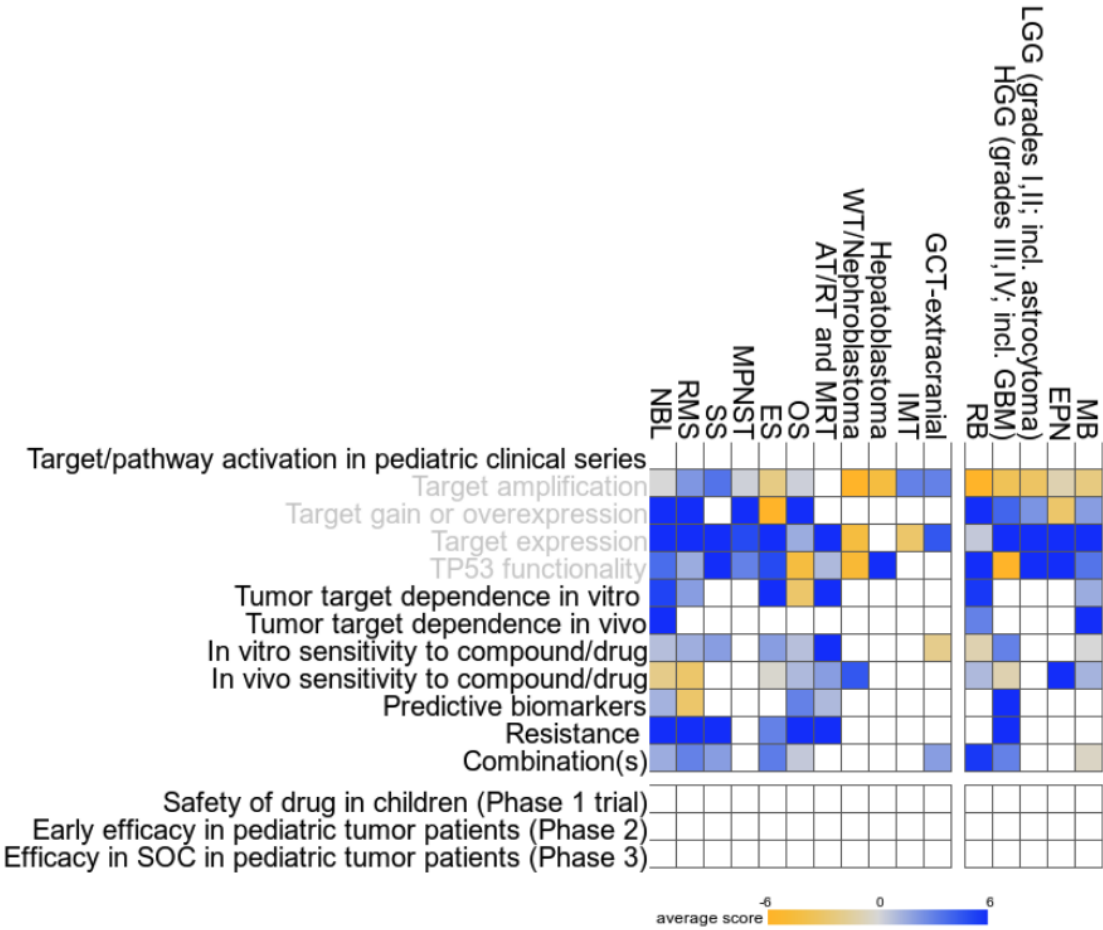
To test our TAR methodology, we conducted a pilot study on MDM2 and *TP53*.

The search terms [*“paediatric cancer type” AND (MDM2 OR HDM2)*] and [*“paediatric cancer type” AND nutlin*] identified 726 unique papers, 161 of which met our inclusion criteria. Another 12 next-generation sequencing studies were added to assess the mutational status of *TP53* in paediatric cancer, as *TP53* mutations generally lead to resistance to MDM2 inhibitors. *MDM2* amplifications were frequently found in rhabdomyosarcoma, whereas they seem to be absent in paediatric brain tumours. Gain or overexpression of *MDM2* occurs across all studied tumour types. *TP53* mutations are frequent in osteosarcoma, Wilms tumour and glioblastoma. *in vitro* inhibition of *MDM2* was relatively effective in all included tumour entities. However, most *in vivo* studies revealed merely reduced tumour growth and only a few reported stable disease or complete response. Long-term nutlin treatment was studied in neuroblastoma, rhabdomyosarcoma and osteosarcoma and resulted in acquired *TP53* mutations. Moreover, this pilot TAR reveals that it may be beneficial to combine *MDM2* inhibitors with chemotherapy or other drugs.

### Summary/Conclusions:

The heatmap generated from analysis of 173 publications on MDM2 and TP53 can be used to identify mechanism-of-action-based matches between MDM2 inhibitors and specific cancer subtypes. This TAR supports clinical evaluation of MDM2 inhibitors in neuroblastoma, rhabdomyosarcoma and high grade gliomas. Our systematic review also revealed a lack of studies investigating the role of MDM2 in paediatric tumourigenesis and predictive biomarkers as well as resistance mechanisms in most indications. Furthermore, our review highlights tumor types (such as malignant peripheral nerve sheath tumours, rhabdoid tumours, hepatoblastoma and inflammatory myofibroblastic tumours) where preclinical data is incomplete or lacking and for which additional preclinical testing is advisable. The TAR strategy is now the standard for systematic reviews on target actionability within the ITCC-P4 consortium.

**MDM2-TP53 Target Actionability Review**  
14 May 2019



Caption 1: MDM2/TP53 TAR heatmap

## **P377 MEK1/2 IS A TARGET OF PRIMA-1<sup>MET</sup>-INDUCED NEUROBLASTOMA CELL DEATH**

*Mrakar, V.*<sup>2</sup>, *Jurkovic Mrakar, S.*<sup>3</sup>, *Lesne, L.*<sup>4</sup>, *Marino, D.*<sup>5</sup>, *Boudal Khoshbeen, M.*<sup>6</sup>, *Maris, J.M.*<sup>7</sup>, *Ansari, M.*<sup>8</sup>, *Gumy-Pause, F.*<sup>9</sup>

Geneva University Medical School, CANSEARCH Research Laboratory, Geneva, Switzerland<sup>2</sup>,  
Geneva University Medical School, CANSEARCH Research Laboratory, Geneva, Switzerland<sup>3</sup>,  
Geneva University Medical School, CANSEARCH Research Laboratory, Geneva, Switzerland<sup>4</sup>,  
Geneva University Medical School, CANSEARCH Research Laboratory, Geneva, Geneva University  
Medical School<sup>5</sup>, Geneva University Medical School, CANSEARCH Research Laboratory, Geneva,  
Switzerland<sup>6</sup>, Children's Hospital of Philadelphia, Division of Oncology and Center for Childhood  
Cancer Research, Philadelphia, USA<sup>7</sup>, Geneva University Hospital, Pediatric Department, Onco-  
Hematology Unit, Geneva, Switzerland<sup>8</sup>, Geneva University Hospital, Pediatric Department, Onco-  
Hematology Unit, Geneva, Switzerland<sup>9</sup>

### Background/Introduction:

Neuroblastoma has a low frequency of *TP53* mutations, and its downstream pathway is usually intact. We previously demonstrated the efficacy of PRIMA-1<sup>MET</sup>, thought to be p53 activator, against neuroblastoma, either alone or in combination with common clinical chemotherapeutics.

### Aims:

The present study assessed p53's involvement in PRIMA-1<sup>MET</sup> action and which other cellular pathways are implicated in the response.

### Methods/Materials:

CellTiter 2.0 was used to determine IC<sub>50</sub>. pcDNA3.1(+) plasmid was used to over-express p53 in LA1-55N (MNA, p53-null cells). JQ1 was used to decrease the levels of *MYCN* and p53 in LA1-55N and CLB-GA (non-MNA) cells, respectively. A Proteome Profiler Array Human Phospho-Kinase Array Kit was used to assess activation of the most common intra-cellular signaling pathways. Subsequently, western blot analysis was used to confirm and detail these results.

### Results:

LA1-55N cells expressing p53 were significantly more resistant to PRIMA-1<sup>MET</sup> and contained significantly more GSH than normal LA1-55N cells. JQ1 decreased the expression of both *MYCN* (LA1-55N) and p53 (CLB-GA), and it decreased intracellular GSH. JQ1 synergized with PRIMA-1<sup>MET</sup> in LA1-55N (p53-null cells) and in CLB-GA (non-MNA) cells. Analysis of cellular signaling pathways demonstrated the involvement of the MEK/ERK pathway. PRIMA-1<sup>MET</sup> inhibited MEK1/2 signaling. Co-treatments with PRIMA-1<sup>MET</sup> and several MEK/ERK pathway inhibitors in BE-2C, CHP212, NBL-S, NGP and SK-N-DZ, demonstrated good synergies for Crizotinib (ALKi) and trametinib (MEK1/2i), and a mixed response for U0126 (MEK1/2i) and SCH772984 (ERK1/2i).

### Summary/Conclusions:

Previous and current results suggest that p53 is indirectly involved in PRIMA-1<sup>MET</sup>-mediated cell death. This occurs through p53's modulation of GSH levels rather than direct interaction between PRIMA-1<sup>MET</sup> and p53. We identified the MEK/ERK pathway as a major target for PRIMA-1<sup>MET</sup> as it is a potent inhibitor of MEK1/2. Synergistic action with other inhibitors of the MEK/ERK signaling pathway was confirmed, and this could present a novel and efficient option for neuroblastoma treatment in the future.

## **P378 ROCK inhibition induces changes in the neuroblastoma tumor microenvironment**

*Pepich, A.<sup>2</sup>, Karin, K.<sup>3</sup>, Tümmler, C.<sup>4</sup>, Dyberg, C.<sup>5</sup>, Andonova, T.<sup>6</sup>, Kogner, P.<sup>7</sup>, Johnsen, J.I.<sup>8</sup>, Baryawno, N.<sup>9</sup>, Näsman, A.<sup>10</sup>, Wickström, M.<sup>11</sup>*

Karolinska Institutet, Women and Children's Health, Stockholm, Sweden <sup>2</sup>, Karolinska Institutet, Medicine, Stockholm, Sweden<sup>3</sup>, Karolinska Institutet, Women and Children's Health, Stockholm, Sweden<sup>4</sup>, Karolinska Institutet, Women and Children's Health, Stockholm, Karolinska Institutet<sup>5</sup>, Karolinska Institutet, Women and Children's Health, Stockholm, Sweden<sup>6</sup>, Karolinska Institutet, Women and Children's Health, Stockholm, Sweden<sup>7</sup>, Karolinska Institutet, Women and Children's Health, Stockholm, Sweden<sup>8</sup>, Karolinska Institutet, Women and Children's Health, Stockholm, Sweden<sup>9</sup>, Karolinska Institutet, Women and Children's Health, Stockholm, Sweden<sup>10</sup>, Karolinska Institutet, Women and Children's Health, Stockholm, Sweden<sup>11</sup>

### Background/Introduction:

We have shown that every fourth neuroblastoma patient presents with at least one mutation or structural aberration associated with the Rho/Rac signaling pathway; a pathway implicated in neural crest differentiation and migration. These mutations promote a more active state of Rho, which is believed to result in an increased downstream Rho-associated kinase (ROCK) activation. ROCK has previously been identified as a promising drug target to inhibit cancer growth and metastatic spread. Furthermore, Rho/ROCK signaling pathway effects on cytoskeleton arrangement and cell shape have been suggested to also be involved in tumor promoted modulation of the tumor microenvironment (TME).

### Aims:

Here we have explored ROCK as a therapeutic target in neuroblastoma with emphasis on its' influence on the TME and immune response.

### Methods/Materials:

We evaluated two different ROCK inhibitors, RKI-1447 and HA-1077, in a 2D cell viability assay (WST-1) and in a multi-cellular tumor spheroid (MCTS) model with co-cultured fibroblasts and tumor cells. We also performed immunohistochemistry (IHC) staining on cells within the TME after treatment with HA-1077 in tumors from the transgenic neuroblastoma model TH-*MYCN* and quantified infiltrating cells.

### Results:

Using two different ROCK inhibitors, we demonstrated that ROCK inhibition suppressed neuroblastoma growth both in a 2D cell viability assay and in a MCTS model with co-cultured fibroblasts and tumor cells. The MCTS 3D tumor model is preferred for better modeling of physiological drug distribution, cell-to-cell interactions, and the TME. In the MCTS model we did not observe an effect of the two ROCK inhibitors on the fibroblasts at concentrations used in this study, but noted evident repressed tumor cell growth. In addition, we monitored populations of T cells (CD3<sup>+</sup>, CD4<sup>+</sup> and CD8<sup>+</sup>), macrophages (F4/80<sup>+</sup>) and fibroblasts ( $\alpha$ -SMA<sup>+</sup>) within ROCK inhibitor, HA-1077, treated and untreated tumor tissue from homozygous TH-*MYCN* mice. Treatment with HA-1077 significantly inhibited tumor growth in homozygous TH-*MYCN* mice. Through quantifying the number of infiltrating cells in ROCK inhibitor treated tumor tissue compared to untreated tumor tissue we observed a significant increase in CD3<sup>+</sup> and CD8<sup>+</sup> T cells following ROCK inhibition.

### Summary/Conclusions:

This shift in CD8<sup>+</sup> T cells indicates a shift toward a cytotoxic T cell immune response following ROCK inhibition in our neuroblastoma tumor tissue samples. Further experimentation is necessary to verify these results; investigations are ongoing. We hope that these studies can reveal new insights into the Rho/ROCK signaling pathway and involvement of the TME in neuroblastoma.





### **P379 Alk mutations - a promessing future in target therapy**

*Hally Neves, N.S.<sup>2</sup>, VINCE, C.S.C.<sup>3</sup>, AZAMBUJA, A.M.P.<sup>4</sup>, BRUMATTI, M.<sup>5</sup>, PEREIRA, P.L.<sup>6</sup>, NOVAK, E.M.<sup>7</sup>, GIMENEZ, T.M.<sup>8</sup>, KREPISCHI, A.V.C.<sup>9</sup>, PARISI-DUTRA, M.<sup>10</sup>, BARRETO, J.S.<sup>11</sup>, DUTRA, A.P.<sup>12</sup>, CRISTOFANI, L.M.<sup>13</sup>, ODONE-FILHO, V.<sup>14</sup>*

ITACI - INSTITUTO TRATAMENTO CANCER INFANTIL-HC FMUSP, Oncology pediatric, Sao Paulo, Brazil <sup>2</sup>, ITACI - INSTITUTO TRATAMENTO CANCER INFANTIL-HC FMUSP, Oncology pediatric, Sao Paulo, Brazil<sup>3</sup>, ITACI - INSTITUTO TRATAMENTO CANCER INFANTIL-HC FMUSP, Oncology pediatric, Sao Paulo, Brazil<sup>4</sup>, ITACI - INSTITUTO TRATAMENTO CANCER INFANTIL-HC FMUSP, ONCOLOGY PEDIATRIC, Sao Paulo, ITACI - INSTITUTO TRATAMENTO CANCER INFANTIL-HC FMUSP<sup>5</sup>, ITACI - INSTITUTO TRATAMENTO CANCER INFANTIL-HC FMUSP, ONCOLOGY PEDIATRIC, Sao Paulo, Brazil<sup>6</sup>, ITACI - INSTITUTO TRATAMENTO CANCER INFANTIL-HC FMUSP, MOLECULAR LABORATORY, Sao Paulo, Brazil<sup>7</sup>, ITACI - INSTITUTO TRATAMENTO CANCER INFANTIL-HC FMUSP, MOLECULAR LABORATORY, Sao Paulo, Brazil<sup>8</sup>, ITACI - INSTITUTO TRATAMENTO CANCER INFANTIL-HC FMUSP, MOLECULAR LABORATORY, Sao Paulo, Brazil<sup>9</sup>, ITACI - INSTITUTO TRATAMENTO CANCER INFANTIL-HC FMUSP, ONCOLOGY PEDIATRIC, Sao Paulo, Brazil<sup>10</sup>, ITACI - INSTITUTO TRATAMENTO CANCER INFANTIL-HC FMUSP, ONCOLOGY PEDIATRIC, Sao Paulo, Brazil<sup>11</sup>, ITACI - INSTITUTO TRATAMENTO CANCER INFANTIL-HC FMUSP, ONCOLOGY PEDIATRIC, Sao Paulo, Brazil, <sup>12</sup>, ITACI - INSTITUTO TRATAMENTO CANCER INFANTIL-HC FMUSP, ONCOLOGY PEDIATRIC, Sao Paulo, Brazil <sup>13</sup>, ITACI - INSTITUTO TRATAMENTO CANCER INFANTIL-HC FMUSP, PEDIATRIC ONCOLOGY, Sao Paulo, Brazil <sup>14</sup>

#### **Background/Introduction:**

A vast array of oncogenic variants has been identified for anaplastic lymphoma kinase (*ALK*). Therefore, there is a need to better understand the role of *ALK* in cancer biology to optimize treatment strategies.

In tumors where the full-length *ALK* itself is mutated, such as neuroblastoma, the picture regarding the role of *ALK* as an oncogenic driver is less clear. The discovery of germline missense point mutations in the intact *ALK* oncogene related to the etiology of familial neuroblastoma, and of somatically acquired mutations that predict for inferior outcome in patients with the most aggressive form of this disease, has positioned *ALK* as the most important mutated oncogene tractable for targeted therapy in this disease.

We described a case of 4-year old child with stage 4 neuroblastoma, amplified *MYCN*, with an at least uncommon fusion *ALK-MYC* and *OTOF-ALK* which may have conferred an extremely aggressive disease. The patient died after 6 months of diagnosis, despite the use of Crizotinib.

#### **Aims:**

To describe an aggressive fusion of *ALK* mutation which may be suitable as a target therapy once it is found in the tumor sample.

#### **Methods/Materials:**

A sample obtained from a cervical lymph node metastasis was analyzed by NGS method using the kit for detection OptiView DAB IHC and amplification OptiView.

#### **Results:**

After diagnosis of Neuroblastoma, patient received chemotherapy as high risk protocol, with continued aggressive progression of disease even after alternative schedules. As soon as the NGS results were available, Crizotinib was started. Unfortunately, the child died with progressive disease after 1 month.

#### **Summary/Conclusions:**

Neuroblastoma is the most common cancer diagnosed during the first year of life. Many clinical, biological, genetic and biochemical factors are associated with worse prognosis. This case addresses many of the current issues surrounding the role of *ALK* mutations and neuroblastoma and discusses the prospects for clinically effective targeted treatments based on mutated *ALK* inhibition.

## **P380 Lutetium-177-DOTATATE treatment for relapsed/refractory neuroblastoma patients**

*Basu, E.<sup>2</sup>, Modak, S.<sup>3</sup>, Pandit-Taskar, N.<sup>4</sup>*

Memorial Sloan Kettering Cancer Center, Pediatrics, New York, USA <sup>2</sup>, Memorial Sloan Kettering Cancer Center, Pediatrics, New York, USA<sup>3</sup>, Memorial Sloan Kettering Cancer Center, Radiology, New York, USA<sup>4</sup>

### Background/Introduction:

Despite improved outcomes for high-risk neuroblastoma (NB) patients, survival is still low for patients with refractory and/or relapsed disease, and additional treatments are needed. Since most neuroblastoma tumors express somatostatin receptor-2 (SSTR-2), SSTR-2 analogues such as DOTATATE can be used for imaging and targeted therapies. Lutetium-177-DOTATATE is FDA approved for treatment of gastrointestinal neuroendocrine tumors in adults which also express SSTR-2 and can be considered for treatment of relapsed/refractory NB patients.

### Aims:

To describe Lu-177-DOTATATE treatment in patients with relapsed/refractory NB.

### Methods/Materials:

Patients with relapsed/refractory NB were identified for possible treatment with Lu-177-DOTATATE based on the following parameters: Gallium-68-DOTATE PET scan avidity, major organ toxicities  $\leq$  grade 2, adequate bone marrow function (platelets  $>50K$ , ANC  $>500/ul$ ), and availability of stored autologous peripheral blood stem cells. Treatment was administered in the outpatient clinic following radiation safety guidelines. Patients received pre-medication of antiemetics followed by an infusion of renal protective amino acids (2.5% Lysine + Arginine) at the rate of 250 mL/hour starting at least 30 minutes prior to Lu-177-DOTATATE. Lu-177-DOTATATE was infused over 30 minutes using a Graseby syringe pump. Patients and their families received verbal and written instructions regarding radiation safety restrictions. Up to four doses of Lu-177-DOTATATE were administered at 8-10 week intervals.

### Results:

Five patients with multiply relapsed NB, (median 4 relapses and 7 prior therapies), ages 4.5, 6, 10, 11 and 38 years, received 9 doses of Lu-177-DOTATATE. One patient received 4 doses, another 2 doses and the other 3 patients received 1 dose each. Administered dose ranged between 100- 200 mCi/administration. The infusions were well tolerated with grade 1 nausea with one of 9 treatments and no episodes of emesis. Weekly laboratory evaluations did not reveal changes in renal function up to the last follow up (3 months - 1.5 years). There was no  $\geq$  grade 2 organ or hematologic toxicity except grade 2 thrombocytopenia noted after 3rd dose in the patient who received 4 doses of Lu-177-DOTATATE. One patient had significant clinical improvement (less pain and less pain medication usage) after initial dose with Ga-68-DOTATATE PET scan that was stable disease (SD) by International Neuroblastoma Response Criteria (INRC) yet had  $>40\%$  reduction of soft tissue masses and decrease in extent and avidity by SUV of all osseous lesions. However, this patient had disease progression after the 4th dose. One patient had a mixed response after the first dose and progression after the second dose. Two patients had progression of disease clinically and on scans after the first dose. The 5th patient had a clinical response with decrease of pain and has yet to have a follow up scan.

### Summary/Conclusions:

Lu-177-DOTATATE is a feasible outpatient treatment with minimal toxicities for patients with relapsed/refractory NB. Further treatments on phase I trials are necessary for dose discovery in children with NB.



### **P381 The dual PLK1-/PI3K inhibitor Rigosertib shows antitumoral activity in preclinical neuroblastoma models**

Szymansky, A.<sup>2</sup>, Witthauer, M.J.<sup>3</sup>, Guergen, D.<sup>4</sup>, Firle, K.A.<sup>5</sup>, Huebener, N.<sup>6</sup>, Dorado García, H.<sup>7</sup>, Ahrens, K.<sup>8</sup>, Künkele, A.<sup>9</sup>, Lissat, A.<sup>10</sup>, Henssen, A.G.<sup>11</sup>, Toedling, J.<sup>12</sup>, Hertwig, F.<sup>13</sup>, Eggert, A.<sup>14</sup>, Schulte, J.H.<sup>15</sup>

Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin, Germany<sup>2</sup>, Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin, Germany<sup>3</sup>, Experimental Pharmacology & Oncology Berlin-Buch GmbH, Neuroblastoma In Vivo Models, Berlin, Germany<sup>4</sup>, Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin, Charité – Universitätsmedizin Berlin<sup>5</sup>, Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin, Germany<sup>6</sup>, Experimental and Clinical Research Center (ECRC) of the Charité and MDC, Clinical research, Berlin, Germany<sup>7</sup>, Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin, Germany<sup>8</sup>, Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin, Germany<sup>9</sup>, Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin, Germany<sup>10</sup>, Experimental and Clinical Research Center (ECRC) of the Charité and MDC, Clinical research, Berlin, Germany<sup>11</sup>, Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin, Germany,<sup>12</sup> Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin, Germany<sup>13</sup>, Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin, Germany<sup>14</sup>, Charité – Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology/BMT, Berlin, Germany<sup>15</sup>

#### Background/Introduction:

High-risk neuroblastoma responds poorly to conventional chemotherapy and overall survival of patients with high-risk neuroblastoma is below 50%. New targeted therapeutics promise to be a solution for individual patients, yet recent studies have shown that tumors can rapidly develop resistance to single-target drugs. The multi-kinase inhibitor Rigosertib suppresses phosphoinositide 3-kinase (PI3K) pathway activation as well as polo-like kinase 1 (PLK1) activity. Both, *PLK1* and *PI3K*, are expressed at elevated levels in high-risk neuroblastoma, and high expression correlates with unfavorable disease outcome. Small molecule inhibitors of either PI3K or PLK1 have previously been shown to have specific antitumoral efficacy in neuroblastoma. These earlier studies report that single inhibition of either target reduced cell viability, proliferation, caused cell cycle arrest, and massively induced apoptosis.

#### Aims:

We have investigated the response of preclinical models of high-risk neuroblastoma to Rigosertib, a dual pathway inhibitor.

#### Methods/Materials:

Rigosertib was applied to neuroblastoma cell lines at various concentrations. Its effect on cell viability was measured applying a luminescence assay. Wound healing and clonogenic assays were used to investigate the effect of Rigosertib on migration and cell-cell interactions. Using qPCR and Western blot experiments, we investigated the effect of Rigosertib on PI3K-PLK1 pathway proteins. Furthermore, we functionally characterize the cellular effects of dual PI3K-PLK1 pathway interference in neuroblastoma cells, focusing on proliferation, apoptosis and differentiation.

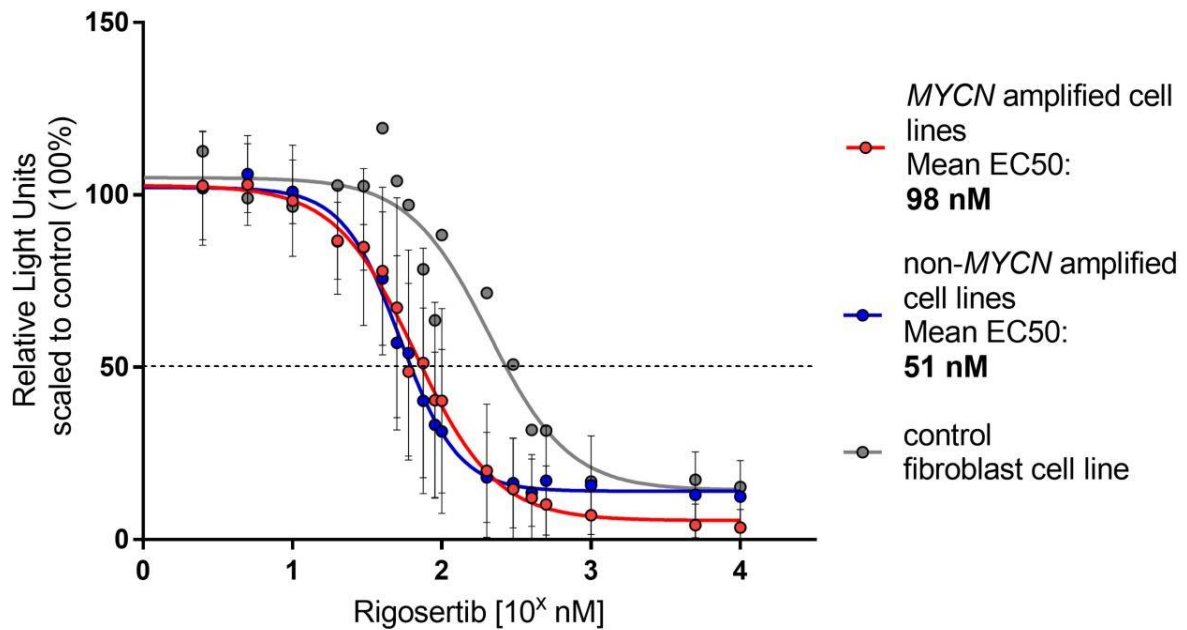
#### Results:

Treatment of a variety of neuroblastoma cell lines with Rigosertib exhibited potent antiproliferative effects at nanomolar concentrations (IC<sub>50</sub>: 30nM - 238nM). Wound healing and colony formation are strongly impeded by application of Rigosertib. Analyses of PLK1, PIK3 and corresponding pathway

components on transcript as well as protein level validated the target-specific mode of action in response to the Rigosertib treatment.

Summary/Conclusions:

Our results indicate that Rigosertib is a highly effective multikinase inhibitor *in-vitro*, which warrants further preclinical evaluation in *in-vivo* neuroblastoma models. Combinations of Rigosertib and classical chemotherapy as well other small molecule inhibitors or targeted CAR-T-cell therapy may be a promising approach for treating high-risk neuroblastoma.



Caption 1: Three days after treatment with Rigosertib, Neuroblastoma cells show reduced cell viability at nanomolar concentrations.

## **P382 Biomarker Analysis for checkpointkinase 1 inhibitor prexasertib in *in vitro* high-risk neuroblastoma models**

Keller, K.M.<sup>2</sup>, Koopmans, B.<sup>3</sup>, Essing, A.<sup>4</sup>, Van den Boogaard, T.L.<sup>5</sup>, Dolman, M.E.M.<sup>6</sup>, Molenaar, J.J.<sup>7</sup>

Prinses Máxima Centrum, Research, Utrecht, Netherlands <sup>2</sup>, Prinses Máxima Centrum, Research, Utrecht, Netherlands<sup>3</sup>, Prinses Máxima Centrum, Research, Utrecht, Netherlands<sup>4</sup>, Prinses Máxima Centrum, Research, Utrecht, Prinses Máxima Centrum<sup>5</sup>, Prinses Máxima Centrum, Research, Utrecht, Netherlands<sup>6</sup>, Prinses Máxima Centrum, Research, Utrecht, Netherlands<sup>7</sup>

### Background/Introduction:

Checkpoint kinase 1 (chk1) is a serine/threonine protein kinase that is essential for DNA damage repair and cell cycle checkpoint regulation. Ultimately chk1 activation plays a role in genomic integrity by helping to prevent propagation of cells with DNA damage. Prexasertib (LY2606368)-a small molecule chk1 inhibitor in clinical development for adult and pediatric solid tumors-has been shown to potentiate DNA damage and induce cytotoxic effects. Additionally, mRNA analysis of high-risk neuroblastoma (HR-NB) tumors indicates that low chk1 expression gives an overall survival advantage for NB patients. Taken altogether, chk1 inhibition by prexasertib is a novel therapeutic approach that demands further *in vitro* and *in vivo* investigation for use in the treatment of HR-NB.

### Aims:

The aims of the project are to a) further validate prexasertib as a therapeutic approach for NB, b) identify synergistic drug combinations with prexasertib and c) establish a reliable clinical biomarker for prexasertib combination therapy to ensure the development of high-quality clinical trials.

### Methods/Materials:

High-throughput screening of 23 NB classical cell lines and 10 NB organoids using a drug library containing 170 compounds that are clinically approved or currently in clinical development was completed to identify new therapeutic approaches. Western blot and flow cytometry were used to further validate prexasertib treatment in NB cells and reporter cell lines were developed to aid in the identification of potential biomarkers. Additional high-throughput screens of 10 compounds combined with prexasertib were completed to identify synergistic combinations.

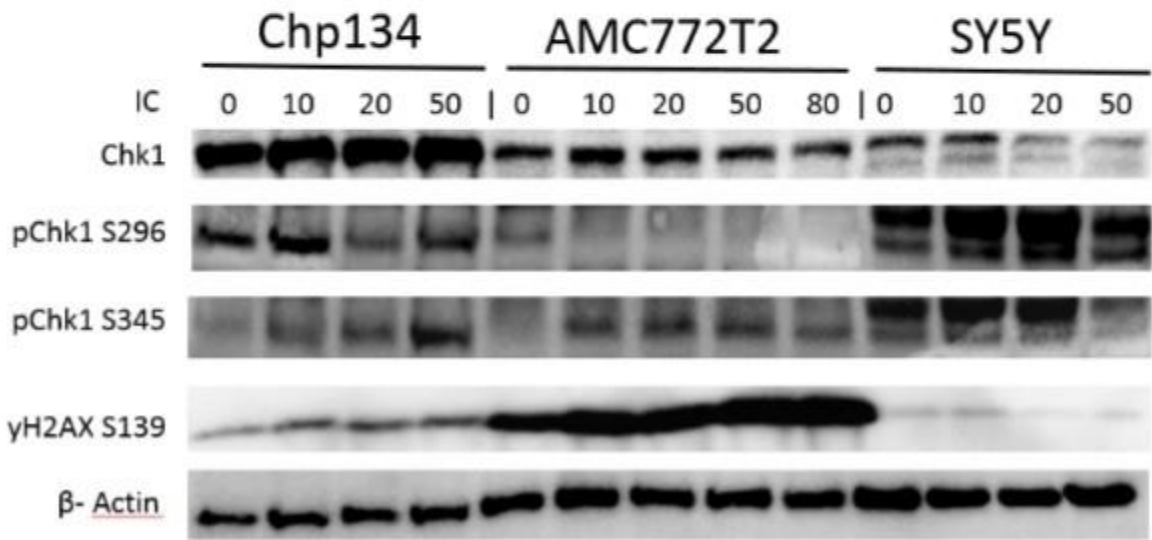
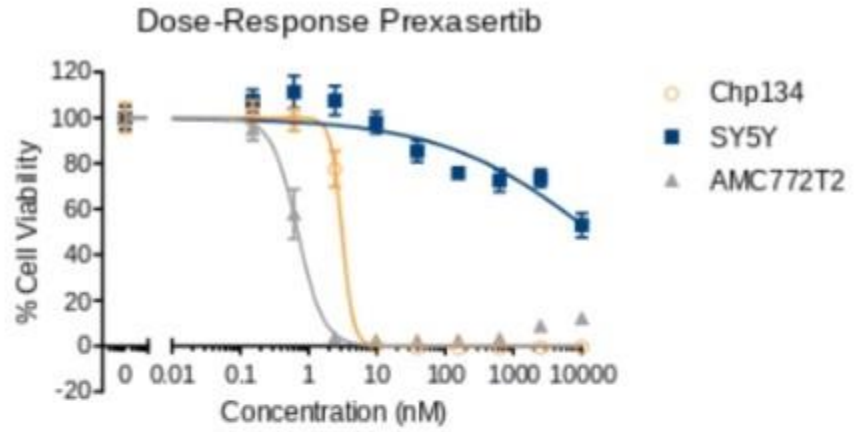
### Results:

High-throughput screening of NB classical cell lines and NB organoids demonstrates high *in vitro* sensitivity to prexasertib treatment. The observed IC50 values for prexasertib are well within the clinically achievable range that has been reported in literature. Further validation of prexasertib demonstrates that NB sensitivity to chk1 inhibition is related to baseline levels of DNA damage and that DNA repair defects/replication stress can potentiate these effects. As such, players within the DNA repair pathways are investigated as potential therapeutic biomarkers and we included DNA damaging agents as well as small molecule inhibitors targeting DNA repair pathways in synergy screens.

### Summary/Conclusions:

Our study supports further pre-clinical development of prexasertib as a therapeutic approach for the treatment of NB. Additionally, we show that existing or induced DNA damage can act synergistically with chk1 inhibition and that this DNA damage could potentially be used as a clinical biomarker.





Caption 1: Figure 1- Prexasertib dose-response curves and western blot analysis of relevant proteins

### **P383 *In vitro* and in vivo validation of combination strategies with Venetoclax for neuroblastoma**

Vernooij, L.<sup>2</sup>, Bate-Eya, L.T.<sup>3</sup>, Eleveld, T.F.<sup>4</sup>, Alles, L.K.<sup>5</sup>, Koopmans, B.<sup>6</sup>, Lee, J.<sup>7</sup>, Schild, G.G.<sup>8</sup>, Ober, K.<sup>9</sup>, Lankhorst, L.H.<sup>10</sup>, Jonus, K.H.C.<sup>11</sup>, Koster, J.J.B.<sup>12</sup>, Goldsmith, K.C.<sup>13</sup>, Molenaar, J.J.<sup>14</sup>, Dolman, M.E.M.<sup>15</sup>

Princess Máxima Center for Pediatric Oncology, Research, Molenaar group, Utrecht, Netherlands <sup>2</sup>, University of Oxford, Department of Oncology, Oxford, United Kingdom<sup>3</sup>, Amsterdam UMC, location VUmc, Cancer Center Amsterdam, Tumor Genome Analysis Core, Amsterdam, Netherlands<sup>4</sup>, Princess Máxima Center for Pediatric Oncology, Research, Molenaar group, Utrecht, Princess Máxima Center for Pediatric Oncology<sup>5</sup>, Princess Máxima Center for Pediatric Oncology, Research, Molenaar group, Utrecht, Netherlands<sup>6</sup>, Emory University School of Medicine, Department of Pediatrics, Oncology, Atlanta, USA<sup>7</sup>, Princess Máxima Center for Pediatric Oncology, Research, Molenaar group, Utrecht, Netherlands<sup>8</sup>, Princess Máxima Center for Pediatric Oncology, Research, Molenaar group, Utrecht, Netherlands<sup>9</sup>, Princess Máxima Center for Pediatric Oncology, Research, Molenaar group, Utrecht, Netherlands<sup>10</sup>, Emory University School of Medicine, Department of Pediatrics, Oncology, Atlanta, USA<sup>11</sup>, Amsterdam UMC, location AMC, Department of Oncogenomics, Amsterdam, Netherlands, <sup>12</sup>, Emory University School of Medicine, Department of Pediatrics, Oncology, Atlanta, USA <sup>13</sup>, Princess Máxima Center for Pediatric Oncology, Research, Molenaar group, Utrecht, Netherlands <sup>14</sup>, Children's Cancer Institute, -, Sydney, Australia <sup>15</sup>

#### Background/Introduction:

The majority of neuroblastoma patients overexpress the anti-apoptotic protein B cell lymphoma/leukemia 2 (BCL-2). We previously showed that the BCL-2 inhibitor Venetoclax causes programmed cell death in BCL-2-dependent neuroblastoma, which contributed to the initiation of a phase I trial to study the safety and pharmacokinetics of Venetoclax in children with relapsed or refractory neuroblastoma. Unfortunately, Venetoclax monotherapy only led to tumor growth stabilization and not to complete tumor regression. This was in line with previous *in vitro* and *in vivo* studies showing resistance development after Venetoclax treatment.

#### Aims:

In the current study, we aim at the identification and validation of more effective targeted combination strategies to prevent or overcome neuroblastoma resistance to Venetoclax.

#### Methods/Materials:

Two strategies were followed to identify effective Venetoclax combination options for neuroblastoma patients. First, we focused on finding more personalized combination therapies for subgroups of BCL-2-dependent neuroblastoma patients with additional ALK mutations or RAS-MAPK pathway aberrations. Neuroblastoma model systems harboring these genomic backgrounds were exposed to a library of targeted compounds in combination with a series of Venetoclax concentrations to determine synergistic interactions with Venetoclax. Secondly, approaches to overcome Venetoclax resistance were studied by directly targeting the MCL-1-driven resistance mechanism and by performing an additional high-throughput drug screen. In the latter study, non-resistant and Venetoclax-resistant neuroblastoma cells were exposed to a drug library to identify compounds that were more effective in Venetoclax-resistant cells. Taken together, these strategies revealed promising combination therapies with Venetoclax that were further validated *in vitro* and *in vivo* using BCL-2-dependent neuroblastoma xenografts or patient-derived xenografts.

#### Results:

High-throughput drug screens on BCL-2-dependent neuroblastoma cell lines harboring additional ALK mutations surprisingly did not yield ALK inhibitors as most effective or synergistic drugs in combination with Venetoclax. Instead, CHK1 inhibitor Prexasertib, PARP inhibitor Talazoparib and dual PI3K/HDAC inhibitor CUDC-907 were the most promising hits for combination therapy with

Venetoclax. In RAS-MAPK-mutated neuroblastoma cells, the FDA-approved MEK inhibitor Trametinib showed synergistic responses with Venetoclax. These promising effects were confirmed *in vivo* and were found to be related to MEK-mediated phosphorylation and consequently inactivation of the pro-apoptotic protein Bim.

In previous preclinical studies, we demonstrated that Venetoclax resistance occurs due to upregulation of the anti-apoptotic protein MCL-1 and sequestration of Bim released from BCL-2 by MCL-1. *in vitro* combination studies confirmed strong synergism between Venetoclax and MCL-1 inhibitors (i.e. S63845, AZD-5991 and AMG-176) in a panel of BCL-2- and MCL-1-dependent neuroblastoma model systems. In addition, high-throughput screening of non-resistant and Venetoclax-resistant neuroblastoma cells identified the MDM2 inhibitor Idasanutlin as a re-sensitizing drug for Venetoclax-resistant neuroblastoma cells. Idasanutlin caused BAX-mediated apoptosis in Venetoclax-resistant neuroblastoma cells in the presence of Venetoclax, while it caused p21-mediated growth arrest in non-resistant cells and Venetoclax-resistant cells in the absence of Venetoclax. *in vivo* combination therapy showed tumor regression and superior efficacy over single-agent treatments in different high BCL-2-dependent neuroblastoma mouse models. Less BCL-2-dependent mouse models were not sensitive to Venetoclax-Idasanutlin combination therapy.

#### Summary/Conclusions:

We identified various targeted combination strategies with Venetoclax that will guide the clinical implementation of BCL-2 inhibition for the treatment of children with BCL-2-dependent neuroblastoma.

## **P384 The synthetic lethality landscape of key drivers and drug targets in Neuroblastoma**

*Schischlik, F.*<sup>2</sup>, *Lee, J.*<sup>3</sup>, *Liu, Z.*<sup>4</sup>, *Thiele, C.*<sup>5</sup>, *Ruppin, E.*<sup>6</sup>

National Institute of Health, Cancer Data Science Laboratory, Bethesda, USA <sup>2</sup>, National Institute of Health, Cancer Data Science Laboratory, Bethesda, USA<sup>3</sup>, National Institute of Health, Pediatric Oncology Branch, Bethesda, USA<sup>4</sup>, National Institute of Health, Pediatric Oncology Branch, Bethesda, National Institute of Health<sup>5</sup>, National Institute of Health, Cancer Data Science Laboratory, Bethesda, USA<sup>6</sup>

### Background/Introduction:

Neuroblastomas (NB) are tumors originating from cells of the neural crest and are driven by few genetic alterations with a limited number of druggable target genes.

### Aims:

Here we apply a novel data-driven approach to identify synthetic lethal (SL) partners of key driver genes and drug targets in NB. These provide a new approach for discovering novel tumor vulnerabilities in NB.

### Methods/Materials:

SL interactions denote the relationship between two genes whose combined inactivation is lethal to the cell, while their individual inactivation is not (and we also considered synthetic dosage lethal (SDL) relations, which denote an analogous functional relation between the activation of one gene and the inactivation of another). We focused on a list of 25 key known driver genes and drug targets of NB patients. To identify their SL and SDL partners in NB, we applied the previously published and validated ISLE computational pipeline (Lee et al., 2018, Nature Communications) to analyze 54 pediatric and 17 neuroblastoma cell lines from the Cancer Dependency Map (*depmap*, Ghandi et al., 2019, Nature) and 523 patients' NB primary tumors omics data (n=142 patients from TARGET, n=190 from the SEQC/MAQC-III Consortium, n=191 from Ackerman et al., 2018). Briefly, ISLE is applied in three steps: First, it identifies putative SL gene pairs from *depmap*. Second, it selects those gene pairs whose co-inactivation is under-represented in primary NB tumors. Thirdly, it prioritizes candidate SL pairs whose co-inactivation is associated with better patient prognosis.

### Results:

Performing this ISLE analysis in NB, we identified 496 significant SL interactions for 18 of the 25 driver or drug target genes. First, we tested, whether the predicted SL interactions can predict gene essentiality by analyzing the results of published experimental *in vitro* CRISPR screens. Reassuringly, we find that for several driver genes/drug targets, one can successfully predict their essentiality in the CRISPR screen and drug validations based on their SL pairs, and that, as expected, the SL inferred are more predictive of gene essentiality and drug response in the NB cell lines than in general pediatric cell lines.

Second, our analysis predicts many novel SL partners of key drivers in NB such as *MYCN*, *ALK* and *CHD5*. For *MYCN*, the SL interactions inferred include *MYCN-ATRX* and *MYCN-BAZ1A* (both *ATRX* and *BAZ1A* are involved in chromatin remodeling) and *MYCN-CDCA4* (cell cycle regulation). Notably, 13 of these *MYCN* SL interactions involve druggable SL partners, providing novel selective putative drug target candidates. Six *MYCN* SL interactions showed evidence of physical interactions with *MYCN*. REACTOME pathway analysis of *MYCN* SL interactions shows high enrichment of pathways involved in cell cycle (eg. M phase, Cell Cycle Checkpoints, Mitotic Prometaphase) and organic cation/anion transport (FDR <0.2). Interestingly, the ISLE workflow identified *MYCN* as a synthetic lethal partner of the tumor suppressor *CHD5*. Indeed, the SL partners of *CHD5* and *MYCN* share many common enriched pathways such as those of cell cycle regulation (Cell Cycle Checkpoints, G2/M DNA damage checkpoint, Mitotic Prometaphase). Other enriched pathways for *CHD5* involve

DNA repair pathways (Homology Directed Repair, DNA Double-Strand Break Repair, DNA Repair)  
(FDR <0.1).

Summary/Conclusions:

Taken together, these results motivate further experimental investigations exploring the feasibility of a new paradigm for whole exome SL-based precision treatments in NB but also for other pediatric tumors.

## **P385 Extracellular domain shedding of the ALK receptor mediates neuroblastoma cell migration**

*George, R.E.<sup>2</sup>, Huang, H.<sup>3</sup>, Kee, L.<sup>4</sup>, Dries, R.<sup>5</sup>, Pfeifer, K.<sup>6</sup>, Sharma, B.<sup>7</sup>, Debruyne, D.<sup>8</sup>, Guan, J.<sup>9</sup>, Yeung, C.<sup>10</sup>, Wang, W.<sup>11</sup>, Hallberg, B.<sup>12</sup>, Irwin, M.<sup>13</sup>, Palmer, R.<sup>14</sup>*

Dana-Farber Cancer Institute, Harvard Medical School, Department of Pediatric Hematology/Oncology, Boston, USA <sup>2</sup>, Dana-Farber Cancer Institute, Harvard Medical School, Pediatric Hematology/Oncology, Boston, USA<sup>3</sup>, The Hospital for Sick Children, University of Toronto, Hematology-Oncology, Toronto, Canada<sup>4</sup>, Dana-Farber Cancer Institute, Harvard Medical School, Pediatric Hematology/Oncology, Boston, Dana-Farber Cancer Institute, Harvard Medical School<sup>5</sup>, Sahlgrenska Academy, University of Gothenburg, 4Department of Medical Biochemistry and Cell Biology, Gothenburg, Sweden<sup>6</sup>, Dana-Farber Cancer Institute, Harvard Medical School, Pediatric Hematology/Oncology, Boston, USA<sup>7</sup>, Dana-Farber Cancer Institute, Harvard Medical School, Pediatric Hematology/Oncology, Boston, USA<sup>8</sup>, Sahlgrenska Academy, University of Gothenburg, 4Department of Medical Biochemistry and Cell Biology, Gothenburg, Sweden<sup>9</sup>, Dana-Farber Cancer Institute, Harvard Medical School, Pediatric Hematology/Oncology, Boston, USA<sup>10</sup>, Dana-Farber Cancer Institute, Harvard Medical School, Pediatric Hematology/Oncology, Boston, USA<sup>11</sup>, Sahlgrenska Academy, University of Gothenburg, Department of Medical Biochemistry and Cell Biology, Gothenburg, Sweden, <sup>12</sup>, The Hospital for Sick Children, University of Toronto, Hematology/Oncology, Toronto, Canada <sup>13</sup>, Sahlgrenska Academy, University of Gothenburg, Department of Medical Biochemistry and Cell Biology, Gothenburg, Sweden <sup>14</sup>

### **Background/Introduction:**

Mutations of the anaplastic lymphoma kinase (ALK) cell surface receptor tyrosine kinase are the most common directly targetable genetic aberrations in neuroblastoma, affecting approximately 10% of these tumors. However, the wild-type receptor is also overexpressed in the vast majority of neuroblastoma tumors, where it undergoes proteolytic shedding of the extracellular domain (ECD), the functional significance of which is unclear. Here we mapped the ALK cleavage site and show that ECD shedding is important for neuroblastoma cell invasion and migration. CRISPR-based editing of the ECD cleavage site of the wild-type ALK receptor in neuroblastoma cells resulted in their decreased migration and invasion, a phenotype that was associated with downregulation of an epithelial-to-mesenchymal signature, decreased nuclear localization of  $\beta$ -catenin and reversal upon reintroduction of wild-type ALK. Importantly, inhibition of ECD cleavage led to significantly decreased metastasis and longer survival in mouse models of neuroblastoma. Retention of the ECD also potentiated retinoic acid-induced differentiation of neuroblastoma cells. We further show that the ALK ECD is cleaved by matrix metalloproteinase 9, whose inactivation leads to cleavage inhibition and loss of neuroblastoma cell migration. Together, our results indicate pivotal roles for ECD cleavage of wild-type ALK in neuroblastoma metastasis and differentiation, and suggest approaches to harnessing this process for therapeutic gain.

## **P386 De-novo germline ALK mutation associated with congenital metastatic (Ms) neuroblastoma, dysmorphism and developmental delay.**

*Herd, F.<sup>2</sup>, Bown, N.<sup>3</sup>, Henderson, A.<sup>4</sup>, Ramesh, V.<sup>5</sup>, Tweddle, D. A.<sup>6</sup>*

Royal Aberdeen Children's Hospital, Department of Paediatric Oncology, Aberdeen, United Kingdom<sup>2</sup>, Northern Genetics Service, Cancer Cytogenetics, Newcastle Upon Tyne, United Kingdom<sup>3</sup>, Northern Genetics Service, Clinical Genetics, Newcastle Upon Tyne, United Kingdom<sup>4</sup>, Great North Children's Hospital, Paediatric Neurology, Newcastle Upon Tyne, Great North Children's Hospital<sup>5</sup>, Great North Children's Hospital, Paediatric Oncology, Newcastle Upon Tyne, United Kingdom<sup>6</sup>

### Background/Introduction:

Anaplastic Lymphoma Kinase (ALK) is a transmembrane receptor tyrosine kinase that plays an important role in nervous system development. Germline *ALK* mutations predispose to neuroblastic tumours and are the most common cause of hereditary neuroblastoma. Within the *ALK* gene are a number of mutational hotspots. F1174 is the 2nd commonest site of somatic mutation with only one previously reported case in the germline, an F1174V mutation in a child with congenital neuroblastoma and fatal encephalopathy / brain stem abnormality.

### Aims:

To report, to our knowledge, the first case of a child now 3 years old, with congenital neuroblastoma and developmental delay with a germline F1174L *ALK* mutation.

### Methods/Materials:

We report the clinical and genetic features of a child (now 3 years old) with congenital neuroblastoma and developmental delay who was found to have a germline F1174L *ALK* mutation by Sanger DNA sequencing. We hypothesised this mutation was responsible for the child's phenotype and the parents consented to enter the child into the Genomics England 100,000 genomes project <http://www.genomicsengland.co.uk>. Whole genome sequencing (WGS) was performed on germline DNA from the child and both parents together with tumoural DNA. Samples were prepared using an Illumina TruSeq DNA PCR-Free library preparation kit and then sequenced on a HiSeq X generating 150 bp paired-end reads. Germline samples were sequenced to produce at least 85 Gb of sequences with sequencing quality of at least 30. Written informed consent was given by the child's family to report this case.

### Results:

A 3 year old boy presented at birth with "blueberry muffin spots" and was found to have congenital Ms neuroblastoma with a right adrenal primary tumour and skin/subcutaneous tissue and liver metastases. Biopsy confirmed International Neuroblastoma Pathology Classification favourable histology neuroblastoma with no evidence of *MYCN* amplification or segmental chromosomal abnormalities. After an initial period of observation the patient required 2 cycles of chemotherapy due to disease progression and poor feeding. Since then his tumour has slowly and continuously regressed. Genetic testing revealed an *ALK* c.3520T>C p.(Phe1174Leu) *ALK* mutation in the tumour which was subsequently found to be a *de-novo* germline mutation. The child had other health problems since birth including feeding difficulty, poor growth, gross motor developmental delay, hypertonia and convergent squint. Fragile X testing was normal. Whole genome germline DNA sequencing from the child and parents showed no other genetic cause for the child's phenotype and cancer whole genome sequencing revealed 8 variants at low allele frequency of unknown clinical significance.

### Summary/Conclusions:

Germline *ALK* mutations predispose to early onset neuroblastoma. To our knowledge this is the first report of a germline F1174L mutation in a child which is likely to be responsible for his phenotype of poor feeding, small stature and developmental delay. Future reports of germline F1174L mutations will

confirm or refute this finding and may lead to identification of a new genetic syndrome which could be termed an "ALKopathy".



## **P387 Targeting Survivin in neuroblastoma at protein level with the Survivin inhibitor S12**

Schulte, S.,<sup>1</sup> Schmidt, A.<sup>2</sup>, Schmidt, A.<sup>3</sup>, Toedling, J.<sup>4</sup>, Hertwig, F.<sup>5</sup>, Klironomos, F.<sup>6</sup>, Henssen, A.G.<sup>7</sup>, Witthauer, M.J.<sup>8</sup>, Winkler, A.<sup>9</sup>, Schulte, J.H.<sup>10</sup>, Eggert, A.<sup>11</sup>

Charité - Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology, Berlin, Germany<sup>1</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology, Berlin, Germany<sup>2</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology, Berlin, Germany<sup>3</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology, Berlin, Germany<sup>4</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology, Berlin, Germany<sup>5</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology, Berlin, Germany<sup>6</sup>, Experimental and Clinical Research Center (ECRC) of the Charité and MDC, Clinical Research, Berlin, Germany<sup>7</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology, Berlin, Germany<sup>8</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology, Berlin, Germany<sup>9</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology, Berlin, Germany<sup>10</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology, Berlin, Germany<sup>11</sup>

### Background/Introduction:

The oncogene *BIRC5* (encoding the protein Survivin) is located on chromosome arm 17q, upregulated by *MYCN*, and was found to be strongly overexpressed in high-risk neuroblastoma (NB). Survivin is involved in suppression of apoptosis, mitosis, cell cycle, metabolism, invasion and several other key cellular functions. Knock-down of *BIRC5* in neuroblastoma cell lines has been shown to result in cell death. Treatment of neuroblastoma cell lines and mice with neuroblastoma xenografts with the Survivin inhibitor YM155 resulted in cell death and tumor regression. However, more recent studies have revealed that YM155 treatment leads to unspecific DNA damage and other unspecific side effects rather than specific inhibition of Survivin.

### Aims:

Our aim is to explore strategies for targeting Survivin on protein level and test the novel Survivin inhibitor S12, which binds to Survivin and prevents it from homodimerization and binding to other proteins.

### Methods/Materials:

For a panel of 20 NB cell lines, mRNA expression data, epigenetic data and exome sequencing data were obtained, and the cell lines were treated with S12 or YM155. The cell lines were stratified according to sensitivity to the inhibitors, and machine learning was applied to generate a predictive signature for sensitivity to S12. The cellular effects of S12 on neuroblastoma cell lines were further analysed using cell-based assays for cell viability, proliferation, apoptosis and differentiation. Survivin expression before and during treatment with S12 was analysed using western blot analysis.

### Results:

Treating 20 NB cell lines with S12 and YM155 at a wide range of concentrations, we found that the sensitivity to S12 treatment did not correlate well with the sensitivity to YM155 treatment. The mean IC<sub>50</sub> of S12 was approx. 10µM in cell lines stratified as sensitive to S12 treatment. Using a machine-learning algorithm, a molecular predictor based on gene expression and epigenetic features could be established and was validated using a leave-one-out cross-validation strategy.

As expected, treatment of neuroblastoma cells with did not alter survivin protein levels. S12 treatment significantly reduced cell viability, which was driven both by reduced cell proliferation and the induction of apoptosis. S12 treatment also induced cellular differentiation, as indicated by cell shape, the outgrowth of neurite-like processes and the upregulation of markers of neuronal differentiation.

### Summary/Conclusions:

Inhibition of Survivin by interfering with Survivin homodimerization and protein-protein interactions using the small-molecule inhibitor S12 outlines a new strategy for targeting Survivin in neuroblastoma. For translation of this strategy into clinical application we propose further development and improvement of S12-based inhibitors that achieve efficacies at nanomolar concentrations.

## **P388 MYCMI-7 - a small MYC-binding compound that inhibits MYC:MAX interaction and tumor cell growth in a MYC-dependent manner**

Larsson, L.G.<sup>2</sup>, Castell, A.<sup>3</sup>, Mohammad, A.<sup>4</sup>, Qinzi, Q.<sup>5</sup>, Fawkner, K.<sup>6</sup>, Wickström, M.<sup>7</sup>, Zhang, F.<sup>8</sup>, Bazzar, B.<sup>9</sup>, Olsen, T.K.<sup>10</sup>, Dyberg, C.<sup>11</sup>, Johnsen2, J.I.<sup>12</sup>

Karolinska Institutet, Dept. of Microbiology, Tumor and Cell Biology, Stockholm, Sweden <sup>2</sup>, Karolinska Institutet, MTC, Stockholm, Sweden<sup>3</sup>, Karolinska Institutet, MTC, Stockholm, Sweden<sup>4</sup>, Karolinska Institutet, MTC, Stockholm, Karolinska Institutet<sup>5</sup>, Karolinska Institutet, MTC, Stockholm, Sweden<sup>6</sup>, Karolinska Institutet, Oncologi-Pathology, Stockholm, Sweden<sup>7</sup>, Karolinska Institutet, MTC, Stockholm, Sweden<sup>8</sup>, Karolinska Institutet, MTC, Stockholm, Sweden<sup>9</sup>, Karolinska Institutet, Oncology-Pathology, Stockholm, Sweden<sup>10</sup>, Karolinska Institutet, Oncology-Pathology, Stockholm, Sweden<sup>11</sup>, Karolinska Institutet, Oncology-Pathology, Stockholm, Sweden, <sup>12</sup>

### Background/Introduction:

Deregulated expression of the MYC family oncogenes/transcription factors MYC and MYCN occurs in many types of adult and childhood tumors including neuroblastoma, and is in the latter case strongly associated with aggressive tumor development, resistance to therapy and poor prognosis. In mouse tumor models, inactivation of MYC often leads to tumor regression with well-tolerated side effects, suggesting that MYC is a potential and suitable target for anti-cancer therapy. Unfortunately, no specific MYC-targeting drugs are available in clinic today. Thus, developing inhibitors that cripple MYC activity is an unmet need in cancer therapy. MYC/MYCN is dependent on interactions with its partner protein MAX to assert its function. Using a cell-based MYC:MAX interaction screen we recently identified several potent small molecule MYC:MAX inhibitors including MYCMI-6 and MYCMI-7 (Castell et al., 2018).

### Aims:

Our long-term goal is to develop compounds that specifically and selectively inhibit MYC:MAX interactions *in vivo* and that can be of clinical use for cancer therapy for patients with MYC-dependent tumors such as neuroblastoma. The specific aim for this project is to characterize and validate the MYC:MAX inhibitor MYCMI-7 with respect to potency and selectivity *in vitro*, in cells and *in vivo*.

### Methods/Materials:

To assay MYC:MAX and MYCN:MAX interactions, Gaussia Luciferase (GLuc) protein fragment complementation assay (PCA), *in situ* proximity ligation assay (isPLA), co-immunoprecipitation, microscale thermophoresis (MST), FRET and surface plasmon resonance (SPR) assays were used. MST and SPR was also used to study direct binding of MYCMI-7 to recombinant MYC. The effect of MYCMI-7 on MYC target gene expression was measured by RT-PCR, microarray, RNA-seq and chromatin immunoprecipitation (ChIP). Cell proliferation/viability was measured by WST-1, resazurin, colony assays, TUNEL and annexin V staining, flow cytometry and other assays. Expression of proteins involved in these processes were studied by immunoblot analysis. The efficacy of MYCMI-7 as anti-tumor agents was further evaluated using a mouse xenograft model of MYCN-amplified neuroblastoma. Tumor volume, tumor cell proliferation (Ki67), apoptosis (TUNEL), MYC:MAX interaction (isPLA) and other markers were measured in tumor tissue.

### Results:

Based on our previous identification of MYCMI-7 (Castell et al., 2018), we here characterized MYCMI-7 in more detail. We show that MYCMI-7 binds to recombinant MYC and efficiently inhibits MYC:MAX and MYCN:MAX protein interactions both in cells and *in vitro*. In addition, MYCMI-7 treatment results in downregulation of the steady state levels of the MYC and MYCN proteins. Further, MYCMI-7 interfered MYC:MAX interaction with its target genes and efficiently downregulated expression of these genes. We show that MYCMI-7 is a potent inducer of apoptosis, affecting growth of a variety of different tumor cell lines, including neuroblastoma, glioblastoma, Burkitt's lymphoma, acute lymphoblastic leukemia, breast and lung cancer and a number of other epithelial tumors in a MYC-

dependent manner, all at low micromolar concentrations. Importantly, normal cells become G<sub>1</sub> arrested without signs of apoptosis upon MYCMI-7 treatment. Finally, treatment with MYCMI-7 in vivo using a mouse model of *MYCN*-amplified neuroblastoma inhibited tumor growth and prolonged survival of the mice.

Summary/Conclusions:

MYCMI-7 can potentially contribute to development of anti-MYC drugs for treatment of cancer.

## **P389 Novel compounds target calcium pathways in drug-resistant Neuroblastoma**

*Lange, I.*<sup>2</sup>, *Zoeller, K.*<sup>3</sup>, *Espinoza-Fuenzalida, I.*<sup>4</sup>, *Sunada, N.*<sup>5</sup>, *Koomoa, D.*<sup>6</sup>

University of Hawaii, Pharmaceutical Sciences, Hilo, USA <sup>2</sup>, University of Hawaii, Pharm Sci., Hilo, USA<sup>3</sup>, University of Hawaii, Pharm Scie, Hilo, USA<sup>4</sup>, University of Hawaii, Pharm Sci, Hilo, University of Hawaii<sup>5</sup>, University of Hawaii, Pharmaceutical Sciences, Hilo, USA<sup>6</sup>

### **Background/Introduction:**

Neuroblastoma (NB) is the most common pediatric extra-cranial solid tumor in infants. Up to 60% of high-risk NB patients are either refractory to treatment or experience relapse after treatment, and the remaining tumor cells are resistant to therapy. This is in part due to specific Ca<sup>2+</sup> defects in NB that inhibit apoptosis. The biggest challenge to treating relapsed high-risk NB is the propensity of NB cells to alter signaling pathways to promote survival in response to cytotoxic stimuli, which results in relapse. Unfortunately, there are currently no effective treatments for relapsed high-risk NB. Our overall goal is to manipulate key pathways to effectively treat high-risk NB.

### **Aims:**

The main goals of this project were to identify novel compounds that reduce the viability of drug-resistant NB, and determine the mechanism of action of these compounds.

### **Methods/Materials:**

The sulforhodamine B assay was used to screen a library of novel compounds for anti-cancer effects in drug-resistant NB cell lines. Western blot analysis was used to determine if the compounds that exhibited anti-cancer effects inhibited cell cycle progression and/or induced apoptosis. Laser scanning confocal microscopy was used in combination with genetically encoded calcium indicators targeted to the cytoplasm, ER or mitochondria to identify calcium signaling pathways that were affected by the compounds. Electrophysiological patch-clamp measurements were recorded in the whole-cell configuration to determine changes in ion channel activity.

### **Results:**

Two novel compounds were identified, compound 248 and compound 249, that exhibited potent anti-cancer effects in drug-resistant NB cells. The compounds inhibited cell cycle progression and induced apoptosis through a mechanism that involved activation of ER-mitochondrial calcium signaling, mitochondrial calcium overload, opening of the mitochondrial permeability transition pore, and calcium-dependent apoptosis. In addition to regulating calcium stores, the compounds also activated TRP-like channels.

### **Summary/Conclusions:**

This study identified two novel compounds that may be effective for treating refractory and relapsed NB. In addition, the study identified a potential treatment strategy that may be targeted for the treatment of refractory and relapsed NB. The knowledge gained from this study may be used to develop more effective treatments for NB.

## **P390 Enhanced targeting of the mitochondrial adenine nucleotide translocase SLC25A5 by modulating p53 dynamics in neuroblastoma**

*Seneviratne, J.*<sup>2</sup>, *Carter, D.*<sup>3</sup>, *Kim, P.*<sup>4</sup>, *Rahmanto, A.*<sup>5</sup>, *Salib, A.*<sup>6</sup>, *Kleynhans, A.*<sup>7</sup>, *Luo, S.*<sup>8</sup>, *Herath, M.*<sup>9</sup>, *Raipuria, M.*<sup>10</sup>, *Murray, J.*<sup>11</sup>, *Cheng, N.*<sup>12</sup>, *Xue, C.*<sup>13</sup>, *Chung, S.*<sup>14</sup>, *Dilda, P.*<sup>15</sup>, *Fletcher, J.*<sup>16</sup>, *Liu, T.*<sup>17</sup>, *Haber, M.*<sup>18</sup>, *Norris, M.*<sup>19</sup>, *Hogg, P.*<sup>20</sup>, *Cheung, B.*<sup>21</sup>, *Marshall, G.*<sup>22</sup>

Children's Cancer Institute, Embryonal Cancer Therapy and Prevention, Randwick, Australia <sup>2</sup>, Children's Cancer Institute, Embryonal Cancer Therapy and Prevention, Randwick, Australia<sup>3</sup>, Children's Cancer Institute, Embryonal Cancer Therapy and Prevention, Randwick, Australia<sup>4</sup>, Children's Cancer Institute, Embryonal Cancer Therapy and Prevention, Randwick, Children's Cancer Institute<sup>5</sup>, Children's Cancer Institute, Embryonal Cancer Therapy and Prevention, Randwick, Australia<sup>6</sup>, Children's Cancer Institute, Embryonal Cancer Therapy and Prevention, Randwick, Australia<sup>7</sup>, Children's Cancer Institute, Embryonal Cancer Therapy and Prevention, Randwick, Australia<sup>8</sup>, Children's Cancer Institute, Embryonal Cancer Therapy and Prevention, Randwick, Australia<sup>9</sup>, Children's Cancer Institute, Embryonal Cancer Therapy and Prevention, Randwick, Australia<sup>10</sup>, Children's Cancer Institute, Experimental Therapeutics, Randwick, Australia<sup>11</sup>, Children's Cancer Institute, Experimental Therapeutics, Randwick, Australia, <sup>12</sup>, Children's Cancer Institute, Molecular Oncology, Randwick, Australia <sup>13</sup>, Adult Cancer Program, Lowy Cancer Research Centre, Cure Brain Cancer Neuro-Oncology Group, Randwick, Australia <sup>14</sup>, Adult Cancer Program, Lowy Cancer Research Centre, Tumour Metabolism Group, Randwick, Australia <sup>15</sup>, Children's Cancer Institute, Experimental Therapeutics, Randwick, Australia <sup>16</sup>, Children's Cancer Institute, Gene Dysregulation, Randwick, Australia <sup>17</sup>, Children's Cancer Institute, Experimental Therapeutics, Randwick, Australia <sup>18</sup>, Children's Cancer Institute, Molecular Oncology, Randwick, Australia <sup>19</sup>, Centenary Cancer Research Centre, Australian Cancer Research Foundation (ACRF), Camperdown, Australia <sup>20</sup>, Children's Cancer Institute, Embryonal Cancer Therapy and Prevention, Randwick, Australia <sup>21</sup>, Children's Cancer Institute, Embryonal Cancer Therapy and Prevention, Randwick, Australia <sup>22</sup>

### Background/Introduction:

Mitochondria-targeted agents are steadily being integrated into cancer therapies as they induce strong apoptotic phenotypes and potentiate treatment responses to cytotoxic chemotherapy in several cancer types. Mitochondria-associated genes (MAGs) encode for mitochondrial proteins and/or mediate mitochondrial signalling pathways. Several MAGs have been strongly associated with increased malignancy and drug resistance in neuroblastoma (NB). Therefore MAGs represent promising therapeutic targets in NB.

### Aims:

We aimed to identify potential therapeutic targets among MAGs in NB. We then aimed to design rational combination therapies around specific MAG targets.

### Methods/Materials:

We took an integrative functional genomics approach to prioritise MAGs, where we leveraged published; RNA-seq, ChIP-seq, ATAC-seq and CRISPR interference data derived from NB patients or cell lines. To design rational combination therapies we further utilised drug response data from the Genomics of Drug Sensitivity in Cancer (GDSC) database. To assess the mechanisms of our prioritised MAG and targeted combination therapy we utilised several *in vitro* methodologies including; cell viability, cell cycle, apoptosis, microarray, protein half-life and western blot assays. To assess the *in vivo* efficacy of the targeted combination therapy we utilised NB cell line xenograft and transgenic NB TH-MYCN mouse models.

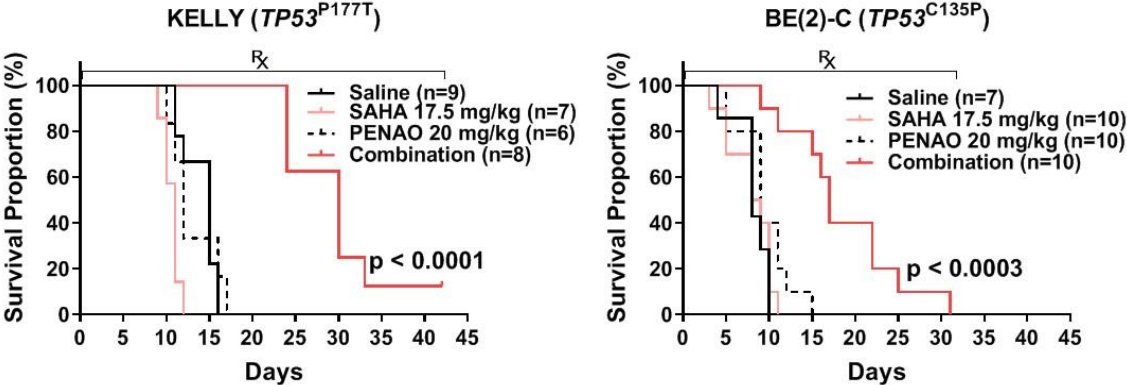
### Results:

Our integrative functional genomics approach identified a subset of MAGs whose expression was associated with higher chromatin accessibility at gene promoters, higher gene dependency for NB cell

survival and poor NB patient survival. Multivariate analysis of this MAG subset identified mitochondrial adenine nucleotide translocase 2 (SLC25A5) to be the strongest independent predictor of poor NB patient prognosis. We found that siRNA mediated knockdown of SLC25A5 reduced NB cell viability and direct inhibition with a selective SLC25A5 inhibitor, PENAO, markedly reduced cell viability in a panel of NB cell lines. We found that PENAO resistance was primarily driven by p53 mutations, so we then used drug response data to model potential combination therapies to overcome PENAO resistance in p53 mutant NB cell lines. This led to the identification of therapeutic synergy between PENAO and SAHA, a histone deacetylase inhibitor, in both mutant and wild type p53 NB cell lines. We found that PENAO combined with SAHA drastically reduced mutant p53 protein levels in NB cell lines and linked this to mutant p53 protein destabilisation. Combining PENAO and SAHA *in vivo* significantly prolonged the survival of mice inoculated with two mutant p53 NB cell lines and in TH-MYCN transgenic mice compared with single agent therapy or the control.

Summary/Conclusions:

PENAO is currently in phase II clinical trials, whilst SAHA is FDA approved for multiple solid tumours. Our promising data on this combination therapy warrants further evaluation for an early phase clinical trial for NB. Furthermore, given that p53 mutations arise more frequently in relapsed NB and confer multi-drug resistance, our proposed combination therapy may offer an effective therapeutic avenue for these patients.



Caption 1: In vivo efficacy of the targeted drug combination in two xenograft models of mutant p53 neuroblastoma

## **P391 Combined replenishment of miR-34a and let-7b inhibits neuroblastoma growth by down-modulation of *MYCN*, *LIN28B* and *ALK* expression in pre-clinical models**

*Perri, P.*<sup>2</sup>, *Di Paolo, D.*<sup>3</sup>, *Pastorino, F.*<sup>4</sup>, *Brignole, C.*<sup>5</sup>, *Corrias, M.V.*<sup>6</sup>, *Emionite, L.*<sup>7</sup>, *Cilli, M.*<sup>8</sup>, *Tamma, R.*<sup>9</sup>, *Priddy, L.*<sup>10</sup>, *Amaro, A.*<sup>11</sup>, *Pfeffer, U.*<sup>12</sup>, *Ribatti, D.*<sup>13</sup>, *Sementa, A.R.*<sup>14</sup>, *Brown, D.*<sup>15</sup>, *Ikegaki, N.*<sup>16</sup>, *Shimada, H.*<sup>17</sup>, *Ponzoni, M.*<sup>18</sup>

IRCCS Istituto Giannina Gaslini, Lab Experimental Therapies in Oncology, Genoa, Italy <sup>2</sup>, IRCCS Istituto Giannina Gaslini, Lab Experimental Therapies in Oncology, Genoa, Italy<sup>3</sup>, IRCCS Istituto Giannina Gaslini, Lab Experimental Therapies in Oncology, Genoa, Italy<sup>4</sup>, IRCCS Istituto Giannina Gaslini, Lab Experimental Therapies in Oncology, Genoa, IRCCS Istituto Giannina Gaslini<sup>5</sup>, IRCCS Istituto Giannina Gaslini, Lab Experimental Therapies in Oncology, Genoa, Italy<sup>6</sup>, IRCSS Ospedale Policlinico San Martino, Animal Facility, Genoa, Italy<sup>7</sup>, IRCSS Ospedale Policlinico San Martino, Animal Facility, Genoa, Italy<sup>8</sup>, University of Bari, Department of Basic Medical Sciences Neurosciences, and Sensory Organs, Bari, Italy<sup>9</sup>, Mirna Therapeutics/Aeglea BioTherapeutics, Laboratories, Austin, Tx, USA<sup>10</sup>, IRCSS Ospedale Policlinico San Martino, Tumor Epigenetic Unit, Genoa, Italy<sup>11</sup>, IRCSS Ospedale Policlinico San Martino, Tumor Epigenetic Unit, Genoa, Italy, <sup>12</sup>, University of Bari, Department of Basic Medical Sciences Neurosciences, and Sensory Organs, Bari, Italy <sup>13</sup>, IRCCS Istituto Giannina Gaslini, Pathology Unit, Genoa, Italy <sup>14</sup>, Mirna Therapeutics, Laboratories, Austin, Tx, USA <sup>15</sup>, University of Illinois at Chicago, Department of Anatomy and Cell Biology, Chicago, Il, USA <sup>16</sup>, Stanford University/University of Southern California, Department of Pathology, Stanford/Los Angeles, Ca, USA <sup>17</sup>, IRCCS Istituto Giannina Gaslini, Lab Experimental Therapies in Oncology, Genoa, Italy <sup>18</sup>

### Background/Introduction:

Neuroblastoma (NB) is a malignant tumor of the sympathetic nervous system that substantially contributes to childhood cancer mortality, mostly due to toxicities and/or chemo-resistance, especially in high-risk patients with relapsed disease. The design of novel drugs to target specific molecular alterations in NB becomes imperative to provide more effective therapies and to ameliorate patients' outcomes. The dysregulated expression of *MYCN*, *ALK* and *LIN28B* as well as the aberrant under-expression of two microRNAs, miR-34a and let-7b are known to drive NB tumorigenesis by maintaining an 'oncogene addiction'.

### Aims:

Due to the ability of miRNA-mimics to recover the tumor suppressor functions of miRNAs which are depleted into cancer cells, in this study we have developed a safe and effective delivery system, selectively targeted to NB cells, to restore the correct activity specific miRNAs in clinically relevant mouse models of NB.

### Methods/Materials:

To evaluate the functional effects of miRNA replenishment and the down-modulation activity over *MYCN*, *LIN28B* and *ALK*, four NB cell lines, IMR-32, HTLA-230, LAN-5 and SH-SY5Y, characterized by different genetic alterations and/or over-expression of these key oncogenes, were chosen. Following *in vitro* cell transfection with ten candidate tumor suppressor miRNA-mimics, functional testing led to the selection of miR-34a and let-7b as the best ones. To investigate the therapeutic efficacy of restoring the tumor suppressor functions miR-34a and let-7b in pre-clinical mouse models, we set-up and characterized a novel formulation of GD<sup>2</sup>-targeted liposomes entrapping miR-mimics for systemic administration of miR-34a and let-7b, individually and in combination. RT-qPCR and IHC techniques were adopted for gene/protein expression analyses. Systemic toxicity and immune-response were evaluated by serum chemistry analyses. Significance of experimental data and mice survival were evaluated by specific statistical tests.

### Results:



The replenishment of miR-34a and let-7b by NB-targeted nanoparticles, individually and more powerfully in combination, significantly reduced cell division, proliferation, neo-angiogenesis, tumor growth and burden, and induced apoptosis in orthotopic xenografts and improved mice survival in pseudo-metastatic models. These results represent the functional consequences of an efficient and cooperative down-modulation of *MYCN* transcription factor and its down-stream targets, *ALK* and *LIN28B*, effected by miR-34a and let-7b, which reactivate the physiological regulatory networks and lastly lead to a favorable therapeutic response. Our targeted liposomes hold physical-chemical features in terms of very high and stable entrapment efficiency, long-lasting stability and very effective binding to NB cells and they do not induce any acute or chronic toxicity nor immune-stimulation, thus resulting safe and efficient carriers for miR-mimics.

#### Summary/Conclusions:

Our findings demonstrate that the combined replacement of miR-34a and let-7b has a promising therapeutic efficacy in itself. Moreover, as miRNA expression affects the response of cancer cells to cytotoxic therapy, the reactivation of crucial pathways following miR-34a and let-7b replenishment by our NB-targeted liposomal formulations is expected to enhance drug sensitivity, providing a viable tool for future clinical applications as adjuvant therapy for high-risk NB patients.

## **P392 A Novel Chimeric Antigen Receptor Targeting Anaplastic Lymphoma Kinase (ALK) for Childhood Cancer Immunotherapy**

*Halliwel, E.*<sup>2</sup>, *Anderson, J.*<sup>3</sup>, *Pataillot-Meakin, T.*<sup>4</sup>, *Chester, K.*<sup>5</sup>, *Straathof, K.*<sup>6</sup>

UCL ICH, DBC Cancer, London, United Kingdom <sup>2</sup>, UCL ICH, DBC Cancer, London, United Kingdom<sup>3</sup>, Imperial, Imperial, London, United Kingdom<sup>4</sup>, UCL CI, CI, London, UCL CI<sup>5</sup>, UCL ICH, DBC Cancer, London, United Kingdom<sup>6</sup>

### Background/Introduction:

Manipulating patient T cells through the insertion of chimeric antigen receptors (CARs) has transformed the treatment of haematological malignancies by enabling T cell redirection. Success rates in solid tumours have been less efficacious. High risk neuroblastoma (NB) is the most common extracranial solid tumour of childhood accounting for ~15% of childhood cancer related deaths. Anaplastic lymphoma kinase (ALK), a tyrosine kinase receptor, is speculated to be involved in healthy neural development but also in NB progression. Both germline and somatic ALK mutations are implicated in constitutively active oncogenic signalling. Previous CAR T cells targeted against NB, such as GD2 and L1-CAM do not yet yield desired results, demonstrating the demand for novel targets.

### Aims:

To evaluate an ALK CAR as potential novel therapy based on *in vitro* cytotoxic activity, cytokine production and proliferative capabilities.

### Methods/Materials:

A panel of second generation ALK-specific CARs was initially investigated; consisting of five unique ALK single chain variable fragments (scFv), two stalks regions of different lengths and a CD28 costimulatory domain with CD3 $\zeta$ , in order to determine a lead combination. Human primary  $\alpha\beta$  T cells were virally transduced to express the CAR on their surface and co-cultured with cell lines of varying ALK surface expression to determine *in vitro* cytotoxicity, cytokine production, short- and long-term proliferative capacity with and without antigen re-stimulation.

### Results:

All ALK CARs created were successfully expressed on the surface of  $\alpha\beta$  T cells and demonstrated robust cytotoxicity and antigen-dependant cytokine generation when co-cultured with ALK positive cell lines but not with ALK negative cell lines. When NB cell lines were co-cultured, with lower ALK surface expression, cytotoxicity and the ability to produce cytokines by the ALK CARs was lowered in a correlative manner. Furthermore, all CARs demonstrated the ability to proliferate at short time points and produce IL-2 and IFN $\gamma$  after re-stimulation. Despite this, their perseverance at longer time points appeared to be limited. Interestingly, differences in stalk regions appeared to influence whether CAR proliferation was antigen-dependant or antigen-independent.

### Summary/Conclusions:

We have successfully demonstrated that CAR T cells directed against ALK show clear antigen dependant cytotoxicity and cytokine production when ALK target expression is high. There was a clear correlative relationship between ALK surface density and the level of cytotoxic activity and cytokine production, shown when NB cell lines were utilised, suggesting ALK may be expressed below the threshold antigen density required to trigger responses. There is hope to combine targeting of ALK with another NB associated antigen in a co-CAR system also.

### **P393 The role of histone deacetylases and lysosomes in neuroblastoma chemoresistance**

*Oehme, I.<sup>2</sup>, Ridinger, J.<sup>3</sup>, Semino, F.<sup>4</sup>, Kronic, D.<sup>5</sup>, Hamacher-Brady, A.<sup>6</sup>, Turunen, L.<sup>7</sup>, Saarela, J.<sup>8</sup>, Berker, Y.<sup>9</sup>, Oppermann, S.<sup>10</sup>, Peterziel, H.<sup>11</sup>, Witt, O.<sup>12</sup>*

KITZ / DKFZ /DKTK, CCU Pediatric Oncology, Heidelberg, Germany <sup>2</sup>, Kitz / DKFZ, CCU Pediatric Oncology, Heidelberg, Germany<sup>3</sup>, Kitz / DKFZ, CCU Pediatric Oncology, Heidelberg, Germany<sup>4</sup>, DKFZ, Light Microscopy Facility, Heidelberg, DKFZ<sup>5</sup>, Johns Hopkins University, Bloomberg School of Public Health, Baltimore, USA<sup>6</sup>, Institute for Molecular Medicine Finland-FIMM, Helsinki Institute for Life Sciences -HiLIFE, Helsinki, Finland<sup>7</sup>, Institute for Molecular Medicine Finland-FIMM, Helsinki Institute for Life Sciences -HiLIFE, Helsinki, Finland<sup>8</sup>, Kitz / DKFZ, CCU Pediatric Oncology, Heidelberg, Germany<sup>9</sup>, Kitz / DKFZ, CCU Pediatric Oncology, Heidelberg, Germany<sup>10</sup>, Kitz / DKFZ, CCU Pediatric Oncology, Heidelberg, Germany<sup>11</sup>, Kitz / DKFZ / DKTK / University of Heidelberg Medical Center, CCU Pediatric Oncology, Heidelberg, Germany, <sup>12</sup>

#### **Background/Introduction:**

The course and clinical outcome of neuroblastoma is highly heterogeneous, ranging from relatively benign disease to highly aggressive and often fatal disease in high-risk neuroblastomas. Lysosomes, acidic vesicular organelles that mainly function as a degradation center for cellular macromolecules as well as aged organelles, promote tumor multidrug resistance by at least three distinct mechanisms. Firstly, they increase tumor cell stress resistance by promoting the turnover of damaged organelles (macroautophagy). Secondly, they trap weakly basic chemotherapeutics, hindering them from reaching their cellular target. Thirdly, they promote secretion of drugs by fusion with the cell membrane (lysosomal exocytosis).

#### **Aims:**

We aim for a better understanding of the underlying resistance mechanisms in these tumors to open up possibilities for novel treatment approaches.

#### **Methods/Materials:**

Several cell models (human neuroblastoma cell lines; non-malignant proliferating human fibroblasts; HDAC10-knockout HAP1 cells) were used. The lysosomal phenotype was characterized by LysoTracker staining (FACS and confocal microscopy) and surface LAMP-1 versus total LAMP-1 expression (FACS and Western Blot). Intracellular doxorubicin levels were measured via flow cytometry and visualized by fluorescent confocal microscopy. Cell viability was determined by trypan blue exclusion and ATP metabolic activity assays.

#### **Results:**

Our studies demonstrate that all three mechanisms contribute to chemoresistance in a panel of highly aggressive neuroblastoma cell lines, suggesting that resistance critically depends on lysosomal function. Specifically, we have demonstrated that the histone deacetylase member HDAC10 promotes fusion of doxorubicin-filled lysosomes with the plasma membrane (lysosomal exocytosis) and thereby clearance of doxorubicin from neuroblastoma cells. Consequently, HDAC10 depletion or inhibition causes intracellular accumulation of doxorubicin and sensitizes highly resistant neuroblastoma cells to chemotherapeutic treatment. To systematically analyze the contribution of lysosomes to therapy resistance in neuroblastoma, we are currently using both established cell lines, as well as patient-derived short-term cultures. In particular, we investigate the correlation between cellular lysosomal content and resistance to a set of 75 FDA/EMA approved anti-cancer therapeutics. We further investigate if specific anti-cancer drugs commonly induce lysosomal adaptation mechanisms during treatment and how such adaptation mechanisms correlate to treatment resistance.

#### **Summary/Conclusions:**

The available information on the full molecular characterization of all our cellular models, including primary patient material, allows us to identify gene expression patterns or genetic aberrations (point

mutations, chromosomal rearrangements) that predict lysosomal adaptation mechanisms and thus possibly treatment resistance. In the future, the identification of lysosomal dependencies could enable the combination of targeted therapies with lysosome-targeting approaches to revert these resistance mechanisms.

## **P394 CD146 is a potential therapeutic target for neuroblastoma**

*Obu, S.<sup>2</sup>, Umeda, K.<sup>3</sup>, Sonoda, M.<sup>4</sup>, Tasaka, K.<sup>5</sup>, Ogata, H.<sup>6</sup>, Kouzuki, K.<sup>7</sup>, Nodomi, S.<sup>8</sup>, Saida, S.<sup>9</sup>, Kato, I.<sup>10</sup>, Hiramatsu, H.<sup>11</sup>, Okamoto, T.<sup>12</sup>, Ogawa, E.<sup>13</sup>, Okajima, H.E.<sup>14</sup>, Morita, K.<sup>15</sup>, Kamikubo, Y.<sup>16</sup>, Kawaguchi, K.<sup>17</sup>, Watanabe, K.<sup>18</sup>, Yagyu, S.<sup>19</sup>, Iehara, T.<sup>20</sup>, Hosoi, H.<sup>21</sup>, Nakahata, T.<sup>22</sup>, Adachi, S.<sup>23</sup>, Umemoto, S.<sup>24</sup>, Heike, T.<sup>25</sup>, Takita, J.<sup>26</sup>*

Kyoto University, Department of Pediatrics, Graduate School of Medicine, Kyoto, Japan <sup>2</sup>, Kyoto university, Department of Pediatrics, Graduate School of Medicine, Kyoto, Japan<sup>3</sup>, Kyoto university, Department of Pediatric Surgery, Graduate School of Medicine, Kyoto, Japan<sup>4</sup>, Kyoto university, Department of Pediatrics, Graduate School of Medicine, Kyoto, Kyoto university<sup>5</sup>, Kyoto university, Department of Pediatrics, Graduate School of Medicine, Kyoto, Japan<sup>6</sup>, Kyoto university, Department of Pediatrics, Graduate School of Medicine, Kyoto, Japan<sup>7</sup>, Kyoto university, Department of Pediatrics, Graduate School of Medicine, Kyoto, Japan<sup>8</sup>, Kyoto university, Department of Pediatrics, Graduate School of Medicine, Kyoto, Japan<sup>9</sup>, Kyoto university, Department of Pediatrics, Graduate School of Medicine, Kyoto, Japan<sup>10</sup>, Kyoto university, Department of Pediatrics, Graduate School of Medicine, Kyoto, Japan<sup>11</sup>, Kyoto university, Department of Pediatric Surgery, Graduate School of Medicine, Kyoto, Japan, <sup>12</sup>, Kyoto university, Department of Pediatric Surgery, Graduate School of Medicine, Kyoto, Japan <sup>13</sup>, Kyoto university, Department of Pediatric Surgery, Graduate School of Medicine, Kyoto, Japan <sup>14</sup>, Kyoto university, Department of Human Health Science, Graduate School of Medicine, Kyoto, Japan <sup>15</sup>, Kyoto university, Department of Human Health Science, Graduate School of Medicine, Kyoto, Japan <sup>16</sup>, Shizuoka Children's Hospital, Department of Hematology and Oncology, Shizuoka, Japan <sup>17</sup>, Shizuoka Children's Hospital, Department of Hematology and Oncology, Shizuoka, Japan <sup>18</sup>, Kyoto Prefectural University of Medicine, Department of Pediatrics, Graduate School of Medical Science, Kyoto, Japan <sup>19</sup>, Kyoto Prefectural University of Medicine, Department of Pediatrics, Graduate School of Medical Science, Kyoto, Japan <sup>20</sup>, Kyoto Prefectural University of Medicine, Department of Pediatrics, Graduate School of Medical Science, Kyoto, Japan <sup>21</sup>, Kyoto university, Drug Discovery Technology Development Office, Center for iPS Cell Research and A, Kyoto, Japan <sup>22</sup>, Kyoto university, Department of Human Health Science, Graduate School of Medicine, Kyoto, Japan <sup>23</sup>, Kyoto university, Department of Pediatric Surgery, Graduate School of Medicine, Kyoto, Japan <sup>24</sup>, Kyoto university, Department of Pediatrics, Graduate School of Medicine, Kyoto, Japan <sup>25</sup>, Kyoto university, Department of Pediatrics, Graduate School of Medicine, Kyoto, Japan <sup>26</sup>

### **Background/Introduction:**

Neuroblastoma (NB) is the most common extracranial solid tumor of the childhood, which is thought to arise from neural crest-derived immature cells. Since the prognosis of patients with high-risk or recurrent/refractory NB remains quite poor despite the intensive multimodality therapy, novel therapeutic interventions are required. CD146 (also known as MCAM), a cell adhesion molecule expressed by neural crest and its derivatives, has been reported to be closely associated with tumor progression and metastasis in various types of malignancies.

### **Aims:**

The objective of the current study was to examine the expression of CD146 in NB cell lines and clinical samples, and to evaluate the anti-tumor effect of CD146-targeting treatment (shRNA knockdown of CD146 or anti-CD146 polyclonal antibody) for NB cells *in vitro* and *in vivo*.

### **Methods/Materials:**

The expression level of CD146 in NB cell lines and clinical samples was investigated by flow cytometric and immunohistochemical analysis. The effect of blocking CD146 was analyzed by our developed rabbit anti-CD146 neutralizing antibody and by shRNA knockdown of CD146 both *in vivo* and *in vitro*. The major pathways downstream of CD146 signaling was examined by western blotting assays in conditional CD146 knockdown NB cells.

### **Results:**

The current study demonstrated that CD146 was expressed by all six NB cell lines and the majority of any stage NB primary tumors. Blocking of CD146 pathway, either by treatment with an anti-CD146 polyclonal antibody or by shRNA knockdown, effectively suppressed tumor growth of NB cells both *in vitro* proliferation and *in vivo* tumor formation, principally due to increased apoptosis by focal adhesion kinase/nuclear factor kappa B signaling pathway.

Summary/Conclusions:

These results suggest that CD146 represents a promising therapeutic target for NB.

## P395 Defining the mechanisms of lorlatinib efficacy against ALK-mutated neuroblastomas

*Matkar, S.*<sup>2</sup>, *Stuhlmiller, T.*<sup>3</sup>, *Witek, G.*<sup>4</sup>, *Farrel, A.*<sup>5</sup>, *Larmour, C.*<sup>6</sup>, *D'Aulerio, A.*<sup>7</sup>, *Krytska, K.*<sup>8</sup>, *Patel, K.*<sup>9</sup>, *Sano, R.*<sup>10</sup>, *Rokita, J.*<sup>11</sup>, *Gerelus, M.*<sup>12</sup>, *Maris, J.*<sup>13</sup>, *Johnson, G.*<sup>14</sup>, *Mossé, Y.*<sup>15</sup>

Children's Hospital of Philadelphia, Oncology, Philadelphia, USA <sup>2</sup>, University of North Carolina at Chapel Hill, School of Medicine, Chapel Hill, USA<sup>3</sup>, Children's Hospital of Philadelphia, Oncology, Philadelphia, USA<sup>4</sup>, Children's Hospital of Philadelphia, Biomedical and Health Informatics, Philadelphia, Children's Hospital of Philadelphia<sup>5</sup>, Children's Hospital of Philadelphia, Oncology, Philadelphia, USA<sup>6</sup>, Children's Hospital of Philadelphia, Oncology, Philadelphia, USA<sup>7</sup>, Children's Hospital of Philadelphia, Oncology, Philadelphia, USA<sup>8</sup>, Children's Hospital of Philadelphia, Biomedical and Health Informatics, Philadelphia, USA<sup>9</sup>, Children's Hospital of Philadelphia, Oncology, Philadelphia, USA<sup>10</sup>, Children's Hospital of Philadelphia, J. Stokes Jr. Research Ins, Philadelphia, USA<sup>11</sup>, Children's Hospital of Philadelphia, Oncology, Philadelphia, USA, <sup>12</sup>, Children's Hospital of Philadelphia, Oncology, Philadelphia, USA <sup>13</sup>, University of North Carolina at Chapel Hill, School of Medicine, Chapel Hill, USA <sup>14</sup>, Children's Hospital of Philadelphia, Oncology, Philadelphia, USA <sup>15</sup>

### Background/Introduction:

Gain-of-function germline and somatic mutations in the anaplastic lymphoma kinase (*ALK*) oncogene has positioned it as the major tractable oncogene for targeted therapy in patients with neuroblastoma. *ALK* mutations are found mostly in three kinase domain hotspots. Although the R1275\* mutation shows sensitivity, F1174\* and F1245\* mutations confer *de novo* resistance to first and second generation *ALK* inhibitors including crizotinib. Lorlatinib, a third-generation *ALK*/ROS inhibitor, overcomes this *de novo* resistance, and is now in Phase 1 testing for patients with relapsed/refractory *ALK*-driven neuroblastoma.

### Aims:

To define the mechanisms underlying the superior efficacy of lorlatinib in *ALK*-mutated neuroblastomas in order to design rationale combination therapies designed to circumvent acquired resistance.

### Methods/Materials:

We employed Multiplexed Inhibitor Beads (MIBs) coupled with quantitative Mass Spectrometry (MS) to quantitatively measure kinase activity dynamics on a proteomic scale following *ALK* inhibition therapy. PDX models harboring F1174L (COG-N-453x), R1275Q (NB1643), R1245C (Felix) and amplified *ALK* (NB1) were treated with either crizotinib (100 mg/kg/day) or lorlatinib (10 mg/kg/day) for 2.5 days or 6.5 days prior to MIB/MS analysis. The results from MIB/MS analysis were validated by immunoblotting and qRT-PCR. We performed RNA sequencing in COG-N-453 and NB1643 PDX models to study the transcriptomic changes following treatment with crizotinib or lorlatinib. We used ChIP seq and ChIP qPCR to study enrichment of *MYCN* and Pol II at promoters of genes of interest.

### Results:

MIB/MS data showed that lorlatinib inhibited *ALK* a thousand-fold more potently than crizotinib in all models studied. In addition to *ALK*, there was an enrichment of kinases involved in G2/M transition that were significantly inhibited by lorlatinib and not altered by crizotinib, verified by qRT-PCR and immunoblotting in all four mouse models. Furthermore, lorlatinib also upregulated several tumor cell intrinsic and extrinsic kinases such as *Csf1r*, and *Pdgrfb* involved in innate immunity and *Ripk2*, *Tie2*, *Tbk1* and *Irak4* involved in inflammatory response. RNA sequencing in COG-N-453 and NB1643 PDX models showed downregulation of multiple *MYCN* target genes involved in cell cycle, DNA repair and metabolism following treatment with lorlatinib, accompanied by decreased expression of G2/M and mitotic genes including *PLK1*, *PLK4*, *CDK1*, *AURKA* and *AURKB*. Moreover, immunoblotting showed downregulation of *MYCN* in COG-N-453 and NB1643 cell lines upon treatment with lorlatinib for 6 hours. ChIP seq analysis of NB1643, Kelly, COG-N-415, LAN-5 and NGP cells showed enrichment of *MYCN* at the promoters of *PLK1*, *PLK4*, *CDK1*, *AURKA* and *AURKB* prior to *ALK* inhibition therapy,

and CHIP qPCR showed displacement of *MYCN* and Pol II from promoters of these G2/M kinases following 6.5 day treatment with lorlatinib in the COG-N-453 PDX model.

Summary/Conclusions:

Lorlatinib exhibits superior efficacy against ALK driven NB models by potent ALK inhibition, as well as direct downregulation of *MYCN* and multiple key G2/M cell cycle transition proteins. Whether and how *MYCN* is regulated by inhibition of ALK signaling and/or by inhibition of other kinases is currently under investigation.



## **P396 Degrading MYC by stabilizing a physiologic form of Aurora Kinase A**

Gustafson, W.C.<sup>2</sup>, Mukherjee, S.<sup>3</sup>, Hansel, T.<sup>4</sup>, Koach, J.<sup>5</sup>, Tu, C.<sup>6</sup>, Davini, M.<sup>7</sup>, Benes, C.<sup>8</sup>, Shokat, K.<sup>9</sup>, Weiss, W.<sup>10</sup>

UCSF, Pediatric Hematology/Oncology, San Francisco, USA <sup>2</sup>, UCSF, Pediatrics, San Francisco, USA<sup>3</sup>, UCSF, Pediatric Hematology/Oncology, San Francisco, USA<sup>4</sup>, UCSF, Pediatrics, San Francisco, UCSF<sup>5</sup>, UCSF, Pediatrics, San Francisco, USA<sup>6</sup>, UCSF, Pediatrics, San Francisco, USA<sup>7</sup>, Massachusetts General Hospital, Medicine, Boston, Mass, USA<sup>8</sup>, UCSF, Pharmacology, San Francisco, USA<sup>9</sup>, UCSF, Neurology, San Francisco, USA<sup>10</sup>

### Background/Introduction:

The MYC family of transcription factors/oncoprotein family members MYC and *MYCN* play key roles in neuroblastoma biology and oncogenesis. Increased expression of MYC proteins delineates a highly aggressive high risk subset of neuroblastoma and MYC proteins are prominent drug targets in cancer, however they are widely regarded as undruggable directly. Overexpression of *MYCN* and MYC is often genetic, by amplification of *MYCN* or enhancer hijacking of the MYC promoter, however MYC proteins are tightly regulated at the level of protein stability. Proteolysis of *MYCN* is blocked by interaction with the mitotic Aurora Kinase A (AURKA).

### Aims:

Here we aim to delineate the mechanism of action of CD532, a MYC targeted inhibitor of Aurora Kinase A, and define physiologic binding partners for MYC and *MYCN* in neuroblastoma.

### Methods/Materials:

Cell lines and tissue culture work were done under standard, previously published methods. CD532 was obtained from an in house generated supply. Proximity ligation assay and co-IP-western blots were done as previously described. Structural clustering of AURKA was done using RStudio suite and principle component analysis using FactoMineR package.

### Results:

We previously described CD532, an AURKA inhibitor that binds to AURKA and alters its tertiary structure to dissociate *MYCN* from AURKA, driving degradation. *MYCN* and AURKA interact in S phase, whereas MYC and AURKA interact in early G2. Nevertheless, we verify here that both MYC and *MYCN* are both dissociated from AURKA by CD532, resulting in degradation of both oncoproteins. Analysis of 140 available human AURKA structures indicates that AURKA bound to CD532 closely resembles the structure of AURKA bound to its physiological interacting partner, TACC3. Through co-immunoprecipitation and proximity ligation assays, we demonstrate using proximity ligation assay and co-immunoprecipitation that CD532 destabilizes interactions between MYC or *MYCN* and AURKA, while in parallel stabilizing interactions between TACC3 and AURKA; and that TACC3 and MYC or *MYCN* interact with AURKA in a mutually exclusive manner. Our studies clarify that CD532 drives AURKA into a conformation found normally in biology. This CD532-stabilized conformation promotes interactions between TACC3 and AURKA and blocks binding of MYC or *MYCN* to AURKA, driving degradation of unbound MYC or *MYCN* in the proteasome.

### Summary/Conclusions:

Here we have shown that AURKA adopts a physiologically relevant structural conformation to stimulate dissociation from both MYC and *MYCN* in neuroblastoma and leukemia cells which is stabilized by a unique tool inhibitor, CD532, to potently promote MYC and *MYCN* degradation.

## **P397 High-throughput drug screening of high-risk neuroblastoma PDX-derived tumor cells**

*Jiménez, J.*<sup>2</sup>, *Voungny, J.*<sup>3</sup>, *Anthony, E.*<sup>4</sup>, *Lescure, A.*<sup>5</sup>, *Gestraud, P.*<sup>6</sup>, *Bhalshankar, J.*<sup>7</sup>, *Bellini, A.*<sup>8</sup>, *Surdez, D.*<sup>9</sup>, *Janoueix-Lerosey, I.*<sup>10</sup>, *Pierre-Eugène, C.*<sup>11</sup>, *Georger, B.*<sup>12</sup>, *Carcaboso, A.M.*<sup>13</sup>, *Marques Da Costa, M.E.*<sup>14</sup>, *Pierron, G.*<sup>15</sup>, *Saint-Charles, A.*<sup>16</sup>, *Lapouble, E.*<sup>17</sup>, *Delattre, O.*<sup>18</sup>, *Del Nery, E.*<sup>19</sup>, *Schleiermacher, G.*<sup>20</sup>

Institut Curie, RTOP team, Paris, France <sup>2</sup>, Institut Curie, Biophenics platform, Paris, France<sup>3</sup>, Institut Curie, Biophenics platform, Paris, France<sup>4</sup>, Institut Curie, Biophenics platform, Paris, Institut Curie<sup>5</sup>, Institut Curie, Bioinformatics platform U900, Paris, France<sup>6</sup>, Institut Curie, RTOP team, Paris, France<sup>7</sup>, Institut Curie, RTOP team, Paris, France<sup>8</sup>, Institut Curie, U830, Paris, France<sup>9</sup>, Institut Curie, U830, Paris, France<sup>10</sup>, Institut Curie, U830, Paris, France<sup>11</sup>, Institut Curie, Département d'Oncologie Pédiatrique, Paris, France, <sup>12</sup>, Hospital Sant Joan de Deu, Institut de Recerca Sant Joan de Deu, Barcelona, Spain <sup>13</sup>, Gustave Roussy, Vectorology and Anticancer Therapies, UMR 8203, Paris, France <sup>14</sup>, Institut Curie, Unité de Génétique Somatique, Paris, France <sup>15</sup>, Institut Curie, RTOP team, Paris, France <sup>16</sup>, Institut Curie, Unité de Génétique Somatique, Paris, France <sup>17</sup>, Institut Curie, U830, Paris, France <sup>18</sup>, Institut Curie, Biophenics platform, Paris, France <sup>19</sup>, Institut Curie, RTOP team, Paris, France <sup>20</sup>

### **Background/Introduction:**

Pediatric precision oncology platforms in Europe have analyzed over 1,000 pediatric cancers by next generation sequencing. While potential targets can be identified in 50% of cases, the remaining patients lack actionable alterations. This occurs particularly in high-risk neuroblastoma (HR-NB), indicating significant, currently unmet needs in precision medicine. Patient-derived tumor xenograft (PDX) mouse models have been established within these platforms. Translating ex vivo drug profiling results in combination with genetic data into clinical application has been successful in small pilot series, providing proof-of-concept for further systematic evaluation of the functional precision medicine approach.

### **Aims:**

The aim of this project is to validate the feasibility of high-throughput drug screening on HR-NB PDX-derived tumor cells (PDTCs). Our ultimate goal is to improve current therapeutic approaches in HR-NB by establishing next generation drug sensitivity and resistance profiling in PDTCs.

### **Methods/Materials:**

We established a biobank of 15 HR-NB PDXs with known genetic profiles and at least 1 actionable mutation (range 1-4). PDTCs were obtained by mechanical/enzymatic dissociation and plated in 384-well plates. The proportion of tumor content in PDTCs was determined by RT-qPCR. Drugs were added 24 hours after plating (D1) at 8 different concentrations each. We screened four HR-NB models using commercially available arrayed drug library representing 182 epigenetic compounds and evaluated their effect on cell survival. We also compared the effect of those drugs to common chemotherapy used in NB and other selected targeted agents. Drugs were administered in monotherapy, unless chemotherapy (monotherapy/combination). Cell viability was measured by Cell-titerGlo® at plating (D0), before drug addition (D1) and at D4. Drug efficacy was assessed by quantitative drug sensitivity scoring and half-maximal effective concentration.

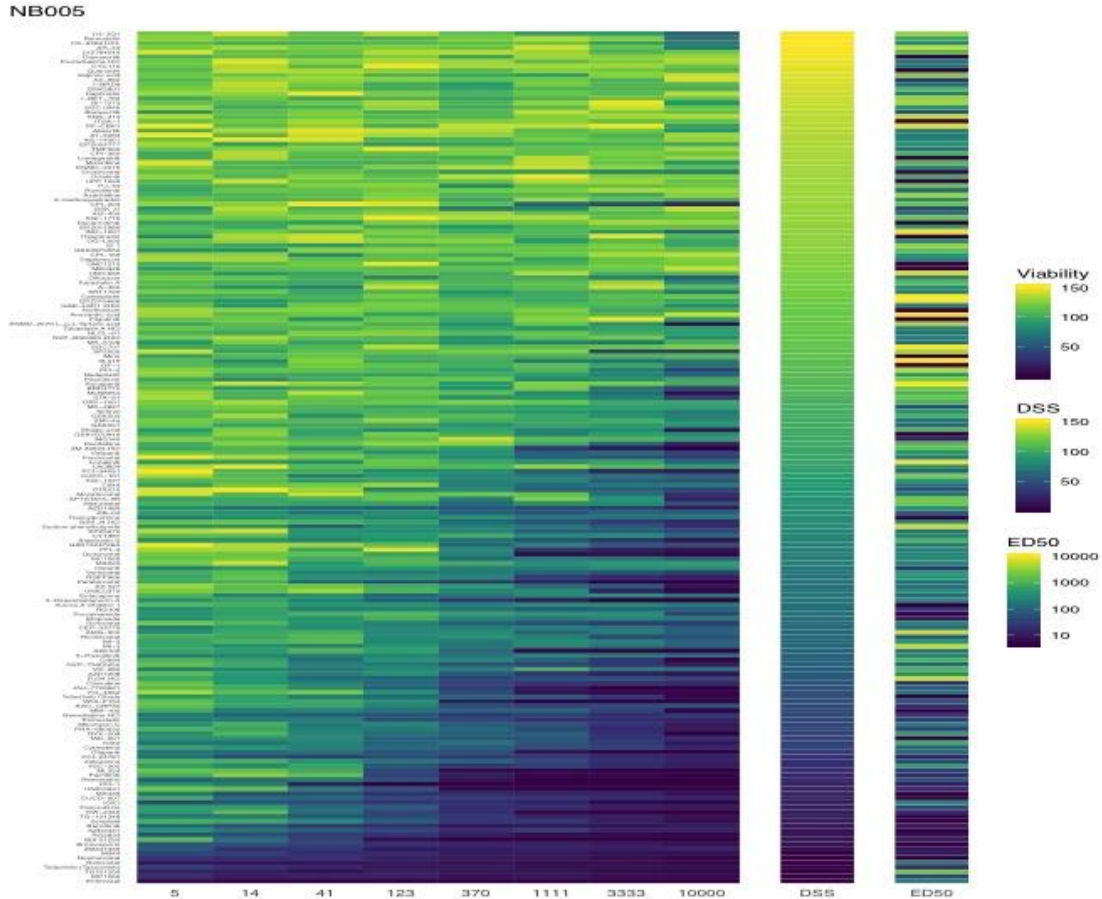
### **Results:**

A median number of  $143.10^6$  [ $42.10^6$ - $376.10^6$ ] cells/tumor was obtained, with a median viability of 83,5% [82-88]. Models HSJD-NB004 and HSJD-NB005, *MYCN* amplified-NB PDX established at diagnosis and relapse from the same patient, harbored *TP53* (c.517G>T), *ARID1B* (c.3968G>A) and *NF1* (c.7669C>T) mutations, with HSJD-NB005 harboring additional *ROS1* (c.1114A>G) mutation. MAP099 model harbored *MYCN/ALK* amplifications, with homologous deletion of *CDKN2A/B*. Model MAP422 showed high mutational load (33 mutations/Mb).

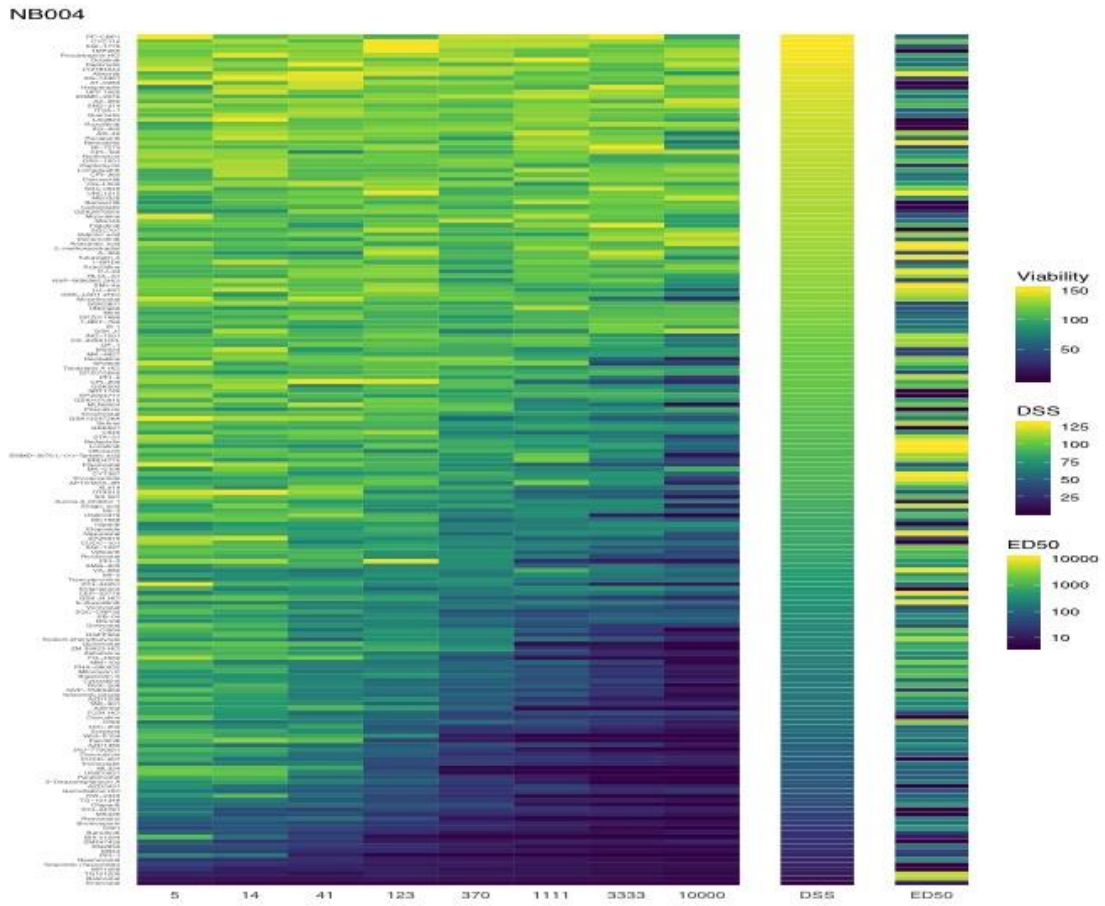
Our drug sensitivity results, available for 2 models, showed differential effects on the proliferation of HSJD-NB004 and HSJD-NB005 models. Surprisingly, both models were resistant to etoposide and carboplatin in monotherapy, and were also relatively resistant to the combined treatment. In contrast, both models responded at relatively high concentrations of doxorubicin. The effect of doxorubicin combined to etoposide was very similar to the effect induced by doxorubicin-carboplatin, in a dose-dependent manner. High sensitivity (top 10 efficacy drugs) was found to HDAC, JAK/STAT and BET inhibitors. Interestingly, lorlatinib showed no effect in HSJD-NB-005. Additional studies are needed to explain the different sensitivity of both models. Taken together, our results suggest that inhibition of HDAC, JAK/STAT pathway or BET proteins may provide an effective strategy to treat a subset of HR-NB.

Summary/Conclusions:

We propose that drug response profiling of PDTs by high-throughput drug screening will provide additional key information for precision pediatric oncology. We think this approach will push forward the development of innovative clinical trials involving personalized drug sensitivity profiles as biomarkers for trial inclusion.



Caption 1: Heat-map of cell viability based on DSS at 8 different concentrations of 200 compounds for PDX model HSJD-NB-004



Caption 2: Heat-map of cell viability based on DSS at 8 different concentrations of 200 compounds for PDX model HSJD-NB-005

## **P398 Addition of Lorlatinib to induction chemotherapy significantly prolongs survival in ALK-mutant murine neuroblastoma models**

*Calton, E.A.*<sup>2</sup>, *Martins da Costa, B.*<sup>3</sup>, *Barker, K.*<sup>4</sup>, *Gao, Q.*<sup>5</sup>, *Tucker, E.R.*<sup>6</sup>, *George, S.*<sup>7</sup>, *Poon, E.*<sup>8</sup>, *Chesler, L.*<sup>9</sup>

The Institute of Cancer Research, Paediatric Tumour Biology Team, London, United Kingdom<sup>2</sup>, The Institute of Cancer Research, Paediatric Tumour Biology Team, London, United Kingdom<sup>3</sup>, The Institute of Cancer Research, Paediatric Tumour Biology Team, London, United Kingdom<sup>4</sup>, The Institute of Cancer Research, Bioinformatics Core, London, The Institute of Cancer Research<sup>5</sup>, The Institute of Cancer Research, Paediatric Tumour Biology Team, London, United Kingdom<sup>6</sup>, The Institute of Cancer Research, Paediatric Tumour Biology Team, London, United Kingdom<sup>7</sup>, The Institute of Cancer Research, Paediatric Tumour Biology Team, London, United Kingdom<sup>8</sup>, The Institute of Cancer Research, Paediatric Tumour Biology Team, London, United Kingdom<sup>9</sup>

### Background/Introduction:

14% of high-risk neuroblastoma tumours carry activating mutations in *ALK* at diagnosis. This genotype carries an increased risk of relapse after first-line treatment, and evidence from paired tumour biopsies shows enrichment of *ALK*-mutant clones at relapse.

*ALK* mutation provides a survival advantage to neuroblastoma cells via both pro-proliferative signalling, and inhibition of apoptosis. Selection for *ALK*-mutant sub-clones at relapse may therefore be caused by their relative resistance to cell death during cytotoxic treatment.

Targeted *ALK* inhibitors have been trialled in relapsed paediatric cancers and the first-generation inhibitor Crizotinib has now entered a Phase III study for newly-diagnosed *ALK*-mutant neuroblastoma. However, our laboratory has previously demonstrated increased efficacy of the third-generation inhibitor Lorlatinib compared to Crizotinib as a single agent *in vivo*.

### Aims:

To evaluate the preclinical efficacy of Lorlatinib in combination with cytotoxic chemotherapy in *in vivo* models of *ALK*-mutant neuroblastoma, and to test the hypothesis that Lorlatinib is more effective than Crizotinib when given in combination with multi-agent induction chemotherapy.

### Methods/Materials:

Mice from the Th-*ALK*<sup>F1174L</sup>/Th-*MYCN* murine neuroblastoma model were enrolled with established intra-abdominal tumours. Each received a single intraperitoneal dose of chemotherapy (Vincristine, Doxorubicin and Cyclophosphamide, 'VAC') on day 1. Oral Lorlatinib (10mg/kg/day) or vehicle was commenced on day 2 and continued until mice required euthanasia. MRI was performed weekly to measure tumour volume.

Syngenic mice were injected subcutaneously with untreated cells derived from the Th-*ALK*<sup>F1174L</sup>/Th-*MYCN* model to provide a predictable, robust *in-vivo* model for multi-arm, multi-agent preclinical studies. When tumours were >8mm mean diameter, they received a single dose of VAC or vehicle, followed by continuous oral dosing of either Lorlatinib (10mg/kg/day), Crizotinib (100mg/kg/day) or vehicle from day 2 until day 56 (or until euthanasia was required).

Frozen tumour tissue was collected at necropsy for RNA-Seq analysis and compared to publically-available data from human neuroblastoma.

### Results:

Addition of Lorlatinib to chemotherapy significantly prolonged median survival in the Th-*ALK*<sup>F1174L</sup>/Th-*MYCN* transgenic mouse from 13 days to 33.5 days ( $p=0.0005$ ). MRI showed prolonged reduction in tumour burden in animals receiving the combination.

In subcutaneous Th-*ALK*<sup>F1174L</sup>/Th-*MYCN* allografts, this effect was confirmed (median survival 22.4 weeks vs 5.9 weeks,  $p=0.0067$ ). 3 of 4 animals receiving VAC and Lorlatinib combined had durable complete responses, with no tumour evident at necropsy. For animals treated with Crizotinib in addition to VAC, no survival benefit was observed (median survival 5.8 weeks vs 5.9 weeks).

RNA-Seq of untreated Th-*ALK*<sup>F1174L</sup>/Th-*MYCN* tumours showed increased expression of anti-apoptotic *BCL2* family members compared to control tumours from Th-*MYCN* single-transgenic murine neuroblastoma. We identified the same anti-apoptotic pattern in human neuroblastomas expressing high levels of *ALK* mRNA, suggesting this is a clinically-relevant effect of ALK activation. We are currently analysing these anti-apoptotic markers in treated murine tumours to confirm their relationship to treatment response.

#### Summary/Conclusions:

The addition of Lorlatinib, but not Crizotinib, to VAC chemotherapy significantly prolongs survival in *ALK*-mutant murine neuroblastoma. This strongly supports development of clinical trials combining Lorlatinib with standard-of-care induction chemotherapy in children with high-risk neuroblastoma that is *ALK*-mutant at diagnosis.

## P399 ALK mutations in primary and relapsed neuroblastoma in a GPOH cohort

*Rosswog, C.*<sup>2</sup>, *Fassunke, J.*<sup>3</sup>, *Schömig-Markiefka, B.*<sup>4</sup>, *Merkelbach-Bruse, S.*<sup>5</sup>, *Ackermann, S.*<sup>6</sup>, *Ortmann, M.*<sup>7</sup>, *Schulte, J.H.*<sup>8</sup>, *Westermann, F.*<sup>9</sup>, *Berthold, F.*<sup>10</sup>, *Simon, T.*<sup>11</sup>, *Hero, B.*<sup>12</sup>, *Fischer, M.*<sup>13</sup>

University Children's Hospital, Experimental Pediatric Oncology, Cologne, Germany<sup>2</sup>, University Hospital of Cologne, Institute of Pathology, Cologne, Germany<sup>3</sup>, University Hospital of Cologne, Institute of Pathology, Cologne, Germany<sup>4</sup>, University Hospital of Cologne, Institute of Pathology, Cologne, Germany<sup>5</sup>, University Children's Hospital, Experimental Pediatric Oncology, Cologne, Germany<sup>6</sup>, University Hospital of Cologne, Institute of Pathology, Cologne, Germany<sup>7</sup>, Charité-Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology, Berlin, Germany<sup>8</sup>, German Cancer Research Center (DKFZ), Neuroblastoma Genomics, Heidelberg, Germany<sup>9</sup>, University Children's Hospital, Pediatric Oncology and Hematology, Cologne, Germany<sup>10</sup>, University Children's Hospital, Pediatric Oncology and Hematology, Cologne, Germany<sup>11</sup>, University Children's Hospital, Pediatric Oncology and Hematology, Cologne, Germany,<sup>12</sup> University Children's Hospital, Experimental Pediatric Oncology, Cologne, Germany<sup>13</sup>

### Background/Introduction:

In sporadic neuroblastoma, *ALK* mutations are found in 8-10% of all cases. Previous studies suggested a higher prevalence of *ALK* mutations in the high- and intermediate-risk group whereas others reported an almost even distribution among subgroups. Reliable information on the prevalence of *ALK* mutations and their association with clinical phenotypes is of increasing relevance to neuroblastoma diagnostics as *ALK* inhibitors are on their way to first-line treatment.

### Aims:

We here aimed to investigate *ALK* mutations frequencies in a large cohort of neuroblastoma samples at diagnosis and relapse and determine their associations with clinical variables.

### Methods/Materials:

A cohort of 841 neuroblastoma samples (683 at diagnosis; high-risk, n=325, non-high-risk, n=358; 158 at relapse; high-risk, n=117, non-high-risk, n=41) was analyzed by dideoxy-sequencing, whole-exome sequencing, or panel sequencing for the presence of *ALK* mutations. For 49 patients, paired samples from diagnosis and relapse were available, which were analyzed by panel sequencing with a minimum sequencing depth of 200x and a minor allele frequency of 5%. Mutations found by next generation sequencing within this cohort were validated by digital droplet PCR (ddPCR) or dideoxy-sequencing.

### Results:

*ALK* mutations were found in 10.5% (72/683) of neuroblastoma samples at diagnosis. In the relapse cohort, *ALK* mutations were detected in 17.1% (27/158). Analyses of paired samples revealed *ALK* mutations in 6 patients both at diagnosis and at relapse (12.2%). In two other paired samples, mutations were detected only at relapse, which were confirmed as *de novo* mutations by ddPCR. The *ALK* mutation frequency of initial high-risk and non-high-risk tumors did not differ (10.8% vs. 10.3%, p=0.90). In the entire cohort, *ALK* mutations were not associated with *MYCN* amplification status or age at diagnosis. In the non-high risk cohort, however, patients whose tumors harbored *ALK* mutations were significantly older than patients with *ALK* wild-type tumors (15.8 vs. 10.5 months, p=0.032). Three-year overall survival of patients with *ALK*-mutated tumors was slightly poorer than that of patients with *ALK* wild-type tumors in the entire cohort (0.72±0.05 vs. 0.84±0.02, p=0.055). This difference in patient survival was due to worse outcome of high-risk patients with *ALK*-mutated tumors as compared to high-risk patients with *ALK* wild-type tumors (0.53±0.09 vs. 0.67±0.03, p=0.036), whereas survival of non-high-risk patients was not associated with the *ALK* mutation status (0.92±0.05 vs. 0.98±0.01, p=0.278).

### Summary/Conclusions:

No differences were observed for *ALK* mutations frequencies in high- and non-high risk neuroblastoma in this cohort, whereas the prevalence of *ALK* mutations is higher at relapse, emphasizing the need of re-sequencing of tumors at relapse. Association of *ALK* mutations with poor overall survival was observed only in high-risk patients, but not in non-high-risk patients.



## **P400 Targeting polyamine addiction in neuroblastoma: optimizing combined inhibition of polyamine biosynthesis and transport as a novel therapeutic approach**

*Haber, M.*<sup>2</sup>, *Murray, J.*<sup>3</sup>, *Xiao, L.*<sup>4</sup>, *Pandher, R.*<sup>5</sup>, *Kusuma, F.*<sup>6</sup>, *Kearns, A.*<sup>7</sup>, *Brand, J.*<sup>8</sup>, *Mosmann, E.*<sup>9</sup>, *Vanhoutte, R.*<sup>10</sup>, *Verhelst, S.*<sup>11</sup>, *Martin, S.*<sup>12</sup>, *Vangheluwe, P.*<sup>13</sup>, *Burns, M.*<sup>14</sup>, *Hogarty, M.*<sup>15</sup>, *Ziegler, D.*<sup>16</sup>, *Norris, M.*<sup>17</sup>

Children's Cancer Institute, Molecular Targets and Cancer Therapeutics, Sydney, Australia <sup>2</sup>,  
Children's Cancer Institute, Molecular Targets and Cancer Therapeutics, Sydney, Australia<sup>3</sup>,  
Children's Cancer Institute, Molecular Targets and Cancer Therapeutics, Sydney, Australia<sup>4</sup>,  
Children's Cancer Institute, Molecular Targets and Cancer Therapeutics, Sydney, Children's Cancer  
Institute<sup>5</sup>, Children's Cancer Institute, Molecular Targets and Cancer Therapeutics, Sydney, Australia<sup>6</sup>,  
Children's Cancer Institute, Molecular Targets and Cancer Therapeutics, Sydney, Australia<sup>7</sup>,  
Children's Cancer Institute, Molecular Targets and Cancer Therapeutics, Sydney, Australia<sup>8</sup>,  
Children's Cancer Institute, Experimental Therapeutics, Sydney, Australia<sup>9</sup>, Katholieke Universiteit  
Leuven, Department of Cellular and Molecular Medicine, Leuven, Belgium<sup>10</sup>, Katholieke Universiteit  
Leuven, Department of Cellular and Molecular Medicine, Leuven, Belgium<sup>11</sup>, Katholieke Universiteit  
Leuven, Department of Cellular and Molecular Medicine, Leuven, Belgium, <sup>12</sup>, Katholieke Universiteit  
Leuven, Department of Cellular and Molecular Medicine, Leuven, Belgium <sup>13</sup>, Aminex Therapeutics,  
Aminex, Boston, USA <sup>14</sup>, Children's Hospital of Philadelphia, Division of Oncology, Philadelphia, USA  
<sup>15</sup>, Children's Cancer Institute, Molecular Targets and Cancer Therapeutics, Sydney, Australia <sup>16</sup>,  
Children's Cancer Institute, Molecular Targets and Cancer Therapeutics, Sydney, Australia <sup>17</sup>

### Background/Introduction:

Polyamines are essential cations frequently upregulated in tumours for survival, proliferation, and immune evasion. *MYCN* directly regulates the rate-limiting enzyme in polyamine synthesis, ornithine decarboxylase 1 (ODC1). Treatment of neuroblastoma cells with the ODC1 inhibitor difluoromethylornithine (DFMO), although a promising therapeutic strategy, is only partially effective at impeding cell growth due to activation of compensatory mechanisms resulting in increased polyamine uptake from the surrounding microenvironment. We have recently identified the solute carrier SLC3A2 as a key transporter responsible for the compensatory polyamine uptake induced by DFMO. We have further shown that the polyamine transport inhibitor, AMXT 1501, which blocks polyamine uptake, can substantially enhance DFMO efficacy both *in vitro* and in neuroblastoma mouse models (Sci. Trans. Med. 2019), opening the way for clinical trials of combined inhibition of polyamine synthesis and uptake. SLC3A2 is known to frequently dimerise with one of a number of light chains to form a transporter complex, but it is unclear if any of these cotransporters are involved in polyamine uptake, and as such, whether further benefit could be obtained by relevant co-transporter inhibition.

### Aims:

To further elucidate the mechanisms underlying polyamine uptake in neuroblastoma by identifying SLC3A2 binding partners that contribute to polyamine uptake.

### Methods/Materials:

Colony and cytotoxic assays were used to determine tumour cell growth. CRISPR-Cas9 technology was used to create SLC3A2 knockout neuroblastoma cell lines. Polyamine uptake was measured by using radiolabelled and fluorescent polyamines.

### Results:

Among the six light-chain proteins that are known to form binding partnerships with SLC3A2, we have shown that high expression of three of these binding partners (SLC7A5, A6 and A10), like SLC3A2, is strongly associated with poor prognosis in primary neuroblastoma ( $P < 0.001$ ). Of these, the L-type amino acid transporter SLC7A5, which is the most extensively studied SLC3A2 cotransporter, is highly expressed in a broad range of cancers. Expression of SLC7A5 is strongly correlated with SLC3A2 in both primary human neuroblastoma tumors ( $R^2 = 0.645$ ,  $P < 0.0001$ ) and PDX samples ( $R^2 = 0.538$ ,

P = 0.0005). Like AMXT 1501, inhibition of SLC7A5 with the selective inhibitor, JPH-203, synergises with DFMO to reduce colony formation in neuroblastoma cell lines (P = 0.0249), with this effect being more pronounced in SLC3A2 knockout cells. We have further developed a rapid, simple FACS-based polyamine uptake assay using fluorescently labelled polyamine species, producing comparable results to those obtained through the traditional radiolabelled polyamine uptake assay, which is potentially valuable for biomarker studies in the clinical context.

#### Summary/Conclusions:

Our results suggest that inhibiting the SLC3A2 co-transporter SLC7A5 may synergise with DFMO to reduce neuroblastoma growth. We are currently conducting *in vivo* studies to determine the optimal combination of polyamine transport and synthesis inhibitors, in preparation for clinical trial.

## **P401 Combined MDM2 and Survivin inhibition as a new therapeutic strategy in neuroblastoma**

*Baum, C.<sup>2</sup>, Schmelz, K.<sup>3</sup>, Proba, J.<sup>4</sup>, Henssen, A.G.<sup>5</sup>, Eggert, A.<sup>6</sup>, Schulte, J.<sup>7</sup>, Hundsdoerfer, P.<sup>8</sup>*

Department of Pediatric Hematology/Oncology/Stem Cell Transplantation, Charité-Universitätsmedizin Berlin, Berlin, Germany <sup>2</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology/Oncology/Stem Cell Transplantation, Berlin, Germany<sup>3</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology/Oncology/Stem Cell Transplantation, Berlin, Germany<sup>4</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology/Oncology/Stem Cell Transplantation, Berlin, Charité - Universitätsmedizin Berlin<sup>5</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology/Oncology/Stem Cell Transplantation, Berlin, Germany<sup>6</sup>, Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology/Oncology/Stem Cell Transplantation, Berlin, Germany<sup>7</sup>, Helios Klinikum Berlin-Buch, Department of Pediatrics, Berlin, Germany<sup>8</sup>

### Background/Introduction:

Inactivation of TP53 is frequently caused by amplification and overexpression of TP53 negative regulators, of which the best studied is MDM2. Targeted activation of the TP53 pathway via inhibition of MDM2-TP53 interaction has been extensively studied in neuroblastoma. The prioritized MDM2 inhibitor Idasanutlin (RG7388) has demonstrated high efficacy in neuroblastoma and entered a pediatric clinical trial. However, single agent treatment with MDM2 inhibitors has been reported to result in therapy resistances through acquisition of *de novo* mutations. Survivin is encoded by the baculoviral inhibitor of apoptosis repeat-containing (*BIRC5*) gene that is mapped to chromosome 17q25, a region with a frequent copy number gain in high-risk neuroblastoma. Survivin overexpression is correlated with poor patient prognosis, metastatic spread and resistance to chemotherapy, making it an attractive drug target. YM155 is a small molecule drug that targets Survivin on a transcriptional level and demonstrates activity against a broad range of cancer entities.

### Aims:

It is of high interest to preclinically investigate effective combination therapies, to achieve more durable therapy responses while reducing toxicity. Therefore, we evaluated the effect of the MDM2 inhibitor Idasanutlin in combination with the Survivin inhibitor YM155 and further characterized the synergistic effects of the combination therapy in order to create a basis for further clinical application.

### Methods/Materials:

The effects of the YM155/ Idasanutlin combination treatment on cell viability, migration and apoptosis was investigated *in vitro* using established neuroblastoma cell lines with varying genetic backgrounds. The effects of the combination treatment on neuroblastoma cell viability was investigated using XTT. Cell viability values were converted to relative compound effect values and CI values were calculated, using CompuSyn software according to Chou & Talalay (1984). Scratch Assays were performed to investigate the effects of the combination treatment on migration. Apoptosis induction was detected by flow cytometry using Annexin/PI and Caspase-3 antibody and by immunoblot based detection of PARP cleavage. TP53 dependency was tested using neuroblastoma cell lines overexpressing a dominant negative TP53 mutant. Mode-of-action analysis was performed using western blotting experiments.

### Results:

We evaluated the effect of the MDM2 inhibitor Idasanutlin in combination with the Survivin inhibitor YM155 and observed high degrees of synergism in a preclinical neuroblastoma model. We demonstrated that the combination of MDM2 and Survivin inhibition consistently reduces viability and resulted in highly synergistic CI values in neuroblastoma cell lines with varying genetic backgrounds. Migration capability of neuroblastoma cells was reduced when cells were treated with the combination therapy. Flow cytometric detection of annexin V/propidium iodide (PI) staining and caspase 3 activity, as well as immunoblotting based detection of cleaved PARP showed that the combination treatment enhances the apoptosis induction in neuroblastoma cell lines in a dose-dependent manner. Stable,

ectopic dn-TP53 expression attenuated the Idasanutlin single agent treatment effect but did not affect the YM155 or combination treatment mediated apoptosis-induction.

**Summary/Conclusions:**

In conclusion, our preclinical data provide a rational framework for a combination therapy consisting of Idasanutlin and YM155 for high risk neuroblastoma treatment.

## **P402 NRAS status determines sensitivity to SHP2 inhibitor-combination therapies targeting the RAS-MAPK pathway in neuroblastoma**

*Irwin, M.*<sup>2</sup>, *Valencia-Sama, I.*<sup>3</sup>, *Ladmour, Y.*<sup>4</sup>, *Kee, L.*<sup>5</sup>, *Adderley, T.*<sup>6</sup>, *Christopher, G.*<sup>7</sup>, *Kano, Y.*<sup>8</sup>, *Ohh, M.*<sup>9</sup>

Hospital for Sick Children, Pediatrics, Toronto, Canada<sup>2</sup>, University of Toronto, Laboratory Medicine and Pathobiology, Toronto, Canada<sup>3</sup>, University of Toronto, Medical Biophysics, Toronto, Canada<sup>4</sup>, Hospital for Sick Children, Paediatrics, Toronto, Hospital for Sick Children<sup>5</sup>, Hospital for Sick Children, Paediatrics, Toronto, Canada<sup>6</sup>, Hospital for Sick Children, Paediatrics, Toronto, Canada<sup>7</sup>, University of Toronto, Laboratory Medicine and Pathobiology, Toronto, Canada<sup>8</sup>, University of Toronto, Laboratory Medicine and Pathobiology, Toronto, Canada<sup>9</sup>

### Background/Introduction:

Survival for high-risk neuroblastoma (NB) remains poor and treatment for relapsed disease rarely leads to long-term cures. Large sequencing studies of NB tumors from diagnosis have not identified common targetable driver mutations other than the 10% of tumors that harbor mutations in the anaplastic lymphoma kinase (*ALK*) gene. However, at NB recurrence more frequent mutations in genes in the RAS-MAPK pathway have been detected. The *PTPN11*-encoded tyrosine phosphatase SHP2 is an activator of the RAS pathway, and we and others have shown that pharmacologic inhibition of SHP2 suppresses the growth of various tumor types harboring KRAS and HRAS mutations such as pancreatic and lung cancers.

### Aims:

We hypothesized that SHP2 inhibitors might inhibit growth of NB cells and that sensitivity may depend on the genetic status of *RAS*, *PTPN11* and other components of the RAS-MAPK pathway signaling. Thus, we assessed the sensitivity of multiple SHP2 inhibitors alone and in combination with drugs targeting RAS-MAPK signaling *in vitro* and *in vivo* using cells with and without different RAS pathway activating mutations.

### Methods/Materials:

The sensitivities of three SHP2 inhibitors (NSC-87877, II-B08, and SHP099) were assessed in a panel of human NB cell lines with diverse genetic backgrounds, including mutations in the neuroblastoma *RAS* oncogene (*NRAS*) as well as *NF1* and *ALK*. We also generated isogenic NB cells with endogenous wild-type *NRAS* (*NRAS*<sup>wt</sup>) expression that stably overexpressed *NRAS*<sup>wt</sup> or mutant *NRAS* (*NRAS*<sup>mt</sup>) proteins. Growth inhibition of ranges of doses of SHP2 inhibitors alone and in combination with trametinib, vemurafenib, and ulixertinib were determined *in vitro* using Alamar blue and bromodeoxyuridine incorporation assays, and synergy was determined using excess-over-bliss methods. Downstream RAS-MAPK signaling was assessed by western immunoblot. NB xenografts were generated in NOD-SCID mice and treated with SHP099 alone and in combination with trametinib and tumor measurements and bioluminescence imaging were analyzed.

### Results:

We observed inhibition of growth and downstream RAS-MAPK signaling in NB cells in response to treatment with the SHP2 inhibitors NSC-87877, II-B08, and SHP099. Cells with RAS pathway alterations, most notably *NRAS*<sup>Q61K</sup>, were less sensitive to all three SHP2 pharmacologic inhibitors. Furthermore, in contrast to cells with wild-type *NRAS*, isogenic NB cells engineered to overexpress *NRAS*<sup>Q61K</sup> were more resistant to SHP099 and II-B08. Interestingly, dual inhibition of SHP2 and RAS effectors MEK, RAF, or ERK with trametinib, vemurafenib, and ulixertinib, respectively, showed synergistic effects *in vitro* in NB cells harboring RAS-activating mutations and reversed SHP2 inhibitor resistance. Using xenograft models we also demonstrated that, in comparison to vehicle or single agent treatment, combinations of SHP099 with the MEK inhibitor trametinib reduced tumor volume and increased survival *in vivo*. Immunoblots of treated tumor lysates showed increased apoptosis and

potent reduction of p-SHP2, p-MEK and p-ERK levels in mice treated with SHP099 and trametinib in comparison to vehicle or either drug alone.

Summary/Conclusions:

These results suggest that combination therapies of SHP2 inhibitors with other drugs that target the RAS-MAPK pathway may be effective in relapsed NB including those that acquire RAS-MAPK pathway mutations.

## **P404 The Emergence of a Novel Scientific Society: Dynamics of Collaboration between Scientists at the Neuroblastoma Research Congresses between 1975 and 2016**

*Wittek, M.*<sup>2</sup>, *Berthold, F.W.*<sup>3</sup>, *Christoph, C.B.*<sup>4</sup>

Institute of Sociology and Socialpsychology, University of Cologne, Cologne, Germany <sup>2</sup>, University of Cologne, Department of Pediatric Oncology and Hematology, Cologne, Germany<sup>3</sup>, University of Cologne, Department of Pediatric Oncology and Hematology, Cologne, Germany<sup>4</sup>

### Background/Introduction:

This study investigates how collaborations among scientists in the field of neuroblastoma research developed throughout 41 years. Based on abstracts submitted to the ANR conference series we constructed a network of collaboration by inferring co-author relationships between scientists appearing together on an abstract.

### Aims:

Our aim was to quantitatively describe the evolution of scientific collaboration, to analyze how network position relates to academic success, and to identify structural tendencies in the formation and maintenance of co-author ties.

### Methods/Materials:

The data set was collected from the published abstracts of the Advances of Neuroblastoma (ANR) congresses from 1975-2016. Based on the co-authors' collaboration network, degree centrality (local connections), closeness (global connections), and betweenness centrality (brokerage) were calculated and related to the neuroblastoma-specific Hirsch index (h-index, Web of Science). In addition, stochastic actor-oriented models (SAOMs) were used to uncover how collaboration in neuroblastoma research changed its structure as the society matured. SAOMs are multivariate network models capable of controlling for individual attributes in modeling the development of networks and rely on a simulation of decisions to start, end or maintain ties.

### Results:

The data set comprised 18 conferences with 8459 authors affiliated at 553 institutions in 53 countries. 74% of authors and 39% of institutions contributed at one congress only. 3993 abstracts were presented in total. Centralization in the distribution of co-author ties increased steadily throughout time (skewness of the degree centrality distribution rose from 0.99 (1975) to 3.69 (2016)). This finding suggests that a small group of researchers attracted many co-authors especially at the later ANR conferences. A high number of presentations and of co-authors (measured by fractionalized counting (weight) and degree centrality) were strongly associated with a high h-index as measure of scientific productivity ( $R^2 = 0.51$  and  $0.47$ , resp.). In addition, the application of SAOMs showed that the number of co-authors a scholar had was already influential for the accumulation of additional co-author ties in an early stage of the field's development. This Matthew effect was statistically significant from the conference held from 1996 onward.

As more authors and institutions entered the field, we observed that scholars who often occurred on abstracts in last author positions attracted more co-authors than researchers who occupied mostly first or middle positions. This parameter for a status effect was observed first in 2008 ( $P < .00001$ ). Moreover, we found that collaborations were more strongly shaped by status signals among authors within the same institution compared to authors who were not affiliated at the same institution (interaction between same institution and status effect,  $P=0.006$ ). In summary, our results indicate the emergence of a global, densely connected core of authors who were highly productive and managed to attract many co-authors primarily from their home institutions.

### Summary/Conclusions:

The 41 year long history of ANR congresses (1975-2016) allowed us to study the emergence of a scientific society. Our data set quantified the growth of neuroblastoma research and indicated that different social processes were at work in earlier compared to later periods of the society.



