ANR 2016

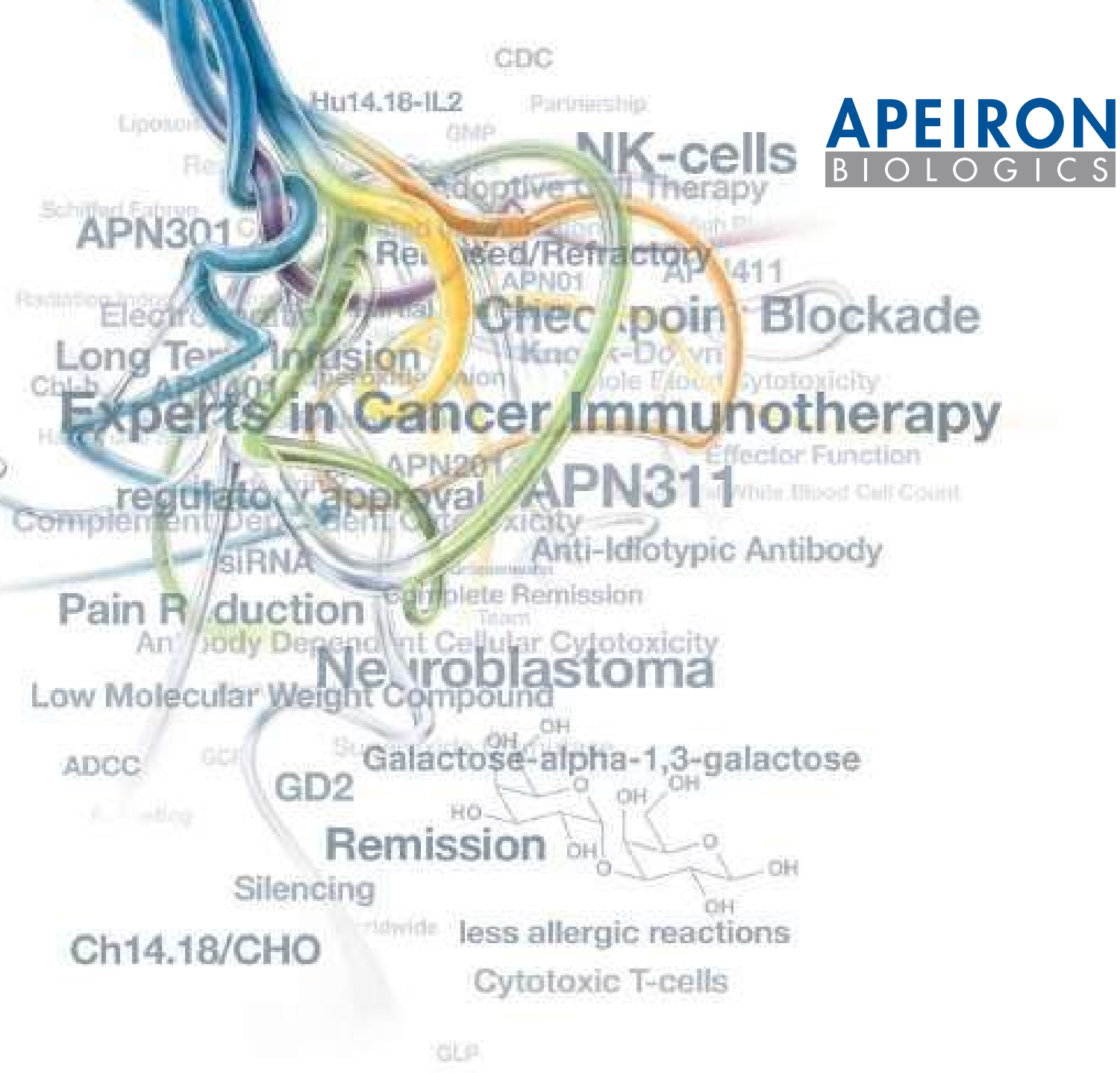
19th – 23rd June
Cairns Convention Centre,
Queensland, Australia

Advances in Neuroblastoma Research Congress

ANRA Advances in Neuroblastoma Research Association



PROGRAM AND ABSTRACT BOOK



APEIRON BIOLOGICS

PROJECTS IN IMMUNO-ONCOLOGY

Anti-GD2 antibody ch14.18/CHO (APN311) for immunotherapy of neuroblastoma awaiting marketing approval in Europe

Additional projects in immunological check-point blockade and antibody targeting, mostly in clinical stage

For more information come and visit us at booth #7/8

APEIRON Biologics AG
Campus-Vienna-Biocenter 5
1030 Vienna, Austria

www.apeiron-biologics.com



WELCOME TO CAIRNS!



The Local Organising Committee of ANR2016 is thrilled to be hosting you here in Cairns, Australia, at the gateway to the Great Barrier Reef. Our Committee has been working since the Cologne meeting in 2014 to put together what we believe is an exciting and vibrant ANR program that will build upon and extend all the other outstanding ANR meetings previously held in other parts of the world.

We have brought together more than 400 delegates, including scientists, clinician scientists, clinician investigators, nurses, allied health professionals, students and parents from 28 countries to this Congress, to share their research and experiences in our battle against neuroblastoma.

I look forward very much to meeting with you over the course of the Congress and, together with our Committee, making you feel welcome and helping you enjoy both the outstanding science and the wonderful surroundings in which the Congress is set this year.

On behalf of the Local Organising Committee, and with special thanks to Murray Norris, Jamie Fletcher, Michelle Henderson, Tao Liu, Loretta Lau and Toby Trahair for their tremendous efforts in putting together the program for this year's ANR meeting, and to Jen Devaliant for her tireless administrative support, I welcome you all to Cairns and to ANR2016!

Michelle Haber

Michelle Haber
Chair, ANR2016

ANRA Advances in Neuroblastoma Research Association

WELCOME TO ANR2016



It is a great pleasure as President of the Advances in Neuroblastoma Association to welcome you to ANR 2016 in Cairns.

This international meeting in Queensland Australia is a fantastic opportunity to share our research into the causes and treatment of neuroblastoma, an enigmatic and often deadly childhood cancer. We will be able to discuss and interact with clinical and laboratory scientists, nurses, parents, patient advocates from all over the globe. The meeting will also host important parallel sessions including a Parents Day symposium, an educational symposium for physicians, a meeting of the SIOPEN European neuroblastoma cooperative trials group, and ANZCHOG, the Australia New Zealand Children's Oncology Group.

We owe thanks to the careful and tireless meeting planning of Michelle Haber and Murray Norris and their local Organizing and Scientific committees, with the support of the ANRA Steering committee and ANRA Advisory Boards. We are very excited to hear from our invited outstanding plenary speakers, and thank them in advance.

We look forward to seeing you all at the exciting scientific sessions, poster sessions, lots of discussions, and Gala dinner!

We hope you will have an interesting and rewarding meeting, and that our ongoing collaborations will result in 100% cure for neuroblastoma!

Sincerely,

K Matthay M.D

Kate Matthay, MD
President, ANRA

MINISTER
FOR TOURISM & MAJOR EVENTS



Welcome to Tropical North Queensland for the 17th Advances in Neuroblastoma Research Conference 2016.

The Cairns Convention Centre is an ideal venue to host the world's leading neuroblastoma forum, the first time it has been held in Australia, or indeed, in the southern hemisphere.

This week's conference will provide a key opportunity for research scientists, clinicians, early career fellows, nurses and allied health care workers to be updated on the latest basic, translational and clinical research developments in the field of childhood neuroblastoma.

Hosting business events such as this helps grow the State's business tourism industry by allowing visitors from around the world to experience the best address on earth – Queensland.
The Palaszczuk Government is proud to support the 17th Advances in Neuroblastoma Research Conference 2016 through Tourism and Events Queensland, which supports visitation and expenditure in Queensland, and creates jobs.

I hope you enjoy your time in beautiful Tropical North Queensland and make the most of the destination's diverse tourism experiences.

The Hon Kate Jones MP
Queensland Minister for Tourism and Major Events

Tourism and Events Queensland

PROGRAM **BOOK**

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ACKNOWLEDGEMENTS AND SPONSORS

Thank you to our sponsors!

The ANR 2016 CONFERENCE WOULD LIKE TO THANK THE FOLLOWING SPONSORS FOR THEIR GENEROUS SUPPORT AND CONTRIBUTIONS:

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Solving Kids' Cancer

DIAMOND CORPORATE SPONSOR

APEIRON BIOLOGICS

PLATINUM PLUS SPONSORS

United Therapeutics ONCOLOGY

Alex's Lemonade Stand FOUNDATION FOR CHILDHOOD CANCER™

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ANR 2016 SUPPORTERS

Cancer Council

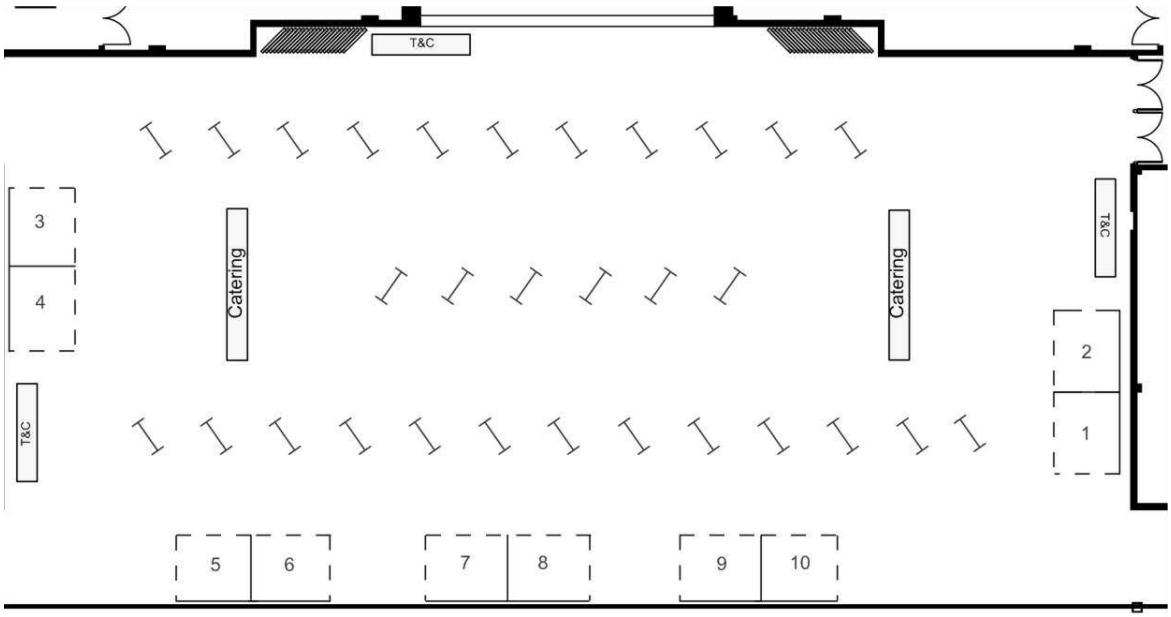
Children's Neuroblastoma Cancer Foundation

Kids Cancer Alliance

NOVOGEN

Tourism and Events Queensland

We would also like to thank the Lerner Family Foujarradndation for their generosity in making it possible for fifteen Young Investigators and three investigators from developing countries to be supported to attend ANR2016 in Cairns and present their research findings. We also thank them for their support of the nine prizes being offered this year for Best Young Investigator Oral presentation, Best Open Oral Presentation and Best Poster, in each of the basic, translational and clinical divisions.



DIAMOND CORPORATE SPONSOR

APEIRON BIOLOGICS

Booths #7&8

APEIRON Biologics AG is based in Vienna, Austria, engaged in projects in immunooncology.
APN311 (ch14.18/CHO): anti-GD2 chimeric antibody for treatment of neuroblastoma, European Marketing Authorization process ongoing.
APN301: anti-GD2 antibody-IL2 fusion protein in Phase II, to deliver IL2 to GD2+ tumors.
APN401: novel individual cellular immunotherapy by silencing the intracellular checkpoint cbl-b in immune cells ex-vivo and re-administration to fight cancer. Phase I study completed.
APN411: low molecular weight compounds to activate the immune system against cancer in preclinical stage (developed jointly with Evotec and Sanofi).
APN01 (recombinant ACE2): licensed to GlaxoSmithKline in 2010, now in Phase II in ARDS.

DIAMOND CHARITY SPONSOR

Solving Kids' Cancer

Booths #3&4

Solving Kids' Cancer is an international parent-led charity based in London and New York that seeks to understand the research landscape, strengthen the science and cure more children. SKC encourages the development of innovative clinical trials and prioritises funding to help bring more effective treatments to children in the US, Europe and beyond. The organisation also supports families and provides information to help parents make important decisions on their child's treatment, as well as facilitating access to clinical trials. SKC hosts an annual Parent Conference on neuroblastoma for families to learn from the world's top paediatric cancer experts.

PLATINUM PLUS SPONSOR

United Therapeutics ONCOLOGY

Booths #1&2

Our company
United Therapeutics Corporation is a biotechnology company focused on the development and commercialisation of unique products to address the unmet medical needs of patients with chronic and life-threatening conditions.
We have four approved products on the market today and as a group we are relentless in our pursuit of Medicines for Life®.

Our entry into paediatric oncology
We are privileged to work in partnership with the paediatric neuroblastoma community and this opportunity has given our mission new depth.
We believe that even the smallest patient populations deserve access to treatment options and we intend to continue that mission.

PLATINUM PLUS SPONSOR

Alex's Lemonade Stand FOUNDATION FOR CHILDHOOD CANCER™

Alex's Lemonade Stand Foundation (ALSF) emerged from the front yard lemonade stand of cancer patient Alexandra "Alex" Scott (1996-2004)
who wanted to raise money to help find a cure for all children with cancer. To date, ALSF, a registered 501(c)3 charity, has raised more than $120 million to fund over 550 pediatric cancer research projects across North America. ALSF's rigorous review process ensures selected projects are going to make the most substantive and critical contributions to childhood cancer research. ALSF grants lead to innovative breakthroughs and promising new treatments by investing in projects at every stage of a researcher's career. ALSFgrants.org

GOLD SPONSOR

GILEAD

Booth #10

Gilead's mission is to advance patient care by developing therapeutics to treat life-threatening diseases. We apply biopharmaceutical science to create medicines to treat conditions including Haematology/Oncology (ZYDELIG® [idelalisib]), HIV (GENVOYA® [elvitegravir/cobicistat/emtricitabine/tenofovir alafenamide], STRIBILD® [tenofovir disoproxil fumarate/emtricitabine/elvitegravir/cobicistat], EVIPLERA® [tenofovir disoproxil fumarate & emtricitabine & rilpivirine], ATRIPLA® [tenofovir disoproxil fumarate & emtricitabine & efavirenz], TRUVADA® [emtricitabine & tenofovir disoproxil fumarate], EMTRIVA® [emtricitabine], VIREAD® [tenofovir disoproxil fumarate]), chronic hepatitis B (VIREAD® [tenofovir disoproxil fumarate], HEPSERA® [adefovir dipivoxil]), chronic hepatitis C (HARVONI® [ledipasvir & sofosbovir], SOVALDI® [sofosbuvir]), and systemic fungal infections (AmBisome® [liposomal amphotericin B])

Company name: Gilead Sciences Pty Ltd
Address: Level 6, 417 St Kilda Road, Melbourne, Victoria, 3004, Australia
Phone: +61 (0)3 9272 4400
Freecall: 1800 806 112
Fax: +61 (0)3 9272 4411

GOLD SPONSOR | CONFERENCE DINNER SPONSOR

The William Guy Forbeck Research Foundation was established in 1984 and has funded scientific think tanks and research for over 30 years. The core of this scientific programming is fostering collaborations among institutions and interdisciplinary research to find new approaches in the fight against cancer. New programs and initiatives such as the Collaborative Research Program and the Interactive International Neuroblastoma Research Group Database (iINRGdb) provide further avenues for collaboration. Significant outcomes of WGFRF think tanks include the International Neuroblastoma Staging System and International Neuroblastoma Risk Group (1985).

SILVER SPONSOR

Over 1 in 6 GP prescriptions written in Australia is for a brand supplied by Aspen Australia making it the number one prescription company in Australia.[1] For product information and an extensive range of clinical resources to assist your practice, please review the Aspen Australia website, access details - see your Aspen representative.

1 Reference 1: IMS AMI Dec 2014 (as measured by scripts generated)

SILVER SPONSOR

Booth #5

Cancer Therapeutics CRC (CTx) is a collaborative partnership of MRIs, Universities and biotechnology companies that translates Australia's innovative research discoveries into new cancer drugs ready for clinical development. The major focus for our drug discovery is early intervention in the metastatic cascade and we bring this focus to not only common adult cancers, but also to the somewhat neglected area of new drugs for children with cancer. CTx's research and development capabilities span the full range of technologies and expertise required to discover novel small molecule cancer drugs and develop them to the clinical candidate stage.

SUPPORTER

The Children's Neuroblastoma Cancer Foundation (CNCF) was founded in 2000 after losing our son Nick to neuroblastoma. He was 10 years old. CNCF is committed to finding a cure for neuroblastoma through research funding, education and awareness programs.

We collaborate with other foundations to help further the research process and hasten the pathways to a cure. We have funded nearly $2.5 million in research and have supported hundreds of families through our Parent and Caregiver Education Program.

Children's Neuroblastoma Cancer Foundation
360 W. Schick Road
Suite 23, # 211
Bloomingdale, IL USA 60106
Phone: 1-866-671-2623
www.cncfhope.org
info@cncfhope.org

SUPPORTER

'The kids cancer alliance (KCA) is an exciting and visionary initiative that will accelerate improvements in the survival rates and quality of life of children diagnosed with cancer in New South Wales and across Australia.'
Professor Glenn Marshall, KCA Director

KCA is a Cancer Institute New South Wales translational cancer research centre and is an alliance of Children's Cancer Institute, Children's Medical Research Institute, Kids Research Institute, Sydney Children's Hospital, The Children's Hospital Westmead, and John Hunter Children's Hospital, with support from the University of NSW and the University of Sydney.
KCA is the ultimate example of "bench-to-bedside" medicine.

SUPPORTER

Novogen is an oncology-focused, Australian-US drug development company and has two proprietary drug discovery platforms, the superbenzopyrans (SBPs) and the anti-tropomyosins (ATMs). Based on initial research at the UNSW, the ATM family has been developed through a rational drug design program to target the Tpm3.1 protein, a critical structural component of cancer cells. Anisina (ATM-3507) is the company's first ATM drug candidate and has been shown to be effective *in vitro* and *in vivo* against a broad range of cancer types, including neuroblastoma. The drug is currently undergoing IND-enabling toxicology studies in preparation for the initiation of clinical trials.

For more information, please contact:
Novogen Ltd
Suites 502, Level 5, 20 George Street, Hornsby NSW 2077, Australia
Phone: +61 (0)2 9472 4101
Website: www.novogen.com

SUPPORTER

Neuroblastoma Australia is run by families and friends affected by neuroblastoma on a volunteer basis. We have three main objectives:-

- **To raise the profile** of neuroblastoma
- **To increase fundraising efforts** targeted specifically at Neuroblastoma research
- To provide **information and support** for those affected by Neuroblastoma

Neuroblastoma Australia was inspired by Sienna Hoffmann who tragically lost her battle against the disease at just 2 years of age. It was her determination that saw her family, and the family of Ciara Flanagan, a neuroblastoma survivor, set up the charity and develop the network that exists today.

SUPPORTER

NEUROBLASTOMA UK
FIGHTING CHILDHOOD CANCER

Neuroblastoma UK is the leading national charity focussed entirely on research to improve diagnosis, treatment and towards a cure for neuroblastoma.
Established some 30 years ago, over £6m has been dedicated to research through our biennial grant round, to cancer institutions and universities and has produced results that have led to new treatments and clinical trials, including

- Immunotherapy - engineering the child's immune cells to treat their cancer ('CAR Immunotherapy')
- Targeted radiation therapy for children (mIBG), in combination with chemotherapy
- Gene therapy, and investigations into a child's genetic susceptibility to neuroblastoma
- Improving treatments for children through studying the action of specific drugs in the body
- Exploiting the embryonic environment in children to re-programme cancer stem cells
- Monitoring disease load during treatment.

Neuroblastoma UK is entirely voluntary, meaning that 95% of funds raised goes directly into those research projects that are rigourously selected through an international Scientific Advisory Body and the charity's Trustees, to ensure the projects meet not only the highest scientific criteria and impact, but also families' priorities.

EXHIBITOR

LINK Healthcare
Part of the Clinigen Group

Booth #6

The Group is the trusted global leader in ethically sourcing and supplying unregistered medicines to hospital pharmacists and physicians for patients with a high unmet need. Idis Managed Access runs early access programs for innovative new medicines. Idis Global Access in partnership with Link healthcare work directly with healthcare professionals to enable compliant access to unregistered medicines and niche essential registered medicines.

Right Medicine. Right Patient. Right Time.

Julie Gosper
BPharm, GradCert Pharm Prac
Medicine Access Business Manager
M +61 498 880 101
E julie.gosper@linkhealthcare.com.au
W www.clinigengroup.com

EXHIBITOR



Booth #9

Save the date for ANR 2018!

Building Bridges for Neuroblastoma Research in San Francisco, California from May 9-12, 2018.

www.ANR2018.org



ANR 2016 **COMMITTEES**

ADVANCES IN NEUROBLASTOMA RESEARCH ASSOCIATION (ANRA) STEERING COMMITTEE

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Andy Pearson: ANRA Past President
Murray Norris: ANRA Incoming President
Garrett Brodeur: ANRA Secretary
Pat Reynolds: Webmaster
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Mike Hogarty (AM)
Gudrun Schleiermacher (EU)
Angelika Eggert (EU)
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Michelle Haber (Local Chair ANR 2016)
Frank Berthold (Local Chair ANR 2014)

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Michelle Henderson
Loretta Lau
Tao Liu
Glenn Marshall
Toby Trahair

THE ANR 2016 CONFERENCE WOULD LIKE TO THANK THE FOLLOWING ABSTRACT REVIEWERS FOR THEIR TIME AND EFFORT.

THANK YOU!

Shahab Asgharzadeh
Rochelle Bagatell
Sylvain Baruchel
Klaus Beiske
Frank Berthold
Garrett Brodeur
Sue Burchill
Godfrey Chan
Lou Chesler
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Alex's Lemonade Stand FOUNDATION FOR CHILDHOOD CANCER™

INNOVATE. RESEARCH. CURE.

Alex's Lemonade Stand Foundation (ALSF) is always looking for innovative and impactful childhood cancer research projects to fund. Go to ALSFgrants.org to become a part of the ALSF Team.

EARLY CAREER RESEARCH PROGRAMS propel promising researchers toward long-term careers in pediatric oncology investigation and set the course for productive careers.

ACCELERATOR PROGRAMS advance the pace of innovation and push research forward to find breakthroughs and ultimately new clinical interventions for children with cancer.

QUALITY OF LIFE AND CARE PROGRAMS empower nurse researchers and psychologists to make clinically significant discoveries that will improve quality of life or behavioral health outcomes for pediatric cancer patients.

To date, ALSF has raised more than $120 million to fund over 550 pediatric cancer research projects at top hospitals and institutions in the United States and Canada.

ALSFgrants.org







SOCIAL **PROGRAM**

WELCOME RECEPTION

The Welcome Reception will be taking place at Cairns Convention Centre on Monday, June 20th at 7:00pm and will include an outdoor cocktail-style reception and entertainment by Australian Indigenous performers.

GALA DINNER (ticketed event)

The Gala Dinner will take place at Cairns Convention Centre on Thursday, June 23rd at 7:00pm. The evening will feature a selection of the finest Australia food and beverages. The evening's entertainment will include world renowned toxicologist Dr Jamie Seymour, the Gondwana National Indigenous Children's Choir and the announcement of the ANRA Lifetime Achievement Award. The evening will conclude with Tony George and band taking the stage to get you moving!

TJAPUKAI CAIRNS DINNER & SHOW (ticketed event)

Delegates who have purchased tickets to attend are asked to refer to the registration pack for further details regarding the Tjapukai Dinner and Show. Relax around the fire with the Tjapukai warriors where there is time for an informative Medicine Talk. You will enjoy Australian meats, seafood and array of salads and desserts featuring tantalizing Indigenous flavours. The night includes Face Painting, a Cultural Welcome with didgeridoo journey, Aboriginal Dancer performance and Fire Making Ceremony with Spectacular Fire Ball. Be immersed into the Tjapukai culture.

REEF TRIP TO GREEN ISLAND (ticketed event)

Delegates who have purchased tickets to attend are asked to refer to the registration pack for further details regarding the Reef Trip to Green Island. Just 27km from Cairns lies Green Island, a 45-minute journey across the Coral Sea with Great Adventures' high-speed catamarans. Revel in an array of activities or relax and let your afternoon unfold in this tropical paradise. The trip includes use of the island's swimming pool, a trip on a glass bottom boat and use of snorkeling equipment.

DELEGATE **INFORMATION**

REGISTRATION DESK OPENING HOURS

Sunday 19th June	11:00am - 1:00pm
Monday 20th June	07:30am – 9:00pm
Tuesday 21st June	07:30am – 7:30pm
Wednesday 22nd June	07:30am – 5:00pm
Thursday 23rd June	07:30am – 5:30pm

BADGES

Each delegate will receive a name badge upon registering onsite at the ANR conference registration desk at the Cairns Convention Centre. For security reasons delegates are required to wear their name tags for the duration of the conference, including all activities and social events.

DISCLAIMER/LIABILITY

The hosts, organisers, venue and Advances in Neuroblastoma Research Association are not responsible for, or represented by, the opinions expressed by participants in either the sessions or their written abstracts. Responsibility for the itinerary and scientific content of abstracts accepted for publication remains with the authors and their sponsoring institutions. Acceptance by ANRA for publication does not imply any acceptance by ANRA of responsibility.

CONFERENCE APP

The APP for the conference is web based. This address, (http://anr-2016.m.asnevents.com.au/) will work on any device, phone, tablet or computer. The program is live and the most up to date version should there be any last minute changes. The functionality on the app is self explanatory but to get full access you will need to log in with your Currinda password. Your device will need to be connected to the internet. Note that the Cairns Convention Centre is providing complimentary WI-FI for all delegates. If you have any queries, come and see us at the registration desk.

INTERNET

Delegates of ANR 2016 will enjoy complimentary high-speed internet at the Cairns Convention Centre. Please select the *ccc-convention* network and enter password *cairns2016* (all lower case). Hotel internet arrangements vary depending upon the hotel. Delegates are invited to consult their in-room hotel compendium or contact the reception desk of their hotel for further information.

SMOKING

The Cairns Convention Centre and all ANR 2016 hotels are required, like all bars, clubs and restaurants in Queensland, to operate as a smoke free environment. If you must smoke, please only use the designated signed smoking area located on the Cairns Convention Centre Outdoor Plaza.

TIPPING

Tipping is entirely voluntary, and there is no obligation to tip. Most restaurants and pubs have tipping jars, and showing appreciation for good service with a tip is not uncommon in cities and at popular tourist destinations. Australian tourist establishments are generally quite honest and will not add anything to a bill that is not clearly specificed. A typical tip for a good meal in a restaurant is 10%.

TOURIST INFORMATION

The Cairns Tourist Information Centre is located on the corner of Shields Street and The Esplanade, adjacent to the Cairns Lagoon. The Centre is open from 7:30am – 9:00pm daily.

SCIENTIFIC **INFORMATION**

POSTER SESSIONS

Posters will be displayed in the Exhibition Area, Halls C and D on Monday June 20th or Tuesday June 21st. Please see abstract book for details on which day specific posters are displayed. Dedicated time is set aside for poster viewing and presenting authors of posters are asked to stand by their posters at this time.

POSTER MOUNTING AND DISMANTLING

Session Date & Time:	5:30pm - 7:00pm **Monday, June 20th**	5:30pm - 7:00pm **Tuesday, June 21st**
Mounting Date & Time:	10:30am - 11:00am **Monday, June 20th**	12:30pm - 1:30pm **Tuesday, June 21st**
Dismantling Date & Time:	10:30am - 11:00am **Tuesday, June 21st**	10:30am - 11:00am **Wednesday, June 22nd**

SPEAKER AND AV INFORMATION

Audio Visual technicians are allocated to each session room to assist with the operation of all equipment. These technicians will also assist with loading your presentation to the PC in your allocated session room. We ask that you please lodge your presentation at least a full session prior to the session in which you are presenting. Please bring your presentation on a USB, saved in PowerPoint format, for display on a PC.

AWARDS

Thanks to the generosity of the Lerner Family Foundation, there will be awards for the Best Young Investigator Oral presentation, Best Open Oral Presentation and Best Poster, in each of the basic, translational and clinical divisions. All these awards will be announced at the Closing Ceremony to be held at 4:45pm on Thursday 23rd June in Hall A. The Advances in Neuroblastoma Association (ANRA) Lifetime Achievement Award will be announced at the Gala dinner which starts at 7:00pm on Thursday 23 June held in Hall 2 (ticketed event).

CAIRNS CONVENTION CENTRE
MEETING ROOM MAPS

The meeting space for the ANR 2016 Conference is conveniently located on two adjacent levels at the Cairns Convention Centre.

The Plenary Room, Hall A, is accessible from both the Exhibition and Mezzanine Levels.

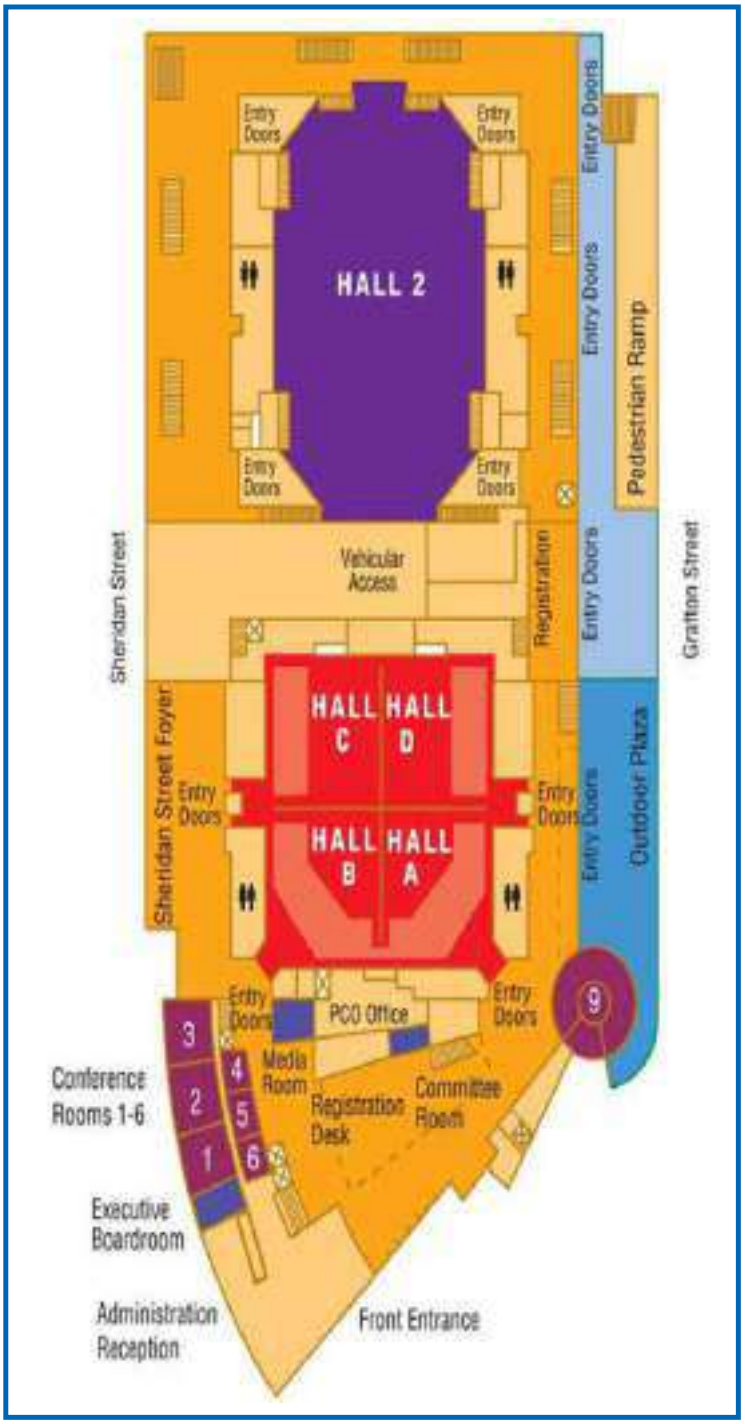
Conference Rooms are located on the Exhibition Level. Meeting Rooms are located on the Mezzanine Level.

The Exhibition Area is located in Halls C & D and is accessible from the Exhibition Level.

Poster sessions, Morning and Afternoon Tea and Lunch will be served in the Exhibition Area.

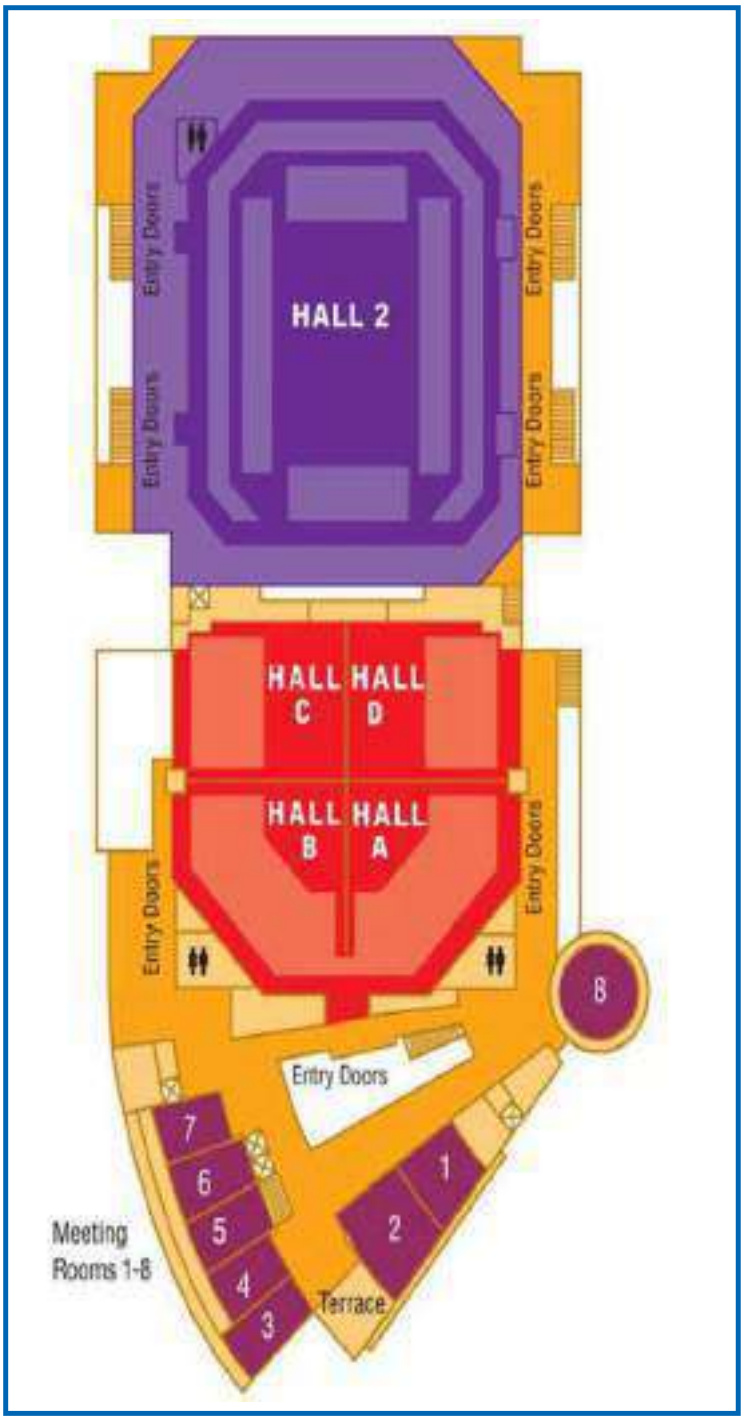
The Welcome Reception will be held on the Outdoor Plaza and is accessible from the Exhibition Level.

The Conference Dinner will be held in Hall 2 and is accessible from the Exhibition Level.



EXHIBITION LEVEL
Ground Floor

CAIRNS CONVENTION CENTRE
MEETING ROOM MAPS



MEZZANINE LEVEL
First Floor

CAIRNS CENTRAL BUSINESS DISTRICT **HOTEL LOCATION MAP**

KEY:

- Rydges Plaza Cairns
- Mantra Esplanade
- Hilton Cairns
- Pullman Cairns International
- Pacific Hotel Cairns
- Shangri-La Hotel – The Marina
- Park Regis City Quays
- Cairns Convention Centre
- Reef Fleet Terminal
- Walking route from Cairns Convention Centre to Reef Fleet Terminal for Green Island Tour



CAIRNS CBD

PROGRAM

INTERNATIONAL **KEYNOTE SPEAKERS**



Prof Martin Eilers

University of Würzburg in Germany

Martin Eilers is professor of biochemistry and molecular biology at the University of Würzburg in Germany. After a Ph.D. at the University of Basel, he joined Michael Bishop's laboratory at the UCSF, where he started working on Myc by generating MycER chimeric molecules, which are widely used as tools to study Myc and N-Myc proteins. Since then, his work focuses on the function and regulation of human Myc proteins. One focus of his recent work is the question of how proteasomal turnover of Myc proteins is coupled to the transcriptional activities. His work on neuroblastoma started from gene expression studies that identified a gene expression profile associated with MYCN amplification. An shRNA screen of these genes identified Aurora-A as being essential for growth of MYCN-amplified cells. The subsequent molecular analysis showed that Aurora-A and N-Myc form a complex that stabilizes N-Myc. Importantly, the complex can be targeted using small molecule inhibitors, leading to degradation of N-Myc and also, for some inhibitors, Aurora-A. In his presentation, Martin will discuss new insights into the composition, the structure and the function of the Aurora-A/N-Myc and Aurora-A/c-Myc complexes and will discuss implications for a targeted therapy.



Dr Stephan Grupp

Children's Hospital of Philadelphia in the USA

Stephan Grupp is an attending physician in the Cancer Center at Children's Hospital of Philadelphia (ChoP), Director of the Cancer Immunotherapy Frontier Program, Director of Translational Research of the Center for Childhood Cancer Research, and the Director of the Stem Cell Laboratory. Within his many roles at CHoP, he is a pediatric oncologist working to improve outcomes for children battling difficult cancers. Stephan trained at Harvard, at Boston Children's and the Dana Farber Cancer Institute, and came to CHOP in 1996. He also has the privilege of working with our Pediatric Hematology/Oncology trainees as Fellowship Director.
Working with colleagues at the University of Pennsylvania, his team have recently opened a phase I clinical trial called CART19, using genetically modified T cells in this trial to treat patients with B cell cancers such as ALL, B cell non-Hodgkin lymphoma (NHL), the adult disease chronic lymphocytic leukemia and other B cell malignancies. T cells have the potential to kill cancer cells, but in patients with cancer, they are not doing their job. By modifying these T cells, cells can be made to behave differently so they will attack cancer cells, using an engineered targeting protein called a chimeric antigen receptor (CAR). Initial results show that this could be an effective therapy for patients with B cell cancers. Indeed, initial results show some of the most powerful activity against cancer of any clinical trial testing engineered cell therapy to date. This has received international attention, and some of this work has been published recently in Science Translational Medicine and the New England Journal of Medicine.



Dr Kimberly Stegmaier

Dana-Farber Cancer Institute in the USA

The Stegmaier laboratory develops and integrates "omic" approaches to identify new protein targets and small-molecule modulators of malignancy with an eye toward clinical translation. The laboratory has focused on pediatric malignancies notable for the aberrancy of differentiation and/or oncogenic activation of transcription factors: the acute leukemias, neuroblastoma, and Ewing sarcoma.
Dr. Stegmaier is an Associate Professor of Pediatrics at Harvard Medical School, a Principal Investigator at Dana-Farber Cancer Institute (DFCI), and an Institute Member of the Broad Institute. She is the Co-director of the Pediatric Hematologic Malignancy Program and an attending physician providing clinical care in Pediatric Oncology at the Dana-Farber Cancer Institute and Boston Children's Hospital. She has won numerous awards, including recently the A. Clifford Barger Excellence in Mentoring Award from Harvard Medical School, the SPR Young Investigator Award, the Sir William Osler Young Investigator Award from the Interurban Clinical Club and a SU2C Innovative Research Grant.
Dr. Stegmaier received her undergraduate degree from Duke University, medical degree from Harvard Medical School, and trained in Pediatrics and Pediatric Hematology/Oncology at Boston Children's Hospital and the Dana-Farber Cancer Institute.



Dr Neal Rosen

Memorial Sloan-Kettering Cancer Center in the USA

Dr. Neal Rosen is the Director of the Center for Mechanism-Based Therapeutics at Memorial Sloan-Kettering Cancer Center, where he is also a Member in the Program in Molecular Pharmacology and Chemistry and the incumbent of the Enid A. Haupt Chair in Medical Oncology.
His major interests include the identification and study of the key molecular events responsible for the dysregulation of growth signaling in carcinomas and the use of this information for the development of effective therapeutic strategies. He has played an important role in the development of multiple inhibitors of receptor mediated signal transduction and has established and validated the concept the Hsp90 protein chaperone is a therapeutic target.
Recently, he has generated the concept that oncoprotein induced feedback inhibition of the signaling network is a major determinant of the transformed phenotype and the response of the tumor to targeted therapy. Currently his laboratory work focuses on using pharmacologic and genetic approaches to develop a detailed understanding of feedback and cross-talk among oncogeneactivated pathways in order to develop rational strategies for combination therapy. Recent work from the Rosen laboratory on ERK, mutant BRAF and PI3K/AKT signaling, the implications of the kinetics of pathway inhibition, and the consequences of relief of negative feedback by oncoprotein inhibitors has led to multiple clinical trials at Memorial Sloan-Kettering and other cancer centers in the United States and internationally.
Dr. Rosen received his undergraduate degree in Chemistry from Columbia College and an MD, PhD in Molecular Biology from the Albert Einstein College of Medicine. He completed a residency in Internal Medicine at the Brigham and Women's Hospital and post-doctoral training and a fellowship in Medical Oncology at the National Cancer Institute. He was on the senior staff of the Medicine Branch at the NCI prior to joining the faculty of Memorial SloanKettering Cancer Center.

INTERNATIONAL **INVITED SPEAKERS**



Dr Lei (Stanley) Qi

Stanford University in the USA

Dr. Lei Qi (Stanley) is an Assistant Professor of Bioengineering and of Chemical and Systems Biology in Stanford University, USA. He is one of the major contributors to the development the CRISPR technologies for sequence-specific genome regulation, as well as live-cell genome imaging. He invented a number of technologies, including CRISPR interference (CRISPRi), CRISPR activation (CRISPRa), CRISPR imaging, and high-throughput gain-of-function or loss-of-function screening. His current lab focuses on developing new technologies relevant to genome engineering, and applying these technologies to the study of stem cell fate determination and cancer immunotherapy. He obtained his Ph.D. in Bioengineering from the University of California, Berkeley, and worked as Systems Biology Faculty Fellow in the University of California, San Francisco. He joined Stanford Bioengineering in 2014. He obtained a number of awards including the NIH Director's Early Independence Award.



Dr Spyros Darmanis

Stanford University in the USA

Spyros Darmanis carried out his PhD in the lab of Dr. Ulf Landegren in Uppsala University in Sweden. During his PhD, he worked on the development of protein detection assays and applied them in the identification of putative protein biomarkers that could serve as early diagnostic indicators of different diseases such as prostate cancer and cardiovascular disease. Continuing as a postdoctoral fellow in the same lab and realizing the importance of single cell studies, he developed the first assay to measure level of expression of large numbers of protein and RNA molecules from the same single cell. He then applied this novel technique to investigate in human glioma the heterogeneity of response to treatment with BMP4. Currently he is a postdoctoral fellow in the lab of Prof. Stephen Quake at Stanford University where he is using single cell RNA sequencing methods to investigate the heterogeneity in the adult and fetal human brain both in healthy and different disease states.



Donna Ludwinski

Solving Kids' Cancer in the USA

Donna is Director of Research Programs at Solving Kids Cancer, an organisation dedicated to selectively funding research and clinical trials for new, inventive treatments and combinations of treatments for aggressive childhood cancers with low survival rates. Admiration for the Solving Kids Cancer mission and method noted while her son Erik battled neuroblastoma led to a productive collaboration after his death in 2010. Donna is a voracious consumer of medical literature and has has spent most of her waking hours the past 8 years reading and reporting on clinical and pre-clinical papers and abstracts on paediatric solid tumours. She attends major oncology conferences and keeps abreast of promising research leads. A lay member of the Therapeutic Development Initiative of Solving Kids Cancer, she assists in identifying, analysing, and cultivating promising therapeutic leads to bring novel therapies into the clinic quickly. Passionate about providing resources and advocating for families dealing with childhood cancer, she also serves as a liaison with other foundations with similar goals and focus

PROGRAM

MONDAY, 20 JUNE, 2016

WELCOME
8:45am - 9:10am — Hall A

KEYNOTE 1 - MARTIN EILERS
9:10am - 9:55am — Hall A
Chair: **Garrett Brodeur**

9:10am **Martin Eilers**
Complexes of N-MYC and MYC with Aurora-A: an inroad to targeting MYC function for neuroblastoma therapy *abs# 1*

PLENARY SESSION 1
9:55am - 10:30am — Hall A
Chairs: **William Weiss & Barbara Hero**

9:55am **Shoma Tsubota**
The role of PRC2 in the early neuroblastoma tumorigenesis in *MYCN*-Tg mice *abs# 2*

10:11am **Kevin Freeman**
Transforming primary neural crest cells to model neuroblastoma reveals a lineage sensitivity to BET inhibitors *abs# 3*

MORNING TEA
10:30am - 11:00am — Exhibition Area

PARALLEL 1 - ONCOGENESIS

11:00am - 12:30pm — Meeting room 1 & 2

Chairs: **Akira Nakagawara & Belamy Cheung**

11:00am	**Miller Huang** Human pluripotent stem cell-based models of *MYCN*-amplified neuroblastoma	*abs# 4*
11:15am	**Bieke Decaesteker** SOX11 acts as part of the MYCN regulatory protein complex implicated in neuroblastoma	*abs# 5*
11:30am	**Shizhen (Jane) Zhu** Aberrant activation of SHP2 cooperates with MYCN in neuroblastoma pathogenesis	*abs# 6*
11:45am	**Annick Mühlethaler-Mottet** The ALK-F1174L activating mutation mediates the upregulation of gene cluster located in the 15qD1 genomic region including the Myc locus in tumors derived from murine neural crest progenitor cells	*abs# 7*
12:00pm	**Isabelle Janoueix-Lerosey** Activated ALK signals through the ERK-ETV5-RET pathway to drive neuroblastoma oncogenesis	*abs# 8*
12:15pm	**Carol Thiele** Reactivation of cAMP /PKA pathway is an early event that relieves EZH2-mediated epigenetic suppression in High-Risk Neuroblastoma(HR-NB)	*abs# 9*

PARALLEL 2 - LIQUID BIOPSIES, CIRCULATING TUMOUR CELLS AND METASTASIS

11:00am - 12:30pm — Hall A

Chairs: **Frank Berthold & Klaus Beiske**

11:00am	**Mathieu Chicard** Whole exome sequencing of circulating free tumour DNA for study of spatial and temporal tumor heterogeneity: accumulation of new mutations at tumor progression of neuroblastoma	*abs# 10*
11:15am	**Martina Morini** Liquid biopsies reveal exosomal miRNA modulation in high-risk neuroblastoma patients after the induction therapy	*abs# 11*
11:30am	**Katleen De Preter** Detection of copy number aberrations in cell free DNA from plasma of neuroblastoma patients using shallow massive parallel sequencing	*abs# 12*
11:45am	**Fikret Rifatbegovic** The transcriptomic landscape of bone marrow-derived disseminated tumor cells of high-risk neuroblastoma patients	*abs# 13*
12:00pm	**Alex BK Seong** A novel neuroblastoma metastatic mouse model identifies genes, pathways and drugs regulating metastasis	*abs# 14*
12:15pm	**Giuseppe Barone** A native and immunocompetent *in vivo* model of chemorefractory, bone-marrow metastatic, "ultra-high risk" neuroblastoma.	*abs# 15*

LUNCH

12:30pm - 1:30pm — Exhibition Area

NEUROBLASTOMA CONSORTIUM MEETING

12:30pm - 1:30pm Conference Room 5

INDUSTRY SPONSORED WORKSHOP: APEIRON

12:45pm - 1:25pm Meeting room 1 & 2

R2 PLATFORM WORKSHOP: ONLINE PUBLIC RESOURCE FOR NEUROBLASTOMA OMICS DATA

12:50pm - 1:20pm Hall A

PARALLEL 3 - TERT AND OTHER NOVEL TARGETS

1:30pm - 3:00pm Hall A

Chairs: **Jo Vandesompele & Arata Tomiyama**

1:30pm	**Andrea Kraemer** The prognostic and therapeutic relevance of *TERT* activation in neuroblastoma	*abs# 16*
1:45pm	**Eiso Hiyama** Telomere biology in neuroblastoma: focusing on alteration of TERT promoter lesion	*abs# 17*
2:00pm	**Balakrishna Koneru** Constitutive activation of ATM kinase in neuroblastoma cell lines with the alternative lengthening of telomeres (ALT) phenotype induces resistance to DNA damaging agents	*abs# 18*
2:15pm	**Rebecca Dagg** A unique mechanism for the continual proliferation of high-risk neuroblastoma cells	*abs# 19*
2:30pm	**Jessica Koach** Targeting a novel MYCN onco-factor, PA2G4, for the treatment of neuroblastoma	*abs# 20*
2:45pm	**Per Kogner** Targeting tumor-promoting neuroblastoma microenvironment; Inhibition of tumor development and progression by therapy targeting mPGES-1 and prostaglandin E_2 expression in cancer associated fibroblasts	*abs# 21*

PARALLEL 4 - NOVEL THERAPIES AND IMMUNOTHERAPY

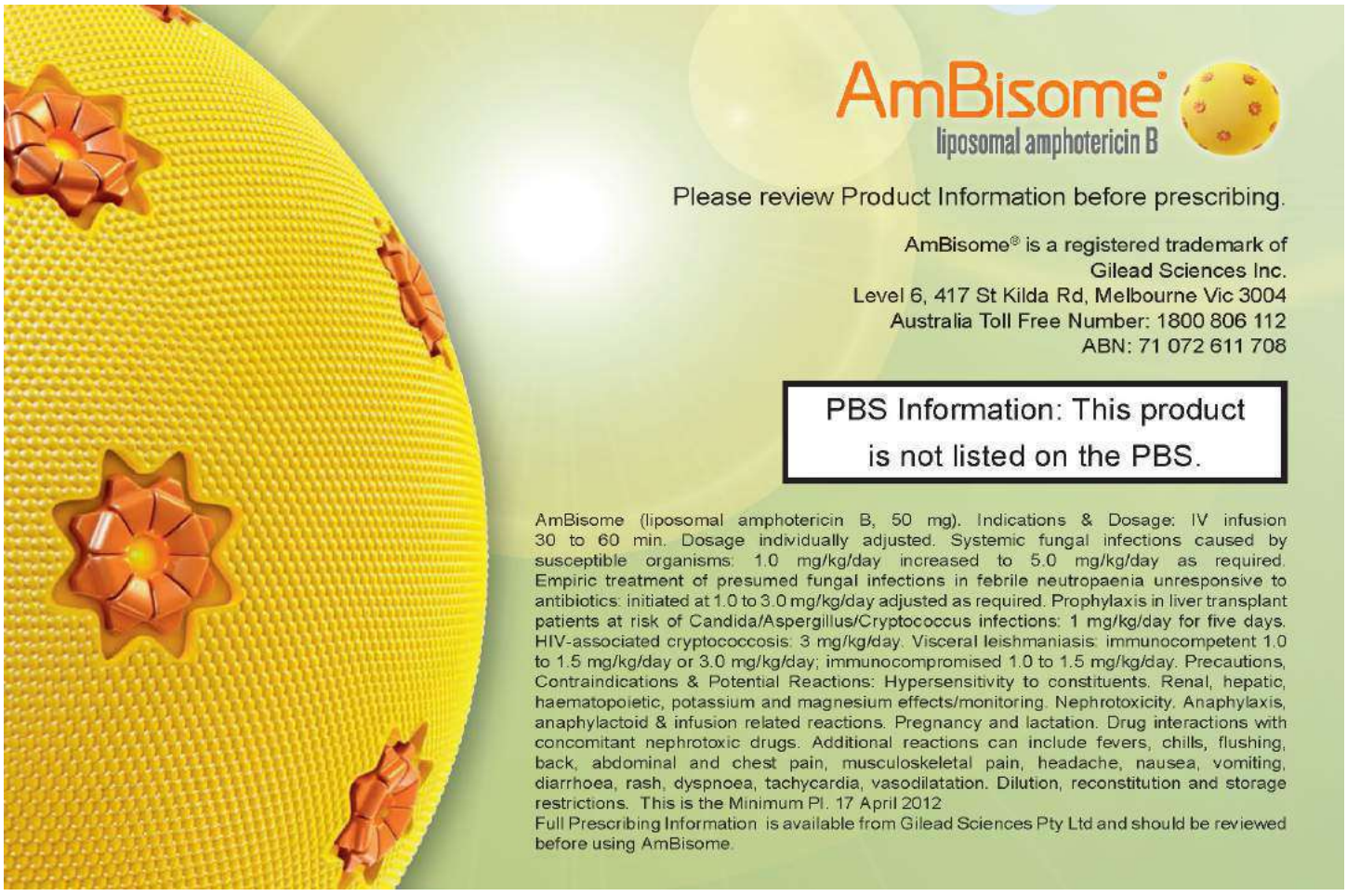
1:30pm - 3:00pm — Meeting room 1 & 2

Chairs: **Alex Swarbrick & Ro Bagatell**

1:30pm	**Alessandro Quattrone** Targeting the LIN28B/let-7 axis by small molecules in neuroblastoma	*abs# 22*
1:45pm	**Meredith Irwin** A small molecule kinome inhibitor screen identifies the TGF-beta-activated kinase 1 (TAK1) as a target for combination therapy in MYC-driven neuroblastoma	*abs# 23*
2:00pm	**Vandana Batra** Preclinical characterization of meta-[^{211}At]astatobenzylguanidine ([^{211}At]MABG) as an alpha particle emitting systemic targeted radiotherapeutic for neuroblastoma	*abs# 24*
2:15pm	**Robyn T Sussman** A CD56 (NCAM1) targeting antibody-drug conjugate is potently effective in preclinical models of high-risk neuroblastoma	*abs# 25*
2:30pm	**Kristopher R Bosse** GPC2 is a putative oncogene and candidate immunotherapeutic target in high-risk neuroblastoma	*abs# 26*
2:45pm	**Shakeel Modak** Phase I study of anti-G_{D2} humanized 3F8 (hu3F8) monoclonal antibody (MAb) plus GM-CSF: High dosing and major responses in patients with resistant high-risk neuroblastoma (HR-NB)	*abs# 27*

AFTERNOON TEA

3:00pm - 3:30pm — Exhibition Area



WORKSHOP 1 - EPIGENETIC REGULATION AND GENOME EDITING IN CANCER

3:30pm - 5:00pm Hall A
Moderator: **Frank Speleman**

The study of epigenetic deregulation of cancer cells has moved to the forefront of cancer research as a result of the recent discovery that across all tumor entities roughly 20% of all mutations affect genes implicated in processes controlling methylation and chromatin modification. At the same time, an increasing number of novel epigenetic drugs are emerging and going through in vitro, pre-clinical and clinical testing. CRISPR/Cas technology has revolutionized controlled genome editing offering new exciting possibilities to investigate the complex epigenetic control of gene expression in cancer cells even down to the single cell level. In this workshop we bring together experts in this field explaining how new insights into the highly complex epigenetic regulatory processes can provide deeper understanding of tumor initiation, progression and therapy resistance and how genome editing can aid to further fuel this discovery process, ultimately providing us the tools and data to identify novel drug targets and therapeutic strategies to combat neuroblastoma.

3:30pm	**Stanley Qi** CRISPR/Cas9 based genome editing
4:00pm	**Tim Mercer** Putting genes under the microscope: an exploration of the human transcriptome
4:20pm	**Kevin Freeman** Exploring the role of chromatin remodeling factors in neuroblastoma oncogenesis using genome-editing of mice neural crest progenitors
4:32pm	**Jason Shohet** Defining epigenetic drug targets in neuroblastoma; structure is function
4:44pm	**Round Table Discussion**

WORKSHOP 2 - NOVEL CLINICAL TRIAL DESIGN FOR ADVANCED NEUROBLASTOMA: A DEBATE

3:30pm - 5:00pm Meeting room 1 & 2
Moderator: **Glenn Marshall**

Much of the pre-clinical and clinical research on neuroblastoma is aimed at discovering more effective therapy for the majority of children who present with clinically advanced disease. Even successful current therapy for advanced neuroblastoma can lead to severe short and longterm side-effects, indicating the need for improved treatment strategies. Recent advances in organic chemistry and structure-aided design have seen a marked increase in the number of available targeted anticancer drugs, but the relative rarity of neuroblastoma may mean that novel trial designs are required to more rapidly incorporate pre-clinical advances into frontline protocols. This significant shift, coupled with more sophisticated methods of minimal residual disease monitoring, techniques for detailed analysis of tumor heterogeneity and 'window therapy' trial designs used to assess treatment efficacy in real-time, suggest that traditional single comparison phase 3 trial designs may no longer be suited to the problem of incorporating promising single agent or combination therapies into the treatment of newly diagnosed patients. The advent of precision or personalised oncologic medicine, aimed at better matching the treatment to the target holds the promise of improved cure rates and lower side-effect profiles. However, the relatively low number of genomic targets in neuroblastoma and the poor availability of novel agents for paediatric patients, means the field faces significant clinical research challenges in the future. We have asked the leaders of frontline international Phase 3 clinical trials for advanced neuroblastoma and early phase trialists to debate the following hypothesis: "That a conventional single comparison, randomised, Phase 3 trial design is no longer appropriate for newly diagnosed advanced neuroblastoma patients".

3:30pm	**Introductory remarks**
3:35pm	**Pre-debate Voting**
3:40pm	**Ruth Ladenstein** Chair, SIOPEN-HR1; St. Anna Kinderkrebsforschung e.V. & Children's Cancer Research Institute Vienna, Austria
3:55pm	**Julie Park** Chair, COG Neuroblastoma Steering Committee; Seattle Childrens Hospital & University of Washington, Seattle, USA
4:10pm	**Angelika Eggert** Chair, German Neuroblastoma Trial Group; Charité Universitätsmedizin Berlin, Germany
4:25pm	**David Ziegler** Co-Chair, NANT Phase I/II DFMO trial; Sydney Children's Hospital, Sydney Australia
4:40pm	**Questions from the floor**
4:55pm	**Post-debate voting**

RAPID FIRE POSTER PRESENTATION 1

5:05pm - 5:25pm Hall A

Christine Gana
New, highly selective MRP1 inhibitors show promising preclinical activity in neuroblastoma *abs# 249*

Hedi Deubzer
The MCM complex is a critical node in the miR-183 signaling network of *MYCN*-amplified neuroblastoma cells *abs# 188*

Lara Riehl
The mitochondrial genetic landscape in neuroblastoma from tumor initiation to relapse *abs# 198*

Sela T Po'uha
Stathmin expression regulates miR-382/PTPN14 expression in neuroblastoma cells *abs# 256*

Giuseppe Giannini
The MRN complex controls replication stress and allows proliferation and survival in MYCN amplified neuroblastoma *abs# 250*

Robyn T Sussman
CAMKV is a candidate immunotherapeutic target in *MYCN*-amplified neuroblastoma. *abs# 226*

Amos HP Loh
Proteomic analysis of high-risk neuroblastoma identifies nuclear distribution protein C as a marker of differentiation and prognosis *abs# 177*

Laurel T. Bate-Eya
EZH2 is highly expressed in neuroblastoma and plays an important role in neuroblastoma cell survival independent of its histone methyltransferase activity. *abs# 187*

Godelieve Tytgat
Circulating tumor DNA for disease monitoring in neuroblastoma *abs# 171*

W. Clay Gustafson
Aurora Kinase A inhibition sensitizes neuroblastoma to ^{131}I -MIBG *abs# 173*

Navin Pinto
Isolation of circulating tumour and associated cells by microfiltration in patients with neuroblastoma *abs# 169*

Barbara Hero
Survival tree analysis of an independent cohort reveals risk factors as proposed in the INRG system *abs# 162*

Claudia Pasqualini
Survival and prognostic factors for children 12 to 18 months of age with stage 4 non-*MYCN* amplified neuroblastoma treated in the SIOPEN high-risk trial *abs# 168*

Shakeel Modak
Phase II study of the combination of bevacizumab plus irinotecan and temozolomide for relapsed or refractory neuroblastoma *abs# 221*

Ulrike Pötschger
Impact of age and *MNA* amplification (MNA) on long-term survival rates: accurate estimation and refined modeling using innovative statistical approaches. A SIOPEN study from the high risk neuroblastoma trial HR-NBL1/SIOPEN. *abs# 170*

Alvin Kamili
Ex vivo drug screening as a strategy for personalised therapy in high-risk neuroblastoma *abs# 174*

POSTER SESSION 1 WITH WINE & CHEESE

5:25pm - 7:00pm Hall C + D

For those with specific interest in abstracts from the Basic, Clinical or Translational categories please use the lists located on page 129 as you walk amongst the posters to find them easily. Posters are arranged in the Exhibition Area in consecutive numerical order. Should you wish to view the entire list of poster abstracts in either Basic, Clinical or Translation divisions, please refer to the 'app' where you will find the posters sorted by category. Further information on the 'app' is available on page 22. Below is a complete listing of all posters in the Monday evening poster session.

Sponsored by aspen AUSTRALIA

M. Reza Abbasi
Impact of bone marrow-derived disseminated neuroblastoma cells on the identification of the relapse seeding clone *abs# 151*

Shifra Ash
Analyzing risk factors for stem-cell collection failure in patients on the High-Risk Neuroblastoma 1 trial (HR-NBL1/SIOPEN) *abs# 152*

Klaus Beiske
Quantification of bone marrow disease in high risk neuroblastoma patients by anti-GD2 immunocytochemistry – impact on survival. A SIOPEN High Risk Study *abs# 153*

Pablo Berlanga
Central imaging review in the SIOPEN high-risk neuroblastoma trial: preliminary data on central nervous system recurrences *abs# 154*

Frank Berthold
Characteristics and risk factors of 517 patients with first recurrence from stage 4 neuroblastoma over 18 months *abs# 155*

Sue A Burchill
Detection of PHOX2B and TH mRNA by RTqPCR in peripheral blood stem cell harvests may identify children with stage 4 neuroblastoma that have an increased risk of an event post reinfusion: a SIOPEN study *abs# 156*

Louis Chesler
Genome-wide analysis of liquid biopsies reveals a novel layer of tumor heterogeneity in neuroblastoma *abs# 157*

Valérie Combaret
Detection of tumor *ALK* Status in neuroblastoma patients using peripheral blood *abs# 158*

Susanne Fransson
Amplification of *CDK4* and *MDM2* is associated with atypical clinical features in high risk neuroblastoma patients *abs# 159*

Mark N Gaze
A comparison of ^{123}I-mIBG planar imaging and SPECT/CT with ^{68}Ga-DOTATATE PET/CT for staging and response assessment of high-risk neuroblastoma *abs# 160*

Jo Lynne Harenza
Development of a targeted sequencing panel for detection of subclonal mutations in neuroblastoma at diagnosis *abs# 161*

Barbara Hero
Survival tree analysis of an independent cohort reveals risk factors as proposed in the INRG system *abs# 162*

MoonSun Jung
An 18-gene Myc activity signature predicts poor clinical outcome in multiple Myc-associated cancer types *abs# 163*

Denis Kachanov
Opsoclonus myoclonus syndrome in children with neuroblastoma *abs# 164*

Natalia M Khranovska
Prognostic and predictive significance of *p53*, *MDM2* and miRNAs gene expression in patients with neuroblastoma *abs# 165*

Teofila Książek
Microarray CGH analysis of genomic imbalances in neuroblastoma FFPE specimens – pilot study *abs# 166*

Noritaka Miyazawa
Clinical characteristics and risk factor of transplantation-associated microangiopathy (TAM) in high-tisk neuroblastoma undergoing autologous peripheral blood stem cell transplantation (auto-PBSCT) *abs# 167*

Claudia Pasqualini
Survival and prognostic factors for children 12 to 18 months of age with stage 4 non-*MYCN* amplified neuroblastoma treated in the SIOPEN high-risk trial *abs# 168*

Navin Pinto
Isolation of circulating tumor and associated cells by microfiltration in patients with neuroblastoma *abs# 169*

Ulrike Pötschger
Impact of age and *MNA* amplification (MNA) on long-term survival rates: accurate estimation and refined modeling using innovative statistical approaches. A SIOPEN study from the high tisk neuroblastoma trial HR-NBL1/SIOPEN. *abs# 170*

Godelieve Tytgat
Circulating tumor DNA for disease monitoring in neuroblastoma *abs# 171*

Jalenka van Wijk
A flow cytometry backbone panel as a first step in detection of circulating tumor cells in neuroblastoma *abs# 172*

W. Clay Gustafson
Aurora Kinase A inhibition sensitizes neuroblastoma to ^{131}I -MIBG *abs# 173*

Alvin Kamili
Ex vivo drug screening as a strategy for personalised therapy in high-risk neuroblastoma *abs# 174*

Daisuke Kaneda
A novel histone deacetylase inhibitor OBP-801 induces apoptosis in neuroblastoma tumor cells *abs# 175*

Loretta MS Lau
Serum C-circles as biomarker of Alternative Lengthening of Telomeres (ALT) in neuroblastoma *abs# 176*

Amos HP Loh
Proteomic analysis of high-risk neuroblastoma identifies nuclear distribution protein C as a marker of differentiation and prognosis *abs# 177*

Ferro Nguyen
Targeted drug delivery using nanoparticles (NPs) in neuroblastoma (NB) xenografts *abs# 178*

Maike Nortmeyer
Bromodomain-inhibition as therapeutic option for *MYCN*-amplified neuroblastoma *abs# 179*

Jed G Nuchtern
The Connectivity Map bioinformatics platform identifies agents that reverse the chemotherapy resistance phenotype in neuroblastoma *abs# 180*

Alessandro Quattrone
A screening for natural products identifies a flavonol as a synergistic compound with 13-cis retinoic acid in neuroblastoma *abs# 181*

Nilay Shah
CYP26-mediated metabolism of retinoids is a putative mechanism of treatment resistance in neuroblastoma *abs# 182*

Justine Stehn
Anti-tropomyosin agents enhance the antitumor effectiveness of microtubule inhibitors in preclinical models of neuroblastoma *abs# 183*

Carol J Thiele
Inhibition of STAT3 with the generation 2.5 antisense oligonucleotide, AZD9150, decreases tumor-initiating potential of neuroblastoma cells and increases their chemosensitivity *abs# 184*

Hiroyuki Yoda
Targeting the *MYCN* oncogene in *MYCN*-amplified neuroblastoma with a novel PI polyamide DNA-alkylating drug conjugate *abs# 185*

Libo Zhang
Combined antitumor therapy with metronomic administration of topotecan and hypoxia-activated prodrug, evofosfamide, in neuroblastoma preclinical models *abs# 186*

Laurel T. Bate-Eya
EZH2 is highly expressed in neuroblastoma and plays an important role in neuroblastoma cell survival independent of its histone methyltransferase activity *abs# 187*

Hedi Deubzer
The MCM complex is a critical node in the miR-183 signaling network of *MYCN*-amplified neuroblastoma cells *abs# 188*

Moritz Gartlgruber
A genome-wide MYCN synthetic lethal screen identifies inhibition of PRC2 as drug target in *MYCN*-amplified neuroblastoma cells *abs# 189*

Venkatadri Kolla
Epigenetic silencing of *CHD5* expression by histone modification in human neuroblastoma *abs# 190*

Jan Koster
R2: A public user-friendly website for integrated analysis of genomic data and associated clinical parameters in neuroblastoma *abs# 191*

Tim Lammens
Non-random pattern of whole chromosome gains and losses in neuroblastoma with numerical chromosomal aberrations *abs# 192*

Koumudi Naraparaju
Role of MiRNAs in the epigenetic silencing of CHD5, a tumor suppressor in neuroblastoma (NB) *abs# 193*

Miki Ohira
Clinical relevance of genomic and epigenomic classification of *MYCN*-non-amplified neuroblastoma *abs# 194*

Miki Ohira
Genomic characterization of high-risk neuroblastoma in Japan: A retrospective study of 537 cases by using updated follow-up data based on INRG variables [Japan Neuroblastoma Study Group (JNBSG)] *abs# 195*

Chi Yan Ooi
MicroRNA-204 suppresses neuroblastoma tumour growth through down-regulation of MYCN oncogene *abs# 196*

Alessandro Quattrone
Exploring m6A mRNA methylation for novel therapeutic chances in neuroblastoma *abs# 197*

Lara Riehl
The mitochondrial genetic landscape in neuroblastoma from tumor initiation to relapse *abs# 198*

Ya-Hui Tsai
CPEB1 down-regulated the expression of MYCN *via* tumor-suppressor miRNA let-7 in human neuroblastoma cells *abs# 199*

Kumiko Uryu
Genetic characteristics of 494 neuroblastomas using genome-wide analysis combined with immunohistochemistry *abs# 200*

David Cantelmi
End of life care for children with neuroblastoma: a retrospective study from the Royal Children Hospital Brisbane *abs# 201*

Angela Cha
Physeal arrest leading to angular deformity after therapy with isotretinoin for high risk neuroblastoma (HR-NBL) *abs# 202*

Hsiu-Hao Chang
A multidisciplinary team care improved outcomes for children with high-risk neuroblastoma *abs# 203*

Matthew D Aldridge
The requirement for accurate standardization and methodology of dosimetry in international trials incorporating molecular radiotherapy (MRT) in the treatment of high-risk neuroblastoma *abs# 204*

Stephane Birkle
Antibodies specific for O-acetyl-GD2 mediates the same efficient anti-neuroblastoma effects as therapeutic ch14.18 antibody to GD2 without antibody induced allodynia *abs# 205*

Tom Boterberg
Importance of quality assurance in radiotherapy for optimal local control. A report from the SIOPEN radiotherapy committee of the High Risk Neuroblastoma Trial (HR-NBL1/SIOPEN) *abs# 206*

Bao C Bui
Stromal collagen type XI alpha 1 COL11A1 expression in neuroblastoma *abs# 207*

Angela Cha
Dinutuximab combined with chemotherapy in patients with multiply relapsed/refractory high risk neuroblastoma (HR-NBL) *abs# 208*

Godfrey CF Chan
Evaluation of genetic modified anaerobic *Salmonella* typhimurium as therapy for neuroblastoma: Comparison of response of orthotopic mouse models with different immunological backgrounds *abs# 209*

Maria V Corrias
Preclinical studies of anti-PDL-1/PD-1-based combination immunotherapy for Neuroblastoma *abs# 210*

Eoin Dodson
Which miRNAs should be developed into novel therapeutics for neuroblastoma? *abs# 211*

Christin Eger
Generation and characterization of a new chimeric human/mouse anti-idiotype antibody ganglidiximab for active immunotherapy against neuroblastoma *abs# 212*

Barbara Hero
Role of Surgery in Patients older than 18 months with localized Neuroblastoma (Stage 1-3) *abs# 213*

Meredith Irwin
More is less: radiation exposure to family caregivers and health care providers of paediatric neuroblastoma patients receiving ^{131}I-MIBG therapy in Canada *abs# 214*

Eugene S Kim
Anti-GD2 antibody combined with activated natural killer cells leads to improved survival and decreased metastasis in a minimal residual disease mouse model of neuroblastoma *abs# 215*

Li-Ling Lin
TLR3-Mediated innate immune response in the treatment of neuroblastoma *abs# 216*

Holger Lode
Interleukin-2 adds toxicity to long term infusion treatment regimen of ch14.18/CHO antibody without measurable additional activity in relapsed/refractory neuroblastoma patients *abs# 217*

Hans Loibner
Galactose-α-1,3-galactose (a-Gal) glycosylation determinant on ch14.18 antibodies produced by CHO- or SP2/0 cell lines – potential clinical impact *abs# 218*

Suzanne P MacFarland
Entrectinib is a potent inhibitor of Trk-driven neuroblastomas in a xenograft mouse model *abs# 219*

Kimikazu Matsumoto
Impact of radiotherapy and curie score on bone relapse in high-risk neuroblastoma *abs# 220*

Shakeel Modak
Phase II study of the combination of bevacizumab plus irinotecan and temozolomide for relapsed or refractory neuroblastoma *abs# 221*

Shakeel Modak
Event-free survival (EFS) and overall survival (OS) of *MYCN*-amplified stage 2/3 neuroblastoma with or without autologous stem-cell transplantation (ASCT) *abs# 222*

Jan Molenaar
The iTHER (individualized THERapy) program; personalized cancer treatment for relapsed pediatric cancer *abs# 223*

Lucas Moreno
The BEACON-Neuroblastoma ITCC/SIOPEN phase 2 trial for children with relapsed and refractory neuroblastoma: a progress report *abs# 224*

Daniel A Morgenstern
Viability Of cryopreserved peripheral blood stem cells (PBSC) does not cuarantee functional activity: important implications for quality assurance of stem cell transplant programmes *abs# 225*

Robyn T Sussman
CAMKV is a candidate immunotherapeutic target in *MYCN*-amplified neuroblastoma *abs# 226*

Yoshiyuki Takahashi
Significantly reduced relapse rate after KIR ligand incompatible allogeneic cord blood transplantation with nonmyeloablative conditioning for primary stage IV neuroblastoma *abs# 227*

Domonique Valteau-Couanet
Validation of a test-dose strategy prior intravenous melphalan in children with renal failure undergoing high-dose chemotherapy with autologous stem cell transplantation *abs# 228*

Orazio Vittorio
Dextran-Catechin conjugate targets copper metabolism in neuroblastoma *abs# 229*

Saurabh Agarwal
Development of a novel transgenic neuroblastoma tumor model using genome editing *abs# 230*

Jessica L Bell
IGF2BP1 harbours prognostic significance by gene gain, diverse expression and interplay with MYCN *abs# 231*

Annick Mühlethaler-Mottet
Aldehyde dehydrogenases activity plays a key role in NB aggressive behavior *abs# 232*

Noriyuki Nishimura
Rab6B mediates the progression of neuroblastoma through the interaction with MTMR5 *abs# 233*

Camilla Persson
Characterization of patient-derived xenograft neuroblastoma cells *abs# 234*

Anna Philpott
Differentiation of Neuroblastoma is controlled by cdk-mediated regulation of the master regulator transcription factor Ascl1. *abs# 235*

Diogo Ribeiro
MYCN-regulated nuclear hormone receptors impact differentiation and survival in neuroblastoma patients *abs# 236*

Hisanori Takenobu
CDX1 regulates cancer stemness pathway in neuroblastoma *abs# 237*

Yasutoshi Tatsumi
BMCC1, a tumor suppressor protein that facilitates DNA-damage response and apoptosis, is associated with favorable prognosis of neuroblastoma *abs# 238*

Nobuyuki Yamamoto
DENN domain protein DENND2A regulates the progression of neuroblastoma *abs# 239*

Saurabh Agarwal
Transmembrane adaptor protein PAG1 is a novel tumor suppressor in neuroblastoma *abs# 240*

Mark A Applebaum
The identification of hypoxia regulated genes that confer a poor prognosis in neuroblastoma patients *abs# 241*

Michael B Armstrong
THe MAD Family members, MXI1 and MXI0, display distinct subcellular localization patterns in neuroblastoma *abs# 242*

Michael B Armstrong
The expression of Mxi1 and Mxi0 lead to differential effects on neuroblastoma pathogenesis and chemosensitivity. *abs# 243*

Eveline Barbieri
Targeting MYCN-amplified neuroblastoma through RORα activation. *abs# 244*

Christina L. Chang
Identification of a novel protein that suppresses the ability of NDPK-A to promote the invasiveness of neuroblastoma cells *abs# 245*

Jorida Coku
Reduced endoplasmic reticulum (ER)-mitochondria tethering as a cause of multidrug resistance in neuroblastoma *abs# 246*

Katleen De Preter
An embryonic stem cell activated FOXM1 transcriptional program marks ultra-high-risk primary neuroblastoma patients for FDI-6 small molecule inhibition *abs# 247*

Han-Fei Ding
Molecular control of neuroblastoma stem cell metabolism *abs# 248*

Christine Gana
New, highly selective MRP1 inhibitors show promising preclinical activity in neuroblastoma *abs# 249*

Giuseppe Giannini
The MRN complex controls replication stress and allows proliferation and survival in MYCN amplified neuroblastoma *abs# 250*

Dana-Lynn Koomoa
TRPM7 promotes Neuroblastoma progression *abs# 251*

Jacqueline M Kraveka
Curcumin mediated apoptosis in human neuroblastoma cells via ROS and inhibition of sphingomyelin synthase and glycosylceramide synthase *abs# 252*

Yuanyuan Li
PPP3CB is a novel prognostic indicator of high-risk neuroblastoma contributing to aggressive behaviors *abs# 253*

Martina Morini
Clinical significance of a seven-gene hypoxia signature in neuroblastoma *abs# 254*

Annick Mühlethaler-Mottet
The CXCR4/CXCR7/CXCL12 axis is involved in a secondary but complex control of neuroblastoma metastatic cell homing *abs# 255*

Sela T Po'uha
Stathmin expression regulates miR-382/PTPN14 expression in neuroblastoma cells *abs# 256*

Rachele Rosati
Functional Genomics identifies novel therapeutic targets for retinoic acid combinations *abs# 257*

Miriam Rosenberg
A multidisciplinary approach to antigen discovery and immune profiling of Opsoclonus-Myoclonus Ataxia Syndrome associated with Neuroblastoma *abs# 258*

Hervé Sartelet
Composite Neuroblastoma: Unique tumours with morphologically and genetically defined intratumoral heterogeneity *abs# 259*

Yuting Sun
The histone H3 lysine 4 presenter WDR5 is a potential therapeutic target in N-Myc-induced neuroblastoma. *abs# 260*

Arata Tomiyama
The signaling complex of tyrosine phosphatase SHP2 and docking protein ShcC regulates oncogenicity of neuroblastoma cells in a tyrosine-phosphorylation dependent manner. *abs# 261*

Catarina Trager
The role of $p75^{NTR}$ during neuronal differentiation of neuroblastoma cells *abs# 262*

WELCOME FUNCTION – FOOD & WINE INCLUDED

7:00pm - 9:00pm Outdoor Plaza



PROGRAM

TUESDAY, 21 JUNE, 2016

KEYNOTE 2 - NEAL ROSEN

8:30am - 9:15am — Hall A
Chair: **Kate Matthay**

8:30am **Neal Rosen**
Not available at time of print *abs# 33*

PLENARY SESSION 2

9:15am - 10:20am — Hall A
Chairs: **Mike Hogarty & Murray Norris**

9:15am **Suzanne Vanhauwaert**
The *BRIP1/FANCJ* DNA helicase is a druggable 17q driver oncogene involved in G-quadruplex induced replicative stress resistance in neuroblastoma *abs# 34*

9:31am **Saurabh Agarwal**
MLL1 and JMJD3 regulate neuroblastoma cancer stem cells *abs# 35*

9:47am **Liselot Mus**
Sensing mutant ALK: capicua and ETV5 as executors of aberrant ALK-driven MAPK signaling in neuroblastoma *abs# 36*

10:03am **Jan Koster**
TERT rearrangements are frequent in neuroblastoma and identify aggressive tumours *abs# 37*

INRG PRESENTATION

10:20am - 10:30am — Hall A

10:20am **Samuel L Volchenboum**
INRG Data Commons – A User Journey *abs# 38*

MORNING TEA

10:30am - 11:00am — Exhibition Area

PARALLEL 5 - EPIGENETICS

11:00am - 12:30pm — Meeting room 1 & 2

Chairs: **Carol Thiele & Takehiko Kamijo**

11:00am	**Frank Westermann** Integrative genome-scale analysis identifies epigenetic mechanisms of transcriptional deregulation in unfavorable neuroblastomas	*abs# 39*
11:15am	**Isabelle Janoueix-Lerosey** Dissecting neuroblastoma specific regulatory networks through epigenome mapping and transcriptional profiling of neuroblastoma and neural crest cell lines	*abs# 40*
11:30am	**Eveline Barbieri** The histone chaperone CHAF1A promotes tumorigenesis and opposes neuroblastoma differentiation via metabolic reprogramming.	*abs# 41*
11:45am	**Bieke Decaesteker** The TBX2 super-enhancer marked transcription factor on 17q is overexpressed in neuroblastoma and infers poor prognosis	*abs# 42*
12:00pm	**Shana Claeys** The HBP1 tumor suppressor is a negative epigenetic regulator of MYCN driven neuroblastoma through interaction with the PRC2 complex.	*abs# 43*
12:15pm	**Tao Liu** Combination therapy with the bromodomain inhibitor JQ1 and the histone deacetylase inhibitor panobinostat synergistically reduce LIN28B gene and N-Myc protein expression and suppress neuroblastoma progression	*abs# 44*

PARALLEL 6 - TRANSLATIONAL AWARDS SESSION

11:00am - 12:30pm — Hall A

Chairs: **John Maris & Matthias Fischer**

11:00am	**Angela Bellini** High frequency of mutations in chromatin remodeling genes in neuroblastoma	*abs# 45*
11:15am	**Melinda Halasz** Identification of spliceosomal components as novel therapeutic targets for the treatment of high-risk, MYCN-driven neuroblastoma	*abs# 46*
11:30am	**Evon Poon** The orally bioavailable small molecule CDK9 inhibitors CYC065 and CCT68127 are potent inhibitors of MYCN transcription	*abs# 47*
11:45am	**Paul J Wood** Long term, continuous exposure to panobinostat induces terminal differentiation and long term survival in the TH-NMYC neuroblastoma mouse model	*abs# 48*
12:00pm	**Renata Sano** A novel antibody-drug conjugate directed to the ALK receptor demonstrates efficacy in models of neuroblastoma	*abs# 49*
12:15pm	**Kellie Haworth** Oncolytic herpes Simplex-1 virotherapy augments chimeric antigen receptor T-Cell (CAR-T) therapy in Neuroblastomas	*abs# 50*

LUNCH

12:30pm - 1:30pm — Exhibition Area

ANRA ADVISORY BOARD MEETING

12:30pm - 1:30pm — Meeting room 1 & 2

PARALLEL 7 - BASIC AWARDS SESSION

1:30pm - 3:00pm — Hall A

Chairs: **Rogier Versteeg & Darrell Yamashiro**

1:30pm	**Joanna Kitlinska** Prenatal stress increases NB tumorigenicity in TH-MYCN mice.	*abs# 51*
1:45pm	**Daniel R Carter** Identifying mechanisms of neuroblastoma tumorigenesis using single cell transcriptomics	*abs# 52*
2:00pm	**Michael Hogarty** *ARID1A* and *ARID1B* mutations in the Swi/Snf BAF chromatin remodeling complex drive poor outcome neuroblastoma	*abs# 53*
2:15pm	**Matthias Fischer** Identification of somatic mutations determining the neuroblastoma phenotype	*abs# 54*
2:30pm	**Hedi Deubzer** MYCN and HDAC5 transcriptionally repress *CD9* to trigger an invasion-metastasis cascade in neuroblastoma	*abs# 55*
2:45pm	**Gonzalo Lopez** *MYCN* amplified neuroblastomas require TEAD4 to orchestrate transcriptional programs, exposing a therapeutic vulnerability	*abs# 56*

PARALLEL 8 - CLINICAL IMAGING, RISK FACTORS AND RESPONSE

1:30pm - 3:00pm — Meeting room 1 & 2

Chairs: **Dominque Valteau-Couanet & Wendy London**

1:30pm	**Steven G. DuBois** Clinical, biologic, and outcome differences according to MIBG avidity in children with neuroblastoma: A report from the Children's Oncology Group (COG)	*abs# 57*
1:45pm	**Shakeel Modak** Discordance in ^{123}I-MIBG (MIBG) and ^{18}FDG positron emitting tomography (PET) scans after multimodality therapy for high-risk neuroblastoma: clinical implications	*abs# 58*
2:00pm	**Yen-Lin Liu** Diagnostic FDG and FDOPA positron emission tomography scans distinguish the genomic type and treatment outcome of neuroblastoma	*abs# 59*
2:15pm	**Daniel A Morgenstern** Towards a model for risk stratification of high-risk neuroblastoma. A report from the HR-NBL-1/SIOPEN study.	*abs# 60*
2:30pm	**Julie R. Park** Revisions to the International Neuroblastoma Response Criteria: A consensus statement from the NCI-Clinical Trials Planning Meeting	*abs# 61*
2:45pm	**Barbara Hero** Risk factors for outcome after relapse or progression of localized Neuroblastoma	*abs# 62*

AFTERNOON TEA

3:00pm - 3:30pm — Exhibition Area

SATELLITE SYMPOSIUM

Meeting rooms 1&2, Thursday 23rd June 2016 **12.30 - 13.15**

An opportunity to examine the clinical application and role of Unituxin® (dinutuximab) in paediatric high-risk neuroblastoma patients, and to share experience in current best-practice approaches to Unituxin® treatment optimisation through a series of case studies.

" ESTABLISHING A NEW STANDARD OF CARE FOR HIGH-RISK NEUROBLASTOMA PATIENTS – OUR PRACTICAL EXPERIENCE "

United Therapeutics Corporation is a biotechnology company focused on the development and commercialisation of unique products to address the unmet medical needs of patients with chronic and life-threatening conditions. As a group, we are relentless in our pursuit of Medicines for Life® and continue our research into treatments for pulmonary arterial hypertension, oncology, and some of the world's most complicated viral illnesses.

We are privileged to work in partnership with the neuroblastoma community and this opportunity has given our mission new depth.

" WE BELIEVE THAT EVEN THE SMALLEST PATIENT POPULATIONS DESERVE ACCESS TO TREATMENT OPTIONS AND WE INTEND TO CONTINUE THAT MISSION. "

United Therapeutics ONCOLOGY

EU/UTX/APR16/049 Date of Preparation: April 2016

Please see Unituxin full Summary of Product Characteristics, including additional monitoring (black triangle designation).

Abbreviated Prescribing Information (API)

Consult the Summary of Product Characteristics (SmPC) in your country before prescribing.

Unituxin® ▼ 3.5 mg/mL concentrate for solution for infusion.

Active Ingredient: Dinutuximab 17.5 mg per 5 mL vial.

Uses: Treatment of high-risk neuroblastoma in patients aged 12 months to 17 years, who have previously received induction chemotherapy and achieved at least a partial response, followed by myeloablative therapy and autologous stem cell transplantation (ASCT). Administered in combination with granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin-2 (IL-2) and isotretinoin.

Dosage and Administration: ***Intravenous (IV) infusion:*** Unituxin is to be administered over five courses at a daily dose of 17.5 mg/m². It is administered on Days 4–7 during Courses 1, 3, and 5 (each course lasting approximately 24 days) and on Days 8–11 during Courses 2 and 4 (each course lasting approximately 28 days). The exact volume of Unituxin concentrate for solution for infusion required for the patient dose must be injected into a 100 mL bag of sodium chloride 9 mg/mL (0.9%) solution for injection. Dilution must be carried out under aseptic conditions. Unituxin should not be administered as an IV push or bolus. It should be administered by IV infusion over 10 hours. Infusion should be started at a dose rate of 0.875 mg/m²/hour and continued at this rate for 30 minutes; the rate should then be increased to 1.75 mg/m²/hour and continued at this rate for the remainder of the infusion, if tolerated. The infusion duration may be extended up to 20 hours to help minimise reactions during infusion that do not respond adequately to other supportive measures. The treatment regimen consists of Unituxin, GM-CSF, IL-2, and isotretinoin, administered over six consecutive courses. Specific clinical criteria must be evaluated prior to the start of each treatment course of Unituxin, and pre-medication should always be considered before starting each infusion. See SmPC for clinical evaluation criteria, pre-medication guidance and full dosing schedule.

Contraindications, Warnings and Precautions etc.:
Contraindications: Hypersensitivity (Grade 4) to the active substance or to any of its excipients (histidine, polysorbate 20 [E 432], sodium chloride and water for injections). ***Warnings and precautions:*** Antihistamine pre-medication (e.g. hydroxyzine or diphenhydramine) should be administered by IV injection approximately 20 minutes before starting each Unituxin infusion. It is recommended that the antihistamine medicinal product be repeated every 4–6 hours as required during infusion of Unituxin. Epinephrine (adrenaline) and hydrocortisone for IV administration should be immediately available at the bedside during administration of Unituxin to manage life-threatening allergic reactions. Capillary leak syndrome is more likely when Unituxin is co-administered with IL-2. It is recommended to administer oral metolazone or IV furosemide every 6–12 hours as required. For severe pain (Grade 3 or 4), which occurs most frequently during the first 4-day course of Unituxin, the infusion rate of Unituxin should be decreased to 0.875 mg/m²/hour. Unituxin should be discontinued if pain is not adequately controlled despite infusion rate reduction and institution of maximum supportive measures. Paracetamol should be administered orally 20 minutes prior to starting each Unituxin infusion, and repeated every 4–6 hours as needed. It is recommended that an opioid, such as morphine sulphate, be administered by IV infusion prior to each Unituxin infusion, and continued as an IV infusion during and until 2 hours after completion of treatment. As a precaution against hypotension, IV sodium chloride 9 mg/mL (0.9%) solution for injection (10 mL/kg) should be administered over one hour just prior to the Unituxin infusion. ***Interactions:*** No interaction studies of Unituxin have been performed.

Adverse Events: (i) Very common (≥1/10): Anaemia, anaphylactic reaction, hypersensitivity, hypokalaemia, hyponatraemia, hypocalcaemia, hypophosphataemia, hypoalbuminaemia, hyperglycaemia, decreased appetite, tachycardia (sinusal, atrial, ventricular), capillary leak syndrome, hypotension, hypertension, hypoxia, cough, dyspnoea, diarrhoea, vomiting, nausea, urticaria, pruritus, pyrexia, pain, face oedema, decreased platelet count, decreased lymphocyte count, decreased white blood cell count, decreased neutrophil count, increased aspartate aminotransferase, increased alanine aminotransferase. (ii) Common (≥1/100 to <1/10): Device-related infection, infection susceptibility increased, bacteraemia, enterocolitis, febrile neutropenia, cytokine release syndrome, hypomagnesaemia, acidosis, hypoglycaemia, neuralgia, peripheral neuropathy, headache, vision blurred, photophobia, mydriasis, bronchospasm, pulmonary oedema, constipation, lower gastrointestinal haemorrhage, maculo-papular rash, urinary retention, proteinuria, haematuria, peripheral oedema, chills, fatigue, irritability, injection site reaction, increased gamma-glutamyltransferase, increased blood creatinine, increased weight. See SmPC for a full list of adverse reactions and dose-modification guidance for the management of treatment-emergent adverse reactions.

Pharmaceutical Precautions: Unituxin should be stored in a refrigerator (2–8°C), protected from light. Diluted solution is stable for up to 24 hours at ambient conditions (less than 25°C).

Legal Category: Prescription-only medicine

Marketing Authorisation Number: EU/1/15/1022/001

Name and Address of Marketing Authorisation Holder:
United Therapeutics Europe, Ltd. Unither House, Curfew Bell Road, Chertsey, Surrey, KT16 9FG, United Kingdom.

Date of SmPC: August 2015

Date of API: September 2015

Adverse events should be reported in accordance with local country requirements and as stated in Appendix V within the SmPC for EU member states.

Adverse events should also be reported to drugsafety@unither.com.

Unituxin® is not licensed in Australia.

WORKSHOP 3 - EMERGING TECHNOLOGIES FOR EXPLORATION OF TUMOR HETEROGENEITY, CLONAL EVOLUTION AND PROGRESSION IN NEUROBLASTOMA

3:30pm - 5:00pm Hall A

Moderator: **Gudrun Schleiermacher**

Sponsored by NOVOGEN

Genetic heterogeneity and clonal evolution have been shown to play a role in progression of neuroblastoma. Recent data from other malignancies suggest that genetic heterogeneity might reflect not only evidence of competing clones, but also cooperating clonal events. This workshop will focus on neuroblastoma genetic heterogeneity, seeking to explore how recent and emerging technologies such as single cell studies and surrogate samples including circulating tumor DNA (ctDNA) and disseminated/circulating tumor cells (DTC/CTC) and can contribute to the understanding of the role of genetic heterogeneity and clonal evolution in tumor progression. While highlighting technical issues, challenges and pitfalls, the important questions of how these findings can be harnessed for clinical management of neuroblastoma patients will be further discussed.

3:30pm	**Spyros Darmanis** Single cell studies of the brain and its malignancies
4:00pm	**John Maris** The biological and clinical relevance of tumoral heterogeneity and clonal evolution in high-risk neuroblastoma
4:15pm	**M. Reza Abbasi** Impact of bome marrow-derived disseminated neuroblastoma cells on the identification of the relapse seeding clone
4:30pm	**Angelika Eggert** Addressing tumor heterogeneity in NB - potential and challenges of liquid biopsies
4:45pm	**Panel Discussion**

WORKSHOP 4 - NEXT GENERATION RISK STRATIFICATION: NEW APPROACHES TO IDENTIFY HIGHEST RISK PATIENTS (OR ULTRA HIGH RISK PATIENTS)

3:30pm - 5:00pm Meeting room 1 & 2
Moderator: **Meredith Irwin**

Risk stratification approaches that rely on robust clinical and biological prognostic factors have been used to predict outcome and tailor therapies for neuroblastoma patients for more than two decades. Current classification systems utilize clinical, histologic, and genetic factors to identify patients with low, intermediate, or high risk neuroblastoma. Recent advances have resulted in improved patient outcomes; however, long-term survival for high-risk patients remains < 50%. Furthermore, current prognostic factors do not predict which high-risk (HR) neuroblastoma patients will fail to achieve remission with current era therapies. There are many efforts aimed at prospectively identifying the subset of HR patients at highest risk of death, or "ultra-high risk (UHR) patients, " for whom novel therapies may be indicated early on in the course of the disease. Currently there is no uniform definition for UHR and to date, no clinical or genetic determinant(s) reliably identify UHR patients. In this workshop we will highlight recent advances in the discovery of germline and somatic genomic alterations that may predict poor outcome or failure to respond to therapy in the setting of high-risk disease. The potential roles for gene expression signatures and detection of minimal residual disease will also be discussed. Following these presentations there will be a panel discussion to consider how to incorporate these novel prognostic factors into upfront clinical trials and how we may use genomic markers and minimum residual disease status together with current prognostic factors to further refine the next generation of risk classification systems.

3:30pm	**Introduction, overview and goals**
3:35pm	**Sharon Diskin** Can germline and somatic mutations (or rare variants) help us identify "ultra-high risk" neuroblastoma and predict patient outcomes?
3:50pm	**Katleen De Preter** Copy number profiles as prognostic marker for high-risk neuroblastoma patients
4:05pm	**Matthias Fischer** Molecular risk stratification of neuroblastoma patients - using RNA, DNA, or both?
4:20pm	**Sue Burchill** Clinical impact and technical application of reverse transcriptase polymerase chain reaction to detect neuroblastoma RNAs in bone marrow and blood
4:40pm	**Panel Discussion**

RAPID FIRE POSTER PRESENTATION 2

5:05pm - 5:25pm Hall A

Sharon J. Diskin
Common germline variants at *MLF1* and *CPZ* loci associated with neuroblastoma susceptibility *abs# 295*

Tao Liu
Suppressing the expression of a single novel long noncoding RNA leads to neuroblastoma regression or eradication *abs# 298*

Shinichi Kiyonari
Identification of new synthetic lethal genes in *MYCN*-amplified neuroblastoma cells *abs# 363*

Zhi Xiong Chen
A non-canonical tumor suppression pathway identified in neuroblastoma – A New Paradigm for Personalized Treatment and Prognosis *abs# 349*

Ji Won Lee
Comprehensive analysis of neuroblastoma using high depth cancer gene panel sequencing *abs# 297*

Michael M Song
Cell lines (CLs) and patient derived xenografts (PDXs) established from post-mortem neuroblastoma samples display heterogeneity in sensitivity to chemotherapeutic agents commonly utilized in the treatment of high-risk neuroblastoma patients *abs# 293*

Keri A Streby
Enhancing ^{131}I-mIBG radiation therapy with oncolytic HSV1716 and NAT gene therapy in high-risk neuroblastoma *abs# 294*

Giuseppe Barone
The CHK1 inhibitor CCT244747, alone and in combination with gemcitabine, is active against p53 deficient models of neuroblastoma resistant to chemotherapy *abs# 283*

Daniel A Morgenstern
Engraftment following busulfan/melphalan (BuMel) high-dose chemotherapy for high-risk neuroblastoma. A report from the HR-NBL-1/SIOPEN trial *abs# 326*

Godelieve Tytgat
131Iodine-metaiodobenzylguanidine (131I -MIBG) and autologous stem cell transplantation harvesting and hematological reconstitution in high-risk neuroblastoma patients *abs# 335*

Ruth Ladenstein
Prognostic Factors in stage 4 neuroblastoma patients treated with Busulphan-Melphalan. Report from the European High Risk Neuroblastoma HR-NBL1/SIOPEN Trial *abs# 324*

Matthew D Aldridge
Establishment of a reproducible methodology and results for molecular radiotherapy dosimetric assessment of ^{177}Lu-DOTATATE in neuroblastoma *abs# 312*

Hiroyuki Shichino
Phase I trial of perifosine monotherapy in patients with relapsed or refractory neuroblastoma *abs# 331*

Gareth Veal
Busulfan and melphalan pharmacokinetics in high-risk neuroblastoma patients treated on the HR-NBL1/SIOPEN trial *abs# 308*

Gudrun Schleiermacher
Genomic profiling using circulating free tumor DNA highlights heterogeneity in neuroblastoma *abs# 301*

Laurel T. Bate-Eya
High efficacy of the BCL-2 inhibitor venetoclax (ABT-199) in neuroblastoma and rational for combination therapy *abs# 284*

Min Kang
Pharmacokinetics (PK) of 13-cis Retinoic Acid in COG Phase III Neuroblastoma Studies *abs# 321*

POSTER SESSION 2 WITH WINE & CHEESE

5:25pm - 7:00pm Hall C + D

For those with specific interest in abstracts from the Basic, Clinical or Translational categories please use the lists located on page 129 as you walk amongst the posters to find them easily. Posters are arranged in the Exhibition Area in consecutive numerical order. Should you wish to view the entire list of poster abstracts in either Basic, Clinical or Translation divisions, please refer to the 'app' where you will find the posters sorted by category. Further information on the 'app' is available on page 22. Below is a complete listing of all posters in the Tuesday evening poster session.

Sponsored by Cancer Therapeutics CRC

Boris Decarolis
Impact of the involvement of the separate body regions in the modified Curie and the SIOPEN mIBG-scoring systems in patients with stage 4 neuroblastoma *abs# 263*

Alexander E. Druy
Prognostic significance of imbalanced chromosomal alterations in primary and recurrent neuroblastoma *abs# 264*

Shakeel Modak
^{18}F-Meta Fluorobenzyl Guanidine (MFBG) Positron Emission Tomography(PET) imaging in patients with Neuroblastoma and other Neuroendocrine Malignancies *abs# 265*

Shakeel Modak
^{124}I-hu3F8 radioimmuno-positron emission tomography (PET) in patients with neuroblastoma and other GD2-positive malignancies: preliminary results on biodistribution, pharmacokinetics and tumor targeting *abs# 266*

Atsuko Nakazawa
A High ALK expression is associated with an unfavorable histology in Neuroblastoma. *abs# 267*

Meng Yao Lu
Feasibility of applying F^{18}-DOPA hybrid MR-PET to follow-up of neuroblastoma patients *abs# 268*

Divya Sahu
Co-expression network analysis reveals long non-coding RNA *SNHG1* as a novel biomarker in neuroblastoma *abs# 269*

Gudrun Schleiermacher
Post surgical 123I-MIBG SPECT/CT in neuroblastoma *abs# 270*

Gudrun Schleiermacher
High-risk neuroblastoma without *MYCN* amplification in patients between 12 and 18 months: Is there a hidden low-risk patient group? *abs# 271*

Katarzyna Szewczyk
The detection and quantification of neuroblastoma metastases in bone marrow using plasmids-targets as standards in QRT-PCR *abs# 272*

Clare J. Twist
Validation of image-defined risk factor (IDRF) assignment in patients with intermediate-risk neuroblastoma: a report from the Children's Oncology Group study ANBL0531 *abs# 273*

Godelieve Tytgat
Epithelial to mesenchymal transition and minimal residual disease monitoring in neuroblastoma *abs# 274*

Sam Volchenboum
Computer-assisted Curie scoring for Metaiodobenzylguanidine (mIBG) Scans in Patients with Neuroblastoma *abs# 275*

Kristoffer von Stedingk
Lack of adaptive immunity markers is associated with early death amongst high-risk neuroblastomas *abs# 276*

Larry L Wang
High-MKI neuroblastomas - MYC-family-driven tumors with augmented expression of MYCN/MYC protein behaves more aggressively than Non-MYC-family-driven tumors: a report from the Children's Oncology Group *abs# 277*

Daniel Weiser
XPO1 is overabundant in patients with neuroblastoma at ultra-high-risk for treatment failure: rationale for refined diagnostic risk stratification and targeted therapy *abs# 278*

Akihiro Yoneda
Incidence of stage IV neuroblastoma patients 2-5 years of age was increased after the cessation of mass screening in Japan *abs# 279*

Elise Young
Molecular Karyotyping in Neuroblastoma – time to stop G-banding. *abs# 280*

Clarke Anderson
Neural stem cell-mediated enzyme/prodrug therapy for neuroblastoma: translation to the clinic *abs# 281*

Giuseppe Barone
A comprehensive preclinical study of ALK inhibitors for the efficacious treatment of *ALK*^{F1174L}*/MYCN*-driven neuroblastoma *abs# 282*

Giuseppe Barone
The CHK1 inhibitor CCT244747, alone and in combination with gemcitabine, is active against p53 deficient models of neuroblastoma resistant to chemotherapy. *abs# 283*

Laurel T. Bate-Eya
High efficacy of the BCL-2 inhibitor venetoclax (ABT-199) in neuroblastoma and rational for combination therapy. *abs# 284*

Jeffrey Bond
Neuroblastoma drug response profiles are associated with gene expression profiles *abs# 285*

Mario Capasso
A high-throughput drug screening of FDA approved anti-cancer compounds suggests candidate tyrosine kinase inhibitors for repositioning in neuroblastoma therapy *abs# 286*

Emmy Dolman
ITCC Biology: pre-clinical targeted drug development for high-risk pediatric cancers *abs# 287*

Andrea Flynn
Effects on tumor cells and the immune microenvironment may both contribute to the anti-tumor activities of DFMO in neuroblastoma pre-clinical models *abs# 288*

Jennifer H Foster
Targeting NEDD8: a novel approach to treating neuroblastoma *abs# 289*

Laura D. Gamble
Targeting the polyamine pathway in combination with conventional chemotherapy for the treatment of childhood neuroblastoma *abs# 290*

Sina Gogolin
Targeting cell cycle and transcriptional CDKs using Roniciclib leads to significant high cell death in MYCN/MYC-activated neuroblastoma cells *abs# 291*

Mitsuteru Hiwatari
Identification of novel pathways and molecules able to down regulate oncogenes expression by *in vitro* drug screening approaches in neuroblastoma cells. *abs# 292*

Michael M Song
Cell lines (CLs) and patient derived xenografts (PDXs) established from post-mortem neuroblastoma samples display heterogeneity in sensitivity to chemotherapeutic agents commonly utilized in the treatment of high-risk neuroblastoma patients. *abs# 293*

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Enhancing ^{131}I-mIBG radiation therapy with oncolytic HSV1716 and NAT gene therapy in high-risk neuroblastoma *abs# 294*

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Common germline variants at *MLF1* and *CPZ* loci associated with neuroblastoma susceptibility *abs# 295*

Angelika Eggert
Mutational dynamics between primary and relapse neuroblastoma involve the Hippo/YAP1 pathway and genes relevant for epithelial-mesenchymal transition *abs# 296*

Ji Won Lee
Comprehensive analysis of neuroblastoma using high depth cancer gene panel sequencing *abs# 297*

Tao Liu
Suppressing the expression of a single novel long noncoding RNA leads to neuroblastoma regression or eradication *abs# 298*

Dries Rombaut
Long non-coding RNAs as novel components in the TP53 pathway *abs# 299*

Gudrun Schleiermacher
Genomic profiling in low and intermediate risk neuroblastoma to refine treatment stratification and improve patient outcome – LINES: a SIOPEN Trial *abs# 300*

Gudrun Schleiermacher
Genomic profiling using circulating free tumor DNA highlights heterogeneity in neuroblastoma *abs# 301*

Robert W Schnepp
The chromatin associated protein JARID2 Is a novel LIN28B-Influenced target in neuroblastoma *abs# 302*

Masatoshi Takagi
Loss of *ATM* function confers risk for advanced stage neuroblastoma but provides a therapeutic target for poly-ADP ribose polymerase inhibitors *abs# 303*

Ya-Hui Tsai
Investigation on the miRNA signature in retinoic acid-resistant neuroblastoma cells as novel therapeutic targets *abs# 304*

Christophe Van Neste
Integrated network analysis of G-quadruplex and replicative stress related genes as sources for neuroblastoma genomic instability *abs# 305*

Jun Yang
The histone demethylase KDM5A regulates p53 function via a translation mechanism *abs# 306*

Alan Van Goethem
Identification of non-invasive biomarkers for treatment response in neuroblastoma by circulating miRNA profiling *abs# 307*

Gareth Veal
Busulfan and melphalan pharmacokinetics in high-risk neuroblastoma patients treated on the HR-NBL1/SIOPEN trial *abs# 308*

Gareth J Veal
Clinical follow-up of high-risk neuroblastoma patients receiving individualised 13-cis-retinoic acid based on pharmacological exposure as part of a national UK study *abs# 309*

Daniel A Morgenstern
Providing information on clinical trials to parents of children with neuroblastoma: a novel liaison in a clinical nurse specialist role. *abs# 310*

Clare J. Twist
Premature physeal closure following prolonged fenretinide administration in patients with neuroblastoma *abs# 311*

Matthew D Aldridge
Establishment of a reproducible methodology and results for molecular radiotherapy dosimetric assessment of ^{177}Lu-DOTATATE in neuroblastoma *abs# 312*

Julien Fleurence
An anti-O-acetylated GD2 ganglioside antibody for the immunotherapy of High – Grade Diffuse Glioma in children *abs# 314*

Mark N Gaze
Immunohistochemical evaluation of target expression in high-risk neuroblastoma samples to facilitate optimisation of molecular radiotherapy *abs# 315*

Tomoro Hishiki
Primary tumor resection after high dose chemotherapy with autologous hematopoietic stem cell transplantation is a safe and feasible option. A report from the Japanese neuroblastoma study group (JNBSG) *abs# 316*

Tomoko Iehara
Opsoclonus-myoclonus syndrome in neuroblastoma: A report from the Japan Neuroblastoma Study Group (JNBSG) *abs# 317*

Minori MI Ishii
Combination therapy of highly activated natural killer cells and anti-disialoganglioside (GD2) antibody for Neuroblastoma: An experimental study *abs# 318*

Merel Jans
Analysis of surgery for Neuroblastoma in The Netherlands *abs# 319*

Denis Kachanov
Low-risk neuroblastoma in Russia: therapy results and prognostic factors *abs# 320*

Min H Kang
Pharmacokinetics (PK) of 13-cis Retinoic Acid in COG Phase III Neuroblastoma Studies *abs# 321*

Shinsuke Kataoka
Long term survival after KIR ligand incompatible allogeneic cord blood transplantation as a salvage therapy for relapsed stage IV neuroblastoma *abs# 322*

Anatoly A P Kazantsev
Treatment high-risk neuroblastoma. *abs# 323*

Ruth Ladenstein
Prognostic Factors in stage 4 neuroblastoma patients treated with Busulphan-Melphalan. Report from the European High Risk Neuroblastoma HR-NBL1/SIOPEN Trial. *abs# 324*

Daniel A Morgenstern
Primary tumour response to busulfan/melphalan high-dose chemotherapy in patients with high-risk neuroblastoma: a pilot study. *abs# 325*

Daniel A Morgenstern
Engraftment following busulfan/melphalan (BuMel) high-dose chemotherapy for high-risk neuroblastoma. A report from the HR-NBL-1/SIOPEN trial. *abs# 326*

Atsushi Narita
Phase I study of anti-GD2 antibody ch14.18/CHO long term infusion in recurrent or refractory neuroblastoma patients in Japan *abs# 327*

Sajid Qureshi
Complication of surgery for abdominal neuroblastoma: Chyle Leak *abs# 328*

Elizabeth Roundhill
Expression, trafficking and biological significance of mitochondrial MRP1 in neuroblastoma. *abs# 329*

Gudrun Schleiermacher
OMS/DES 2011: a Multinational European Trial for Children with Opsoclonus Myoclonus Syndrome *abs# 330*

Hiroyuki Shichino
Phase I trial of perifosine monotherapy in patients with relapsed or refractory neuroblastoma *abs# 331*

Nikolai Siebert
Generation of a new bicistronic DNA vaccine encoding for tyrosine hydroxylase and IL-15 to induce an active immune response against neuroblastoma *abs# 332*

Stefania Sorrentino
Spinal canal invasion in peripheral neuroblastic tumors. Study design and preliminary results of a prospective SIOPEN Study Registry. *abs# 333*

Ryota Souzaki
Creating Three-Dimensional full size model based on preoperative CT images for laparoscopic adrenalectomy and liver biopsy in a case demonstrating adrenal neuroblastoma with liver metastasis *abs# 334*

Godelieve Tytgat
131Iodine-metaiodobenzylguanidine (131I -MIBG) and autologous stem cell transplantation harvesting and hematological reconstitution in high-risk neuroblastoma patients. *abs# 335*

Keith Wheatley
BEACON-2: design of a SIOPEN/ITCC multi-arm multi-stage (MAMS) trial for relapsed neuroblastoma *abs# 336*

Aleksandra Wieczorek
The role of image defined risk factor (IDRF) in evaluation of the risk of post-surgical kidneys dysfunction in children with neuroblastoma *abs# 337*

Darrell J Yamashiro
Targeting activating transcription factor 5 (ATF5) in neuroblastoma with a novel dominant negative inhibitor *abs# 338*

Jinhua Zhang
A research of the induction and differentiation therapy for neuroblastioma in children *abs# 339*

Maxi Zumpe
Generation of new DNA- and protein vaccines for active immunotherapy against *MYCN*-expressing neuroblastoma *abs# 340*

Carmen Dorneburg
Boolean modeling identifies Greatwall/*MASTL* as an important regulator in the AURKA network of neuroblastoma *abs# 341*

Simon Durand
Growth advantage and oncogene addiction of neuroblastoma cells bearing an ALK mutation *abs# 342*

Marco Gualandi
Impact of Neuroblastoma recurrent mutations on embryonic sympatho-adrenal development *abs# 343*

Naonori Kawakubo
Natural antibody against neuroblastoma of the TH-MYCN transgenic mice has CDC activity *abs# 344*

Venkatadri Kolla
MYCN Amplicon in Neuroblastoma (NB) Cell Lines *abs# 345*

Zhihui Liu
Identification of CASZ1 nuclear export signal (NES) reveals potential mechanism for loss of CASZ1 tumor suppressor activity in neuroblastoma (NB) *abs# 346*

Jelena Milosevic
PPM1D/Wip1, the candidate gene on 17q contributing to neuroblastoma development *abs# 347*

Katharina Batzke
Modulation of immune responses and radioresistance by neuroblastoma-derived and host-derived TrkB-target Galectin-1 *abs# 348*

Zhi Xiong Chen
A non-canonical tumor suppression pathway identified in neuroblastoma – a new paradigm for personalized treatment and prognosis *abs# 349*

Johanna Dzieran
MYCN-induced miR-18a interferes with estrogen and NGF signaling to maintain an undifferentiated and more aggressive phenotype in neuroblastoma *abs# 350*

Selene Elifio-Esposito
NPY/NPY5R copy number increases in relapsing neuroblastoma *abs# 351*

Mona Friedrich
MYCN-dependent regulation of gene expression during the cell cycle in neuroblastoma cells *abs# 352*

Jixuan Gao
The ABC transporter ABCE1 is a therapeutic target in neuroblastoma *abs# 353*

Giuseppe Giannini
The Poly (ADP-ribose) polymerase inhibitor olaparib causes mitotic catastrophe in MYCN amplified neuroblastoma by enhancing replication stress *abs# 354*

Sabine Hartlieb
Alternative lengthening of telomeres in primary neuroblastoma specimens – a genomic, epigenomic & proteomic approach *abs# 355*

Charlotte Haunch-Smith
Characterisation of neuroblastoma cells isolated from bone marrow aspirates of children with stage 4 disease at diagnosis: an NCRI CCL CSG Neuroblastoma Group Study. *abs# 356*

Zhongyan Hua
PI3K and MAPK pathways mediate the BDNF/TrkB-increased migration and invasion in Neuroblastoma cells *abs# 357*

Shinya Ikematsu
Inhibition of the growth factor midkine in neuroblastoma by an Okinawan agricultural product *abs# 358*

Niloufar Javanmardi
Neuroblastoma: telomere elongation is responsible for aggressive behavior *abs# 359*

Maria Kavallaris
Stathmin mediates neuroblastoma metastasis in a tubulin-independent manner via RhoA/ROCK signalling and enhanced transendothelial migration *abs# 360*

Patrick Kim
Combination of HDAC and mitochondrial-targeted metabolism inhibitors exhibits strong therapeutic synergy *in vitro* and *in vivo* against neuroblastoma *abs# 361*

Satoshi Kishida
The involvement of Midkine, a growth factor exacerbating cisplatin-induced nephrotoxicity, in cisplatin resistance of neuroblastoma cells *abs# 362*

Shinichi Kiyonari
Identification of new synthetic lethal genes in *MYCN*-amplified neuroblastoma cells *abs# 363*

Jayne Murray
Suppression of Multidrug resistance protein 4 inhibits neuroblastoma growth both *in vitro* and *in vivo* *abs# 364*

Ganna Oliynyk
MYCN mediates metabolic plasticity in childhood neuroblastoma *abs# 365*

Annalisa Pezzolo
GOLPH3 regulates Golgi shape and is activated by DNA damage in neuroblastoma cell lines *abs# 366*

Navin Pinto
Patterns of PD-1, PD-L1 and PD-L2 Expression in Neuroblastoma *abs# 367*

María Victoria Ruiz Pérez
Targeting fatty acid synthesis to induce neuroblastoma differentiation *abs# 368*

Alica Torkov
Blinding the CYCLOPS – Neuroblastoma vulnerabilities unveiled by genomic loss *abs# 369*

Sieu L Tran
Elevated expression of dyskerin is a potential therapeutic target with a telomerase-independent role in Myc/N-Myc-driven neuroblastoma *abs# 370*

Veronica Veschi
Epigenetic siRNA and chemosensitivity screens identify a vulnerability to SETD8 inhibition through reactivation of p53 canonical pathway in Neuroblastoma *abs# 371*

My D Vu
The expression of AT Rich Interactive Domain 1A (ARID1A) in Neuroblastoma *abs# 372*

Amber K Weiner
Integrative approach to define the cell surface landscape in neuroblastoma *abs# 373*
Pei-Yi Wu
Calreticulin-dependent VEGF expression promotes neuroblastoma differentiation *abs# 374*
Denise Yu
MYCN promotes neuroblastoma malignancy by establishing a regulatory circuit with transcription factor AP4 *abs# 375*
Tina Zheng
Human stem cell models for relapse neuroblastoma *abs# 376*

TRANSPORT FROM CAIRNS CONVENTION CENTRE TO TJAPUKAI
7:15pm - 7:45pm

TJAPUKAI CAIRNS DINNER & SHOW OR DINNER ALONE
7:45pm - 9:45pm Tjapukai Aboriginal Cultural Park

TRANSPORT FROM TJAPUKAI TO CONFERENCE HOTELS
9:45pm - 10:15pm



PROGRAM

WEDNESDAY, 22 JUNE, 2016

KEYNOTE 3 - KIMBERLY STEGMAIER

8:30am - 9:15am Hall A
Chair: **Andy Pearson**

8:30am **Kimberly Stegmaier**
Emerging epigenetic targets in *MYCN*-amplified neuroblastoma *abs# 74*

PLENARY SESSION 3

9:15am - 10:20am Hall A
Chairs: **Angelika Eggert & Godfrey Chan**

9:15am **Rogier Versteeg**
Neuroblastoma is bi-phasic and includes classical neuro-epithelial cells and chemo-resistant mesenchymal cells *abs# 75*

9:31am **Pauline Depuydt**
Distal chromosome 6q-deletion defines a subgroup of ultra-high risk neuroblastoma patients *abs# 76*

9:47am **Yael P Mosse**
Chemical proteomics defines kinome responses to ALK inhibition in neuroblastoma *abs# 77*

10:03am **Shahab Asgharzadeh**
Enhancing efficacy of immune checkpoint blockade with anti-macrophage targeted therapy *abs# 78*

MORNING TEA

10:20am - 11:00am Exhibition Area

PARALLEL 9 - MYCN AND TUMOUR BIOLOGY

11:00am - 12:15pm Hall A
Chairs: **Isabelle Janoueix-Lerosey & Kenji Kadomatsu**

11:00am **Giovanni Perini**
Altering the MYCN/MAX ratio in a drosophila MYCN model leads to homeotic transformation of the eye to wing through deregulation of specific HOX genes *abs# 80*

11:15am **Johan van Nes**
Identification and reprogramming of mesenchymal-type cells in neuroblastoma *abs# 81*

11:30am **Marie Arsenian-Henriksson**
Induction of a metabolic switch in neuroblastoma and in other human cancer types upon targeting MYC *abs# 82*

11:45am **Andrew E Tee**
The histone methyltransferase DOT1L induces neuroblastoma progression by regulating gene transcription *abs# 83*

12:00pm **Angela Bellini**
Frequency of high and low level clonal *ALK* mutations in high risk neuroblastoma patients. A SIOPEN study *abs# 84*

PARALLEL 10 - HIGH RISK NEUROBLASTOMA

11:00am - 12:15pm Meeting room 1 & 2

Chairs: **Purna Kurkure & Lisa Diller**

11:00am	**Thorsten Simon** I-131-meta-Iodobenzylguanidine therapy improves survival in high-risk neuroblastoma patients with mIBG positive residual metastatic disease	*abs# 85*
11:15am	**Adela Cañete** Did we improve results in infants with MYCN Amplified Neuroblastoma? Comparison of treatment strategy and outcomes in INES 99.4 and HR-NBL1/ SIOPEN. A SIOPEN Study	*abs# 86*
11:30am	**Meaghan Granger** Myeloablative busulfan/melphalan (BuMel) consolidation following induction chemotherapy for patients with high-risk neuroblastoma. A Children's Oncology Group (COG) study	*abs# 87*
11:45am	**Giselle Sholler** DFMO maintains remission and increases overall survival in high risk neuroblastoma: results of a phase II prevention trial	*abs# 88*
12:00pm	**Thorsten Simon** The benefit of myeloablative chemotherapy with autologous stem cell transplantation in high-risk neuroblastoma patients is stable during long term follow-up. Results of the NB97 trial	*abs# 89*

FREE AFTERNOON

OPTIONAL REEF TRIP TO GREEN ISLAND 12:15 – 17:20

12:15pm - 5:20pm

Lunch boxes can be collected in the ground floor foyer.

DINNER ALONE

PROGRAM

THURSDAY, 23 JUNE, 2016

ANZCHOG WELCOME

9:00am - 9:10am Hall A

PLENARY SESSION 4

9:10am - 10:30am Hall A

Chairs: **Gudrun Schleiermacher & Sue Cohn**

Sponsored by APEIRON BIOLOGICS

9:10am **Julie R Park**
A Phase 3 randomized clinical trial (RCT) of tandem myeloablative autologous stem cell transplant (ASCT) using peripheral blood stem cell (PBSC) as consolidation therapy for high-risk neuroblastoma (HR-NB): A Children's Oncology Group (COG) study *abs# 90*

9:26am **Rajen Mody**
Phase II randomized trial of irinotecan/temozolomide (I/T) with temsirolimus (TEM) or dinutuximab plus granulocyte colony stimulating factor (DIN/GMCSF) in children with refractory or relapsed neuroblastoma: a report from the Children's Oncology Group (COG) *abs# 91*

9:42am **Mark A Applebaum**
Second malignancies in patients with neuroblastoma: a report from the international neuroblastoma risk group project *abs# 92*

9:58am **Ulrike Pötschger**
The way towards an international mIBG skeletal score for high risk neuroblastoma: the statistical perspective *abs# 93*

10:14am **Ruth Ladenstein**
Final results of the randomised short term infusion (STI) of ch14.18/CHOmAB immunotherapy in combination with Aldesleukin: a report on outcome and toxicities from the HR-NBL1/SIOPEN trial *abs# 94*

MORNING TEA

10:30am - 11:00am Exhibition Area

PARALLEL 11 - BIOMARKERS AND NOVEL APPROACHES

11:00am - 12:30pm Meeting room 1 & 2

Chairs: **Per Kogner & Pat Reynolds**

11:00am **Iedan Verly**
Catecholamine metabolites: novel diagnostic insight, correlations with biological features and prediction of clinical outcome in patients with neuroblastoma *abs# 95*

11:15am **Anne Hakkert**
High frequency of Cytosine to Adenine mutations in neuroblastoma correlates with genomic aberrations in 8-Oxo-Guanine repair pathway *abs# 96*

11:30am **Geertrui Denecker**
The FOXM1 target gene BIRC5 (survivin) is a top ranked dosage sensitive gene located on the common large copy number 17q gained segment in neuroblastoma. *abs# 97*

11:45am **Belamy B Cheung**
Identification of novel small molecule compounds to restore sensitivity to trophic factor withdrawal in MYCN-initiated death resistant cells *abs# 98*

12:00pm **Sabine Taschner-Mandl**
Metronomic Topotecan impedes tumor growth of *MYCN*-amplified neuroblastoma cells *in vitro* and *in vivo* by therapy induced senescence *abs# 99*

12:15pm **Michael M Song**
High MYCN, low MYC, low CERS4, and low anti-apoptotic BCL2 gene family expression are associated with sensitivity to fenretinide in neuroblastoma cell lines and PDXs *abs# 100*

PARALLEL 12 - IMMUNOTHERAPY AND EARLY PHASE TRIALS

11:00am - 12:30pm Meeting room 3 & 4

Chairs: **Daniel Morgenstern & Rajen Mody**

11:00am **Annette Kuenkele**
Preclinical assessment of CD171-directed CAR T cell adoptive therapy for childhood neuroblastoma: CE7 epitope target safety and product manufacturing feasibility *abs# 101*

11:15am **Holger N Lode**
Phase II clinical trial with long-term infusion of anti-GD_2 antibody ch14.18/CHO in combination with interleukin-2 (IL2) showed clinical efficacy and improved toxicity in patients with high risk neuroblastoma. *abs# 102*

11:30am **Sara M. Federico**
Humanized anti-gd2 antibody (hu14.18k322a) with Chemotherapy +/- parental natural killer (nk) cells in children with recurrent/ refractory Neuroblastoma *abs# 103*

11:45am **Shahab Asgharzadeh**
Tumor-associated macrophage polarization state and the dynamic nature of PDL1 expression in neuroblastomas *abs# 104*

12:00pm **Julie R Park**
Engineered Neuroblastoma Cellular Immunotherapy (ENCIT)-01: A phase 1 study of autologous T-cells lentivirally transduced to express CD171-specific Chimeric Antigen Receptors (CAR) for recurrent/refractory high-risk neuroblastoma (HR-NB) *abs# 105*

12:15pm **Steven G. DuBois**
Phase II study of alisertib, irinotecan, and temozolomide in children with relapsed and refractory neuroblastoma: A report from the New Approaches to Neuroblastoma Therapy (NANT) consortium *abs# 106*

NEUROBLASTOMA UPDATE - 1

11:00am - 12:30pm Hall A

Chairs: **Sue Cohn & Andy Pearson**

11:00am	**Sharon Diskin** Overview of neuroblastoma epidemiology and genetic predisposition	*abs# 107*
11:20am	**Frank Speleman** Actionable genomic mutations	*abs# 108*
11:40am	**Susan Cohn** Updates on the International Neuroblastoma Risk Group (INRG). Classification System and Interactive INRG Database (iINRGdb)	*abs# 109*
12:00pm	**Gudrun Schleiermacher** Overview of treatment for low- and intermediate-risk patients	*abs# 110*

LUNCH

12:30pm - 1:30pm Exhibition Area

FOLLOW-UP ANRA ADVISORY BOARD MEETING

12:30pm - 1:30pm Meeting room 3 & 4

INDUSTRY SPONSORED WORKSHOP: UNITED THERAPEUTICS

12:45pm - 1:25pm Meeting room 1 & 2

PARALLEL 13 - GENOME WIDE ANALYSIS AND GENETIC VARIATION

1:30pm - 3:30pm Meeting room 3 & 4

Chairs: **Frank Westermann & Katleen De Preter**

1:30pm	**Mario Capasso** Whole exome and deep targeted sequencing of clinically aggressive neuroblastomas reveal recurrent somatic mutations in pathways involved in cancer progression	*abs# 111*
1:45pm	**Sharon J. Diskin** Identification of germline mutations in 776 children with neuroblastoma	*abs# 112*
2:00pm	**Fakhera Ikram** Fusion-transcripts are associated with an unfavourable phenotype in neuroblastoma	*abs# 113*
2:15pm	**Paul Deveau** Clonal reconstruction in neuroblastoma shows enrichment of mutations in cell survival and DNA-repair pathways at relapse	*abs# 114*
2:30pm	**Frank Westermann** Chromosomal rearrangements juxtapose active enhancer elements to oncogenes in high-risk neuroblastoma	*abs# 115*
2:45pm	**Carolina Rosswog** Molecular risk assessment of neuroblastoma patients eliminates the necessity of clinical prognostic markers	*abs# 116*
3:00pm	**Mark A Applebaum** Genetic variants in *BARD1 and KIF15* are associated with *MYCN*-amplification in neuroblastoma	*abs# 117*
3:15pm	**Navin Pinto** Pharmacogenetics of treatment response in patients with high-risk neuroblastoma, a Children's Oncology Group study	*abs# 118*

PARALLEL 14 - CLINICAL AWARDS SESSION

1:30pm - 3:30pm Meeting room 1 & 2

Chairs: **Holger Lode & Jed Nuchtern**

Sponsored by GILEAD

1:30pm	**Lucas Moreno** Predicting "early" relapse/progression/death in children with INRGSS Stage M neuroblastoma using clinical and biologic factors: An INRG database analysis	*abs# 119*
1:45pm	**Meredith S Irwin** Revised Children's Oncology Group (COG) risk stratification incorporating the international neuroblastoma risk group staging system	*abs# 120*
2:00pm	**Ji Won Lee** High-dose ^{131}I-MIBG treatment incorporated into tandem HDCT/auto-SCT for high-risk neuroblastoma: Results of SMC NB-2009 study	*abs# 121*
2:15pm	**Kelly Huibregtse** Incidence and risk factors for secondary malignancy in patients with neuroblastoma after treatment with ^{131}I-metaiodobenzylguanidine	*abs# 122*
2:30pm	**Pablo Berlanga** Central nervous system relapses in patients with high-risk neuroblastoma: the SIOPEN experience	*abs# 123*
2:45pm	**Andras Heczey** Autologous T cells expressing a GD2 specific chimeric antigen receptor with CD28 and OX40 costimulatory endodomains for children with neuroblastoma	*abs# 124*
3:00pm	**Holger N Lode** Killer-cell Ig-like receptor (KIR) haplotypes and Fcγ-receptor polymorphisms correlate with antibody-dependent cell-mediated cytotoxicity levels and survival of high-risk relapsed/refractory neuroblastoma patients treated by long-term infusion of anti-GD_2 antibody ch14.18/CHO in combination with interleukin-2 (IL-2).	*abs# 125*
3:15pm	**Araz Marachelian** A Phase I Study of Lenalidomide in Combination with ch14.18 and Isotretinoin in Patients with Refractory/Recurrent Neuroblastoma (RR-NB): New Approaches to Neuroblastoma Therapy (NANT) Consortium Trial	*abs# 126*

NEUROBLASTOMA UPDATE - 2

1:30pm - 3:30pm Hall A

Chairs: **Sue Cohn & Andy Pearson**

1:30pm	**Julie Park** Overview of treatment for high-risk patients	*abs# 127*
2:00pm	**Lucas Moreno** Overview of treatment for relapsed disease	*abs# 128*
2:30pm	**Lisa Diller** Long-term effect of treatment	*abs# 129*

AFTERNOON TEA

3:30pm - 4:00pm Exhibition Area

KEYNOTE 4 – STEPHAN GRUPP

4:00pm - 4:45pm Hall A

Chair: **Julie Park**

4:00pm	**Stephan Grupp** The CAR T cell revolution in cancer therapy	*abs# 130*

ANR CLOSING CEREMONY

4:45pm - 5:15pm

Hall A

Chair: **Michelle Haber**

CONFERENCE DINNER

7:00pm - 11:30pm

Hall 2

Cancer Therapeutics CRC

CTx discovers develops and commercialises novel small molecule cancer drugs for adults and children

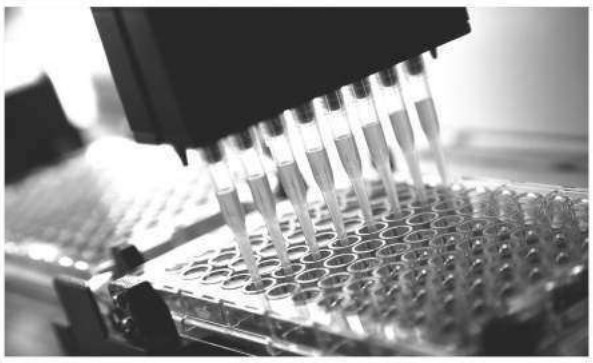
A collaborative partnership of leading Australian MRIs, Universities and biotech companies, CTx is supported by the Australian Government Cooperative Research Centre (CRC) Programme. To date, CTx has successfully commercialized four novel drugs from its pipeline.

CTx has a major focus on targeting early intervention in the cancer metastatic cascade. 90% of cancer deaths result from advanced metastatic disease yet limited attention is being placed on novel adjuvant therapies to treat or prevent the progression. We are bringing this focus to common adult cancers, and also to the often neglected area of new drugs for children with cancer.

CTx's research and development capabilities span the full range of technologies and expertise required to discover novel small molecule cancer drugs and develop them to the clinical candidate stage, and it maintains an extensive and robust patent estate covering its small molecule leads and preclinical candidates. Our business model allows us routes to commercialisation through our internal commercial partners or by partnering with international pharmaceutical or biotechnology companies.

CTx has partners in Melbourne, Sydney, Brisbane, Adelaide, Singapore and an ongoing collaboration with a major international pharmaceutical company. We are always interested in expanding our collaboration to additional organisations that can bring novel cancer biology or who wish to work within our innovative business model.

Find out more at www.cancercrc.com or by contacting our CEO, Dr Warwick Tong (warwick@cancercrc.com) or CSO, Dr Ian Street (ian@cancercrc.com).



Australian Government
Department of Industry, Innovation and Science

Business Cooperative Research Centres Programme

INDUSTRY SPONSORED WORKSHOPS

"EFFICACY AND SAFETY OF ch14.18/CHO IN NEUROBLASTOMA"

Monday 20th June 2016
12:45pm – 1:25pm
Meeting Room 1&2

Holger N. Lode, MD
Professor and Chair of Pediatrics University Medicine Greifswald Germany Monday,

APEIRON BIOLOGICS

Immunotherapy directed against ganglioside GD2 emerges as an important cornerstone in multimodal treatment regimen of neuroblastoma. GD2 is ranked by the national cancer institute of the United States on position 12 of 79 tumor associated antigens and the clinically most advanced concept to exploit GD2 expression on neuroblastoma for therapeutic purposes is passive immunotherapy with monoclonal antibodies (MAB). Clinical efficacy of human/mouse chimeric MAB ch14.18 specific for GD2 was demonstrated in large multi-center clinical trials conducted by independent cooperative groups either used as monotherapy or in combination with cytokines. In Europe ch14.18 was remanufactured in Chinese hamster ovary cells (ch14.18/CHO) and was investigated in multi-center Phase I, II and III clinical trials in frontline treatment regimen and in patients with relapsed and refractory disease. In order to improve tolerability of the treatment, lon term continuous infusion was evaluate suggesting that this is the preferred method of ch14.18 delivery. A summary of results of ch14.18/CHO therapy across clinical trials will be reviewed and discussed underlining efficacy and safety of this MAB for children with NB.

"Establishing a New Standard of Care for High-Risk Neuroblastoma Patients – our practical experience"

Thursday 23rd June 2016
12:45pm – 1:25pm
Meeting Room 1&2

United Therapeutics ONCOLOGY

We are delighted to invite you to attend the symposium sponsored by United Therapeutics Europe, Limited at this year's Advances in Neuroblastoma Research (ANR) 2016 Congress, entitled "Establishing a New Standard of Care for High-Risk Neuroblastoma Patients – our practical experience".
The symposium will raise knowledge of Unituxin® (dinutuximab) as the only approved immunotherapy for the treatment of high-risk neuroblastoma, demonstrating the clinical risks and benefits of this therapeutic advance versus previous standard of care. It is an opportunity to examine the clinical application and role of Unituxin immunotherapy in
patients, and to share experience in current best-practice approaches to Unituxin treatment optimisation through a series of case studies.
The meeting will be co-chaired by two leading European experts in the management of high-risk neuroblastoma who also have experience using Unituxin immunotherapy.

- Dr Jaume Mora (Department of Hematology and Oncology. Hospital Sant Joan de Deu (HSJD) Barcelona, Spain)
- Dr Stergios Zacharoulis (Paediatric Oncology Consultant, Royal Marsden Hospital NHS Trust, Sutton, Surrey, UK)

We look forward to your attendance in what we hope will be an insightful, practical and enjoyable session.

Unituxin is not registered for use in Australia. Unituxin is approved by the European Commission and the US Food and Drug Administration for the treatment of high-risk neuroblastoma in paediatric patients.

A satellite symposium organised by United Therapeutics Europe, Limited (not included in the main event CME/CPD credit offering). ANR 2016 has provided space for this industry session. The programme was independently produced, not subject to review by ANR, and is not part of the scientific/educational programme offered by ANR 2016.

NEUROBLASTOMA PARENTS DAY

FRIDAY 24TH JUNE, 2016

TEA & COFFEE
08:30am - 09:00am

WELCOME AND OVERVIEW OF THE DAY'S AGENDA
09:00am - 09:10am Meeting room 6
Chair: **Donna Drew**

HIGH STAKES, DIFFICULT CHOICES: NAVIGATING THE MAZE OF CANCER THERAPY
09:10am - 10:00am Meeting room 6
Chair: **Donna Drew**

Donna Ludwinski, Solving Kids' Cancer
Making informed and strategic decisions about treatments creates a huge burden of responsibility for parents of children diagnosed with neuroblastoma. Information sources now range from oncologists and web-based medical journals to social media. The key elements of clinical research, and perspectives on past, present and future therapies provide the context for understanding current advances in research. With mutual trust and respect in the doctor-parent relationship, informed parents are best equipped for the difficult task of making choices for their child.

GENETICS
10:00am - 10:40am Meeting room 6
Chair: **Donna Drew**

John Maris, The Children's Hospital of Philadelphia

MORNING TEA
10:40am - 11:00am Mezzanine Level Foyer

PARALLEL - IMMUNOTHERAPY AND NEUROBLASTOMA
11:00am - 11:40am Meeting room 6
Chair: **Toby Trahair**

Holger Lode, University of Medicine Greifswald

PARALLEL - NURSING SYMPOSIUM "BREAKING BAD NEWS"
11:00am - 11:40am Meeting room 5
Chair: **Donna Drew**

Donna Ludwinski, Solving Kids' Cancer
Delivering bad news, and helping parents (and patients) absorb and cope with the bad news is a major challenge for medical professionals. This requires understanding the parent perspective of diagnosis, disease trajectory, informed consent, and managing family expectations in phase I and phase II trials. Nurses play an integral role in supporting parents transitioning from curative intent to palliative care in hospice and in decisions made in end-of-life care. Nursing delegates will have opportunity to share scenarios they faced with families receiving bad news for open discussion and learn from others' experience.

NEW THERAPIES
11:40am - 12:20pm — Meeting room 6
Chair: **Toby Trahair**
Julie Park, Seattle Children's Hospital

LUNCH
12:30pm - 1:30pm — Mezzanine Level Foyer

TARGETED RADIATION
1:30pm - 2:10pm — Meeting room 6
Chair: **Chris Williams**
Mark Gaze, University College Hospital London

SURVIVORSHIP AND LATE AFFECTS
2:10pm - 2:50pm — Meeting room 6
Chair: **Chris Williams**
Richard Cohn, Sydney Children's Hospital

AFTERNOON TEA
3:00pm - 3:20pm — Mezzanine Level Foyer

A FAMILY TELLS THEIR STORY
3:20pm - 4:00pm — Meeting room 6
Chair: **Donna Drew**

PANEL DISCUSSION
4:00pm - 6:00pm — Meeting room 6
Chair: **Glenn Marshall**
Panel: Donna Ludwinski, John Maris, Julie Park, Richard Cohn, Glenn Marshall, Sydney Children's Hospital, Toby Trahair, Sydney Children's Hospital

ROUND UP AND CLOSE
6:00pm - 6:05pm — Meeting room 6
Chair: **Donna Drew**

DINNER
6:05pm



ABSTRACT **BOOK**

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1

Complexes of N-MYC and MYC with Aurora-A: an inroad to targeting MYC function for neuroblastoma therapy

Martin Eilers[1]
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Amplification of *MYCN* is a driver of neuroblastoma development, necessitating strategies to target the protein for effective therapy. The N-MYC protein, like its counterpart, MYC, is a globally acting transcription factor that binds to virtually all active promoters and thousands of enhancers. Current data show that MYC proteins have unusual and potentially targetable ways to engage the transcription machinery. For example, we have recently shown that a critical domain in MYC (MycBoxI) recruits the PAF1c transcription elongation complex to promoters and catalyzes a Brd4- and Cdk9-dependent transfer of PAF1c onto RNA polymerase, providing a model how bromodomain and Cdk inhibitors can target MYC-dependent transactivation.
In neuroblastoma, N-MYC is stabilized by association with the Aurora-A protein. As a consequence, Aurora-A ligands that disrupt the complex destabilize N-MYC. This strategy shows therapeutic efficacy in mouse models of several N-MYC driven tumors and in (c)MYC-driven hepatocellular carcinoma and is being explored in human clinical trials. In contrast, the biological function of the N-MYC/Aurora-A complex has not yet been clarified. We have therefore purified N-MYC protein complexes from neuroblastoma cells and identified novel co-activator complexes that link N-MYC with proteins involved in chromatin topology. These co-activator complexes are specifically required for activation of cell cycle regulated target genes by N-MYC. Association of N-MYC with Aurora-A remodels the co-activator complexes during progression through the cell cycle, arguing that the regulated association of N-MYC with Aurora-A links N-MYC function with the cell cycle machinery. We will present these findings and discuss how they may open new strategies to target N-MYC function.

2

The role of PRC2 in the early neuroblastoma tumorigenesis in *MYCN*-Tg mice

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[Introduction] The aim of this study is to better understand the regulatory mechanisms of early neuroblastoma (NB) tumorigenesis. To this end, we used *MYCN*-Tg mice and primarily looked at sympathoadrenal progenitors during embryogenesis.
[Methods] We established a spheroid culture method, in which undifferentiated neuroblasts could form spheres, and evaluated sphere-forming and tumor-forming ability of neuroblasts. We further analyzed transcriptome and epigenome of E13.5 spheres from wild-type and *MYCN*-Tg mice using microarray and methyl-CpG binding domain (MBD) protein-enriched genome sequencing (MBD-seq).
[Results] Firstly, we observed that E13.5 spheres from *MYCN*-Tg but not from wild-type mice were passageable and were able to develop subcutaneous tumors, suggesting that NB tumorigenesis has started as early as E13.5 in *MYCN*-Tg mice. *MYCN* expression was confirmed by means of *in situ* hybridization in a subset of neuroblasts at E13.5 in *MYCN*-Tg mice. Consistent with this, microarray expression analysis and GSEA revealed that *MYCN* target genes were upregulated in E13.5 *MYCN*-Tg spheres, validating the usefulness of the spheroid culture as a tool reflecting *in vivo* tumorigenesis. Importantly, it was also revealed that polycomb repressive complex 2 (PRC2) target genes were broadly downregulated while its components were slightly upregulated. Moreover, MBD-seq revealed that the number of promoter-methylated genes was increased in *MYCN*-Tg spheres. Genes related to development were enriched in the promoter-methylated genes. We identified a group of genes whose promoters were methylated and expressions were simultaneously downregulated in *MYCN*-Tg spheres. Expressions of these genes were downregulated in high-risk and stage 4 NBs, and could predict patient's prognosis.
[Conclusion] Our study demonstrates the importance of epigenome, particularly PRC2 activation, in impaired developmental program in the early NB pathogenesis. Investigation of the epigenetic intervention potential is currently being undertaken in our laboratory to cure NB. Further study about PRC2 and its epigenetic regulatory mechanism may open a new therapeutic avenue.

3

Transforming primary neural crest cells to model neuroblastoma reveals a lineage sensitivity to BET inhibitors

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Neural crest cells (NCCs) are the embryonic precursor cell population to neuroblastoma. To study neuroblastoma oncogenesis we have established an assay based on the oncogenic transformation of primary mouse NCCs. Since N-Myc amplification is a prominent category of high-risk neuroblastoma, we first determined if N-Myc overexpression was sufficient to transform NCCs. Primary NCCs were isolated from neural tube explants from day 9.5 embryos. We confirmed isolation of NCCs by immunostaining for NCC markers and their ability to form tyrosine hydroxylase (TH) positive neurons. NCCs were retrovirally transduced with MSCV-N-Myc and ~10,000 cells were injected subcutaneously per mouse resulting in two tumors out of 16 mice. Both tumors were uniformly positive for synaptophysin, MAP2 and Phox2B with sporadic staining for TH. These tumors contained neuropil and ganglion cells, and were characterized as neuroblastoma. To determine if loss of p53 could increase the efficiency of transformation, NCCs from p53-compromised mice were infected with N-Myc. This led to 100% tumor penetrance in 19 mice. These tumors were a mix of PNET and other cancer types, and were characterized as primitive neuroectodermal tumors with divergent differentiation. The NCC approach we have established is a promising new tool for investigating the molecular causes and contribution of impaired development to neuroblastoma.
Using our NCC system we can test treatment response along neuroblastoma ontogeny. Stem/progenitor cells, like NCCs, are believed to be the cell of origin for many cancers and a source for the cancer-initiating cells that are speculated to cause relapse. BET (bromodomain and extraterminal domain) inhibitors, like JQ1, target shared lineages in some cancers like SCLC. We too found a lineage-dependent sensitivity in neuroblastoma with superimposable JQ1 responses seen from non-transformed wild-type NCCs through NCC-derived neuroblastomas. We found no correlation of JQ1-sensitivity with proliferation rates, N-Myc expression, transformation status or p53 status. This suggests BET bromodomain inhibitors can target the immature cell populations of neuroblastoma and should be explored as a therapy for preventing relapse.

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Not available at time of print

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Not available at time of print

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The *BRIP1/FANCJ* DNA helicase is a druggable 17q driver oncogene involved in G-quadruplex induced replicative stress resistance in neuroblastoma

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Chromosome 17q gain is by far the most common DNA copy number alteration in aggressive neuroblastoma (NB) but the causal 17q drivers remain to be identified due to the large size of the recurrently involved chromosome segments. Integrated mRNA/CNV analysis of 211 NBs with the CONEXIC algorithm identified the DNA helicase *BRIP1* (alias *FANCJ*) as top-ranked candidate 17q driver gene. Stable BRIP1 knock down in NB cell lines significantly reduced cell viability and colony forming capacity. In keeping with its role in DNA damage repair, knock down induced DNA damage as evidenced by increased γH2AX. Given that BRIP1 also unwinds G-quadruplex (G4) DNA structures, we hypothesized that increased BRIP1 levels could protect NB cells from MYCN induced replicative stress and install what we call "replicative stress resistance". Knock down increases RPA32 protein levels and decreases sensitivity to hydroxy urea induced replication fork stalling as measured by DNA combing. Next, we assumed that the dependency to G4 unwinding of cancer cells would render them sensitive to G4 stabilising ligands and indeed observed strong effects on viability upon treatment with TMPYP4. Gene expression profiling after BRIP1 knock down confirmed enrichment for gene sets implicated in DNA replication and repair. Next, overexpression of BRIP1 in dβh-MYCN-eGFP transgenic zebrafish caused accelerated tumor formation. We are now using this model to screen for synergism between G4 stablising ligands such as TMPYP4 and pyridostatin by oral gavage in zebrafish to assess possible synergistic effects as a prelude to novel therapies for high risk NB. In conclusion, we propose BRIP1 as a major 17q cooperative driver oncogene in NB by providing replicative stress resistance to highly replicative NB cells at G4s, offering a new entry point for drugging of aggressive high risk NB.

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MLL1 and JMJD3 regulate neuroblastoma cancer stem cells

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Neuroblastoma displays marked intratumoral heterogeneity and is composed of multiple subpopulations including the G-CSF receptor expressing (CD114+) cancer stem cells (CSC). These highly tumorigenic G-CSF responsive cells are in part distinguished by stem-cell like epigenetic alterations at microRNA and other oncogenic loci.
Since CSCs are well-known to be regulated by histone modifications, we evaluated the epigenetic regulation of the G-CSF receptor encoded by the *CSF3R* gene. In addition to elevated CSF3R expression, we found high levels of MLL1 (encoded by KMT2A), and Jumonji-D3 (JMJD3, encoded by KDM6B) in CD114+ NB CSCs. MLL1 is an H3K4 histone methyltransferase that catalyzes H3K4me1, H3K4me2 and H3k4me3 methylations, while JMJD3 is an H3K27me3 demethylase. Chromatin immunoprecipitation (ChIP)-qPCR analysis for H3K4me3 and H3K27me3 epigenetic marks at the *CSF3R* locus revealed increased activating H3K4me3 and reduced repressing H3K27me3 marks in CD114+ cells consistent with increased CD114 surface expression. In CD114- bulk tumor cells, we found opposite pattern of increased H3K27me3 and reduced H3K4me3 marks.
We hypothesized that inhibition of these epigenetic regulators would block *CSF3R* expression and inhibit CSC-mediated tumor growth and metastasis. Therefore, we evaluated the *in vivo* efficacy of MM-102 (MLL1/WDR5 inhibitor) and GSK-J4 (JMJD3 inhibitor) against NB xenografts. Remarkably, NB CD114+ cells proved *highly sensitive* to both compounds, leading to rapid apoptosis of CD114+ subpopulations and minimal death of CD114- cells. Apoptosis of CD114+ cells *strongly and significantly correlated with decreased tumor size* ($p<0.001$) *and metastatic burden* ($p<0.001$). Furthermore, exogenous G-CSF partially reversed these effects, and increased CD114+ percentages in resected tumors ($p<0.01$). Overall, we demonstrate that specific histone modifications are essential for survival of the NB CSCs, and specific epigenetic inhibitors can be highly potent anti-tumor agents for high-risk neuroblastoma. Our findings have direct translational impact and support the development of epigenetic stem cell targeted therapies for neuroblastoma.

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Sensing mutant ALK: capicua and ETV5 as executors of aberrant ALK-driven MAPK signaling in neuroblastoma

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Activating ALK mutations occur in 10% of neuroblastomas (NB) and represent an important novel druggable target for more effective treatment of high-risk patients. Importantly, ALK mutations emerged as an important event in relapsed cases. In order to design effective novel targeted therapeutic approaches, gaining detailed insights into downstream ALK signaling is crucial. We and others identified PI3K/mTOR and RAS/MAPK as major downstream signaling axes. Also, we connected FOXO3a controlled RET expression to the PI3K/mTOR axis. Here, using multiple ALK activating and inhibiting cell models, we firmly establish ETV5 as a major RAS/MAPK downstream target upregulated through mutant ALK. ETV5 is known to act as a regulator of epithelial-mesenchymal transition (EMT) and controls stem cell properties and neuronal cell fate decisions. Knockdown of ETV5 reduced the clonogenic potential and growth of NB cells *in vitro* and *in vivo*. RNAseq transcriptome profiling following ETV5 knockdown provided an ETV5 signature score which identifies patients with poor overall survival and showed enrichment in gene sets controlling EMT, in keeping with observed reduced invasive properties in ETV5 depleted NB cell lines. Of further interest, transcription factor target enrichment analysis shows that downregulated genes following ALK inhibition are significantly enriched in targets of Capicua (CIC). Essentially, CIC is a RAS/MAPK responsive gene, negatively regulating ETV5 in melanoma, and acting as general sensor for RTK signalingby repressing gene expression. CIC is located on a 19q13.2 segment of recurrent loss in NB and we found two putative inactivating insertions in the CIC gene in NB cell lines. In conclusion, our data highlight CIC and ETV5 as intrinsic components of ALK - RAS/MAPK signaling in NB with immediate potential relevance given that deregulation of this axis can provide NB cells with EMT plasticity leading to stemness features, improved migratory capacity and therapy resistance.

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TERT rearrangements are frequent in neuroblastoma and identify aggressive tumors

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Recently, we have presented the genomic landscape of 87 neuroblastoma tumors. As recurrent somatic mutations are rare in this cancer and enhancer hijacking has been demonstrated in medulloblastoma, we hypothesized that recurrent structural variations could be identified in our neuroblastoma cohort extended to 108 cases.
Methods
Whole genome sequencing was performed on a total of 108 tumor/lymphocyte DNA samples. Somatic structural variations were identified and analyzed for recurrent locations. Coverage based breakpoint, mRNA expression, telomere length, and super-enhancer analyses were performed.
Results
For each Mb region in the genome, we calculated the number of tumors with structural events. The second-most frequently affected region after *MYCN* was located around the *TERT* locus in 23% of the high stage tumors. *TERT* was the only gene in the vicinity with a significantly increased expression in the rearranged cases as compared to normal cases ($p=8.51x10^{-5}$, Wilcoxon Ranksum). Most of the rearrangements occurred TERT. We identified neuroblastoma-specific super-enhancers in seven of the translocation partners, a significant enrichment over random breaks ($p<0.003$). TRF analysis showed increased telomere lengths for rearranged cases, confirming increased telomere repeat counts in the corresponding sequence data. TERT rearrangements were associated with poor prognosis (p=0.04 Logrank) and almost mutually exclusive with MYCN amplification and ATRX defects. In multivariate analyses all 3 showed independent prognostic significance.
Conclusions
TERT rearrangements form the second-most frequent gene defect in neuroblastoma,
after *MYCN*. *TERT* defects are almost mutually exclusive with *ATRX* and *MYCN* defects, and each identifies a separate group at very high risk. These tumors have elevated *TERT* expression due to rearrangement of the upstream 30 kb or downstream 40 kb regions and in over half of the informative breakpoints, *TERT* was ostensibly activated by hijacking a super-enhancer. Pharmacological inhibition of *TERT* might in future improve the outcome for this patient group.

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INRG Data Commons – A User Journey

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Established in 2004, the International Neuroblastoma Risk Group (INRG) brought together international consortia to collect and standardize data on over 8,000 patients. New and updated information have been periodically added, and in 2013, an online cohort discovery tool was built and made available to investigators from around the world. In addition to allowing users to query the 18,000 patients with combinations of 32 clinical and other phenotypic variables, the site provides a real-time link to pull information from the COG Biopathology Center and neuroblastoma nucleic acid bank.
In order to expand the usability and functionality of the database, the INRG Data Commons was established in 2016 as a way to link the phenotypic information to available genomic data. In a collaboration between the University of Chicago's Center for Research Informatics and Center for Data Intensive Science, a neuroblastoma data commons has been established that permits users to perform complex cohort discovery followed by genomic analysis, all in a protected, high-performance environment. These innovations, along with future expansion plans, have the potential to transform the discovery process for neuroblastoma and other malignancies.
In this presentation, you will be taken on a user journey from cohort discovery and project initiation through the analysis of phenotype and genotype data.
Learning objectives:

- What are the capabilities of the INRG Data Commons?
- What governance is in place for ensuring the privacy of the patients and the appropriate use of the data?
- How do I request access to the data?
- How can I contribute data?

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Emerging epigenetic targets in *MYCN*-amplified neuroblastoma

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The genomics revolution has revealed remarkably simple genomes as a defining feature of many pediatric cancers. These cancers are often driven by key initiating events involving aberrant transcription factors or abnormalities of an epigenetic regulator. For example, *MYCN* amplification in neuroblastoma is a well described recurrent abnormality and poor prognostic marker, EWS/ETS rearrangements are a defining feature of the pediatric solid tumor Ewing sarcoma, *MLL*-rearrangements are commonly found in infant leukemia, and mutations in histone H3.3 have been recognized as initiating events in pediatric glioblastomas. We are taking a collaborative, integrated genomics approach to identifying new dependencies in pediatric cancers through the application of functional and chemical genomic approaches. Specifically, genomically characterized pediatric cancer cell lines are screened using genome-wide shRNA and CRISPR/Cas9 and bioactive chemical libraries to connect disease lineage or genotype with response to either gene suppression/knockout or small-molecule inhibition. High-risk pediatric cancers, including neuroblatoma, were nominated as top priority diseases for screening. Emerging targets scoring in this screening effort in *MYCN*-amplified neuroblastoma will be presented.

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Neuroblastoma is bi-phasic and includes classical neuro-epithelial cells and chemo-resistant mesenchymal cells

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A major question in neuroblastoma research is why most high stage neuroblastoma initially respond to chemotherapy, but ultimately relapse as therapy-resistant tumor. We detected in new neuroblastoma cell lines two cell types with shared genetic defects but highly divergent phenotypes. One type expresses all classical neuroblastoma markers and has a neuro-epithelial (NE) phenotype. The other type lacks all neuroblastoma markers, is motile and has a mesenchymal (MES) character. Immunohistochemistry detected a small fraction of MES cells in most primary neuroblastoma. In four isogenic cell line pairs, MES cells were more chemo-resistant than their NE-type counterparts. Indeed, in primary tumors, viable MES-type cells accumulated after chemo-therapy. Moreover, relapses emerging 5 years after diagnosis were highly enriched for MES cells as compared to the primary pre-treatment samples of the same patients. As these data suggest a role for MES-type cells in relapse development, we analyzed their key regulatory pathways. In isogenic cell line pairs, MES and NE-type cells widely differed in major signaling routes, transcription factors and histone modifications. MES cells had high NOTCH and PRRX1 pathway activity. Induced expression of NOTCH or PRRX1 converted multiple NE-type cell lines into MES-type cells, including chemo-resistance. These pathways activated MEK and PDGFRβ, which were successfully targeted by small molecules to specifically kill MES cells *in vitro*.

Conclusions

Our data suggest that neuroblastoma is a bi-phasic tumor. MES and NE cells differ in many characteristics, but can transdifferentiate into each other. MES and NE cells may correspond to developmental stages, i.e. mesenchymal migratory cells delaminated from the neural crest and more differentiated cells of the adrenergic lineage. MES cells accumulate after chemo-therapy and in relapses. They may survive classical therapy and seed relapses, that ultimately become heterogeneous again. Elimination of MES cells with small molecule inhibitors shows how they are amenable to therapy.

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Distal chromosome 6q-deletion defines a subgroup of ultra-high risk neuroblastoma patients

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G.Schleiermacher/K.De Preter:shared last authors

Prognostic classification of high-risk neuroblastoma patients remains challenging. The aim of this study was to define genomic biomarkers for upfront identification of ultra-high-risk patients that will develop resistance to chemotherapy.

High-resolution array-CGH profiles of diagnostic tumor samples from 570 high-risk neuroblastoma patients were collected from 8 collaborative groups. To correct for possible inter-platform variability, raw data were transformed to a common genome build and segmented using SegAnnDB.

First, datasets from seven European centres were used for training (n=317), while the COG-cohort (n=253) was used for validation of the classifier. We built a classifier using the regions with differential copy numbers (logistic regression analysis) in two extreme training groups, i.e. high-risk patients that die within 18 months and high-risk patients that survive with at least 5 years event-free follow-up. However, this classifier could not discriminate patients with ultra-high-risk in the validation cohort. A possible explanation is that the different cohorts have slightly different population characteristics and treatment protocols.

Second, when we investigated the prognostic power of individual aberrations in both cohorts, we identified regions that are prognostic in one cohort but not in the other, including 1p-deletion and 1q-gain which do not predict poor outcome in the COG cohort. Data from other cohorts will be collected to validate a classifier based on regions that are prognostic across different cohorts.

Indeed, both cohorts also share copy number aberrations that are prognostic, including *MYCN*-amplification, 4p-deletion and distal 6q-deletion (below -0.3 log relative copy number). Most significant results were observed for 6q-deletion which defines a small subgroup of ultra-high-risk patients with extremely poor outcoma as 92% (12/13) and 100% (9/9) of the training and validation patients with this aberration died of disease.

In conclusion, we identified distal 6q-deletions (more distal to ARID1B previously described in neuroblastoma) that mark neuroblastoma patients for fatal outcome. Further studies are ongoing to identify the 6q-gene/regulatory element that drives the aggressiveness of this disease.

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Chemical proteomics defines kinome responses to ALK inhibition in neuroblastoma

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The discovery of mutations in the ALK oncogene as the etiology of familial neuroblastoma and as somatically acquired in 14% of high-risk patients provided an opportunity to develop rational therapeutic strategies. Despite major advances in the clinical development of ALK inhibitors (Infarinato, 2015), it is unclear the exact mechanisms by which ALK inhibition abrogates tumorigenesis or, in some cases, activates compensatory pathways to promote drug resistance. The objective of our work was to identify immediate and adaptive changes in the kinome upon ALK inhibition. We used Multiplexed Inhibitor Beads coupled with Mass Spectrometry (MIB/MS) to quantitatively measure kinase activity dynamics on a proteomic scale. SY5Y cells that harbor one of the three hotspot mutations (F1174L) in the *ALK* gene were treated with either crizotinib or PF-06463922 at 10 nM, 100 nM, 1000 nM for 6 hours, 24 hours and 48 hours prior to MIB/MS analysis. Additionally, three patient-derived xenograft (PDX) models harboring F1174L (COG-N-453x) or the other two most-common mutations, R1275Q (NB-1643) and R1245C (Felix) were treated with either crizotinib (100 mg/kg a day) or PF-06463922 (10 mg/kg twice a day) for 2.5 days or 6.5 days prior to MIB/MS analysis. Although both crizotinib and PF-06463922 inhibited ALK, PF-06463922 preferentially inhibited a series of other kinases, correlating with the superior efficacy of PF-06463922 in pre-clinical studies. Across all three PDX models, Focal Adhesion Kinases (FAK1/2) were among the most statistically different from crizotinib and most strongly-inhibited by PF-06463922. Immunoblotting analysis of FAK phosphorylation (Y397) in cultured cells and tumors treated with both ALK inhibitors showed P-FAK downregulation, a finding that paralleled P-ALK (Y1278) levels in a dose-dependent manner and validated MIB/MS findings. FAK has a postulated role in controlling tumorigenesis in neuroblastoma, and we hypothesize that the preferential inhibition of FAK activity by PF-06463922 may play an important role. Whether FAK is regulated primarily downstream of ALK or jointly through alternative or compensatory signaling networks warrants further investigation.

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Enhancing efficacy of immune checkpoint blockade with anti-macrophage targeted therapy

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Background: Tumor-associated macrophages (TAMs) with alterations in patterns of pro-inflammatory genes are associated with poor prognosis in neuroblastoma (NB). TAM can promote tumor growth and create and anti-inflammatory environment, while expression of the immune checkpoint protein PDL1 can act to block T-cell response via the PD1-PDL1 axis. We set out to identify compounds that inhibit tumor-promoting effects of TAM and assess their efficacy in enhancing immune checkpoint therapy using a transgenic MYCN non-amplified neuroblastoma murine model (NB-Tag).
Methods: Human and murine neuroblastoma cell lines (NBT2 and NBT3L) were used for *in vitro* studies. For *in vivo* tumor growth models, combinations of cyclophosphamide, topotecan, trametinib, anti-CTLA4, and anti-PD1 therapies were used in NB-Tag transgenic, transplantable subcutaneous (NB-SQ), and orthotopic models.
Results: Co-culture of M2-like macrophages with human or murine neuroblastoma cell lines increased tumor proliferation and upregulated MYC expression *in vitro*. Trametinib, a MAPK inhibitor, was identified among a small drug screen with the exceptional ability to inhibit the tumor-proliferative advantage provided by macrophages in the co-culture system. Treatment of 15 week-old NB-Tag mice (visible tumor by MRI) with trametinib after chemotherapy administration significantly impaired tumor regrowth (volume four weeks post-chemo, 491 vs. 42 mm^3, p=0.037). Trametinib treatment also showed similar results in NB-SQ model. Further immune checkpoint blockade of NB-SQ mice with anti-CTLA4 and anti-PD1 in combination with Trametinib treatment post chemotherapy significantly increased survival compared to controls (OS 60% vs. 0%; p<0.0001), and led to long term cures. Tumors and macrophages collected from mice treated with Trametinib showed effective block in phosphorylation of ERK, and attenuation of COX2 expression in macrophages. Transgenic animals that grew despite combination treatment showed STAT3 phosphorylation suggesting this pathway as an escape mechanism.
Conclusions: Our results provide strong evidence that blocking macrophages ability to promote tumor growth through targeted therapies combined with checkpoint blockade can significantly inhibit tumor formation. These findings indicate opportunities to enhance antitumor immunity with the potential to produce durable clinical responses in children with neuroblastomas.

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Withdrawn

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A Phase 3 randomized clinical trial (RCT) of tandem myeloablative autologous stem cell transplant (ASCT) using peripheral blood stem cell (PBSC) as consolidation therapy for high-risk neuroblastoma (HR-NB): A Children's Oncology Group (COG) study

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Background: ASCT improves event-free survival (EFS) for HR-NB. Pilot studies suggest that intensification of myeloablative therapy using tandem ASCT further improves outcome for HR-NB. We conducted a multicenter RCT comparing tandem vs. single consolidation in patients with HR-NB.

Methods: Between 11/2007 and 2/2012, 652 eligible patients (pts) with newly diagnosed HR-NB received induction therapy: 6 cycles of chemotherapy including initial 2 cycles of dose-intensive cyclophosphamide/topotecan followed by PBSC collection. Randomization occurred at end induction to single ASCT with carboplatin-etoposide-melphalan (CEM) or tandem ASCT with thiotepa–cyclophosphamide ASCT followed by a modified CEM (TC:CEM). HR pts with non-MYCN amplified Stage 3 (age>18mos) or Stage 4 (age 12-18 mos) tumors were non-randomly assigned to single ASCT (CEM). EFS and overall survival (OS) were analyzed as intent-to-treat.

Results: Median age at study entry was 3.1 yrs, 88% (n=574 pts) had Stage 4 disease and 38.2% (n=249 tumors) had MYCN amplification. A total of 355 pts were randomized (CEM n=179 pts; TC:CEM n=176 pts) and 27 patients were non-randomly assigned to CEM. Of randomized pts, 249 patients received post-consolidation immunotherapy on COG trials. Treatment-related mortality was 2.6% (Induction n=7 [1%]; Consolidation n=10 [2.8%; n=8 CEM, n=2 TC:CEM]). Rates of severe mucosal, infectious or liver toxicities were similar between arms. 3-year EFS and OS from diagnosis were 51.1±2.0% and 68.2±1.9%, respectively. EFS and OS for randomized cohort are noted in the Table.

Cohorts	N	3-yr EFS +/- SE (%)	2-sided p-value	3-yr OS +/- SE (%)	2-sided p-value
CEM	179	48.8 ± 4.0		69.0 ± 3.6	
TC:CEM	176	61.8 ± 4.1	0.0082	73.8 ± 3.7	0.2563
Immunotherapy					
CEM	129	55.4 ± 4.6		75.7 ± 3.9	
TC:CEM	120	73.7 ± 4.4	0.0009	86.3 ± 3.4	0.0158

Conclusions: Tandem myeloablative consolidation therapy improves survival probability in patients with high-risk neuroblastoma, especially in the setting in post-consolidative immunotherapy.

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Phase II randomized trial of irinotecan/temozolomide (I/T) with temsirolimus (TEM) or dinutuximab plus granulocyte colony stimulating factor (DIN/GMCSF) in children with refractory or relapsed neuroblastoma: a report from the Children's Oncology Group (COG)

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Background: Outcomes for children with relapsed and refractory neuroblastoma are dismal. The combination of I/T has activity in patients with relapsed disease, and the toxicity profile of I/T makes it an excellent backbone for study of new agents. Temsirolimus (TEM) and dinutuximab (DIN) were selected for testing in combination with I/T in subjects with relapsed, progressive or refractory neuroblastoma.

Methods: COG ANBL1221, a randomized Phase II selection design trial, compared response and toxicity in subjects treated with I/T in combination with either TEM (Arm A) or DIN/GM-CSF (Arm B). Patients were eligible at first relapse/progression or first designation of primary refractory disease. Randomization was stratified based on prior therapy and *MYCN* status.

Cycles were administered every 21 days. Partial and complete responses (PR, CR) were confirmed centrally.

Results: Thirty-five eligible patients were enrolled. Median age was 5.7 years (range 2.1-16.2), 24 pts had measurable disease (12 per arm). The 18 subjects randomized to Arm A received 89 total courses (median 3); 1 PR was observed (5%). The 17 patients randomized to Arm B received 136 total courses (median 6). Nine (53%) had objective responses (4 PR, 5 CR), including responses in 5 of 10 patients with relapsed/progressive disease and 4 of 7 with refractory disease. Among Arm B responders, prior therapy included high dose chemotherapy with stem cell rescue in 5 and anti-GD2 therapy in 3. One patient with rapidly progressive thoracic disease experienced Grade 4 hypoxia during Arm B therapy; no other subject experienced unanticipated toxicity. Arm B met protocol-defined criteria for selection as the optimal combination for further study.

Conclusion: I/T/DIN/GM-CSF shows significant anti-tumor activity in patients with relapsed/progressive or refractory neuroblastoma. Additional study of this combination will include evaluation of clinical and biological markers that may identify patients most likely to respond to this active chemo-immunotherapeutic regimen.

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Second malignancies in patients with neuroblastoma: a report from the international neuroblastoma risk group project

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Background: Radiation and chemotherapy exposure are associated with the development of second malignant neoplasms (SMN) in neuroblastoma survivors. However, whether the incidence of SMN has changed with modern, intensive treatment is unknown. Further, the role genetic factors play in the etiology of SMN remains unclear.

Methods: The International Neuroblastoma Risk Group (INRG) database of patients diagnosed from 1974-2013 was analyzed. SMN risk was measured by cumulative incidence, standardized incidence ratios (SIR) and absolute excess risk (AER). Poisson regression estimated incidence rate ratios (IRR). Genes previously linked to SMN were evaluated in a candidate association study identifying germline polymorphisms associated with this phenotype.

Results: 9,269 of 16,520 patients in the INRG database had SMN data. 81 (0.87%) patients developed SMN, including leukemia/lymphoma (n=40), sarcomas (n=19), carcinomas (n=10), CNS tumors (n=10), hepatoblastoma (n=1), and nephroblastoma (n=1). The 10-year cumulative incidence of SMN for high-risk patients was 1.8% (95% CI: 1.2-2.5%) compared to 0.43% (95% CI: 0.19-0.89%) for low-risk patients (p=0.002). Compared to 1990-1997, those treated after 1997 when stem cell transplant became standard practice had an IRR of 1.97 (95% CI: 1.16-3.55; p=0.007). The SMN incidence of the entire cohort was 9-fold higher than expected (SIR=9.2 (95% CI: 7.3-11.4), AER=13.7). High-risk patients had a 20-fold higher incidence of SMN than expected (SIR=20.5 (95% CI: 15.0-27.4), AER=33.3). Intermediate-risk patients showed a 6-fold rise in SMN (SIR=6.2 (95% CI: 3.3-10), AER=8.3) and low-risk patients showed a 3-fold increase (SIR=3.0 (95% CI: 1.2-6.2), AER=3.5). rs861539 in XRCC3 (p=0.004) and rs17036651 in MSH2 (p=0.009) were most associated with developing SMN though neither retained significance after multiple testing corrections.

Conclusions: Exposure to modern, high-risk neuroblastoma therapy increases the SMN risk. Genomic variation in DNA repair genes may influence SMN susceptibility. Further understanding of treatment exposures and genomic variables enhancing SMN risk is critical for optimizing survivorship care.

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The way towards an international mIBG skeletal score for high risk neuroblastoma: the statistical perspective

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Purpose:

A collaborative effort was undertaken to derive an internationally agreed semi-quantitative meta-iodobenzylguanidine (mIBG) scoring method in neuroblastoma by harmonising previously established scoring systems, which were each found to have prognostic value at the end of induction (Yanik JNM 2013). The aim of this analysis was to investigate the individual effect on event-free-survival (EFS) of the two components of the scoring system: 1) the number of involved anatomic regions and 2) the scoring value within each segment and to evaluate the prognostic value of the new proposed scoring system.

Patients and Methods:

COG and SIOPEN merged data of children with stage 4, mIBG avid, neuroblastoma entered on the COG-A3973 (216pts) and the SIOPEN/HR-NBL1 trial (341 pts). Two independent nuclear medicine review teams scored mIBG scans pre- and post-induction according to Curie- and SIOPEN-methodologies. Here, the SIOPEN score evaluating the skeletal (mIBG) uptake on a 0-6 scale in 12 anatomical regions was chosen for the statistical analysis due to the greater range of values. The two study cohorts were investigated separately and a bootstrap-based internal validation was performed.

Results

In 557 pts the cumulative SIOPEN-score post induction had a significant impact on EFS with 5-years EFS of 41%, 33% and 15% for total scores of 0, 1-3 and >3, respectively. However, no increasing hazards with increasing scores per segment were observed. In contrast, the number of positive segments alone had a highly significant impact on EFS with 5-year EFS of 41%, 32% and 14% for patients with 0,1-2 and >2 positive segments post induction.

Conclusion

The number of positive segments was the most important prognostic factor. Weighting the involvement within segments did not improve the prognostic value of the scoring system. These results suggest a possible simplification of MIBG scoring, facilitating future international collaborations.

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Final results of the randomised short term infusion (STI) of ch14.18/CHOmAB immunotherapy in combination with Aldesleukin: a report on outcome and toxicities from the HR-NBL1/SIOPEN trial

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Aim

After the first important results of the antiGD2/SP02 combination immunotherapy (Alice Yu, NEJM, 2010), this trial investigated the potential and tolerance of ch14.18/CHO monoclonal antibody immunotherapy(IT)± subcutaneous interleukin 2(scIL2) in high-risk neuroblastoma (HRNBL1) front-line patients (pts).

Methods

Between 2009-2011,the HR-NBL1/SIOPEN Phase III trial (APN311-302)(EudraCT:2006-001489-17) randomized 406pts (all stage 4 but if <1yr only with MycN amplification (MNA) and MNA stages 2&3 all ages up to 21yrs). Pts were randomised for IT(R2) at start of maintenance. Eligibility included a COJEC induction ± 2TVD; enrolment on the HDC randomisation (R1, BUMEL vs CEM) with R1response criteria. Local treatments aimed at gross surgical resection and radiotherapy (21Gy). Pts receiver either 100mg/m² ch14.18/CHO (d8-12) as 5 daily 8-hour short-term infusion (STI) alone (STIA) or combined with $6x10^6$IU/m^2 scIL2 (d1-5;8-12) (STIB) for a total of 5 IT cycles; both had 6 cyles of oral 13-cis-RA (160mg/m^2,d19-32). The median age at diagnosis was 3yrs(1month-19yrs) and the median observation time 3.1yrs. Outcomes are reported as 3-yrs event free/overall survival rates (EFS/OS).

Results

The EFS/OS for pts treated with ch14.18/CHO with and without scIL2 was 0.60±0.04/0.66±0.04(206pts) and 0.57±0.04/0.65±0.04/(200pts)(NS). The EFS for CR-pts (or VGPR/PRpts) on STIA was 0.68±0.05 (0.46±0.06) and with STIB 0.65±0.05 (0.53±0.06) indicating no benefit for scIL2. Early termination of IT occurred in 18% in STIA but in 44% in STIB (36% toxicity-related, 8% progressions). A Lansky performance status of ≤30% was found 17% STIA-pts but in of 39% STIB-pts (p<0.001). CTC-grade 3&4 allergic reactions was observed in 9% and 20% of

STIA-pts and STIB-pts (<0.001). Incidence of capillary leak and CTC-grade 3&4 fever significantly lower without scIL2 STIA (1% and 14%) vs. STIB (9% and 40%).

Conclusion The EFS/OS rates at 3yrs show a clear improvement to previous SIOPEN experience. A markedly reduced toxicity without IL2 and equivalent outcome suggest a ch14.18/CHO only approach.

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The CAR T cell revolution in cancer treatment

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Chimeric antigen receptors (CARs) combine a binding fragment of an antibody with intracellular signaling domains. We have reported exciting data on CTL019 cell therapy expressing an anti-CD19 CAR. Infusion of these cells results in 100 to 100,000x in vivo proliferation, durable anti-tumor activity, and prolonged persistence in patients with B cell tumors, including sustained complete responses (CRs) in adults and children with acute lymphoblastic leukemia (ALL; Grupp et al., NEJM 2013, Maude et al., NEJM 2014). This talk will update the audience on pediatric engineered cell therapy. Recent updates of our data in ALL show a 93% complete response rate and 79% overall survival at 1 year.

In addition, we will discuss potential other application in pediatric diseases such as neuroblastoma, which may be targeted with GD2 CARs. Preclinical data from ongoing studies will be presented showing the potential efficacy of alternative targets that may extend this therapy outside of B cell malignancy.

CTL019 cells can undergo robust in-vivo expansion and can persist for over 4 years in patients with relapsed ALL, allowing for the possibility of long-term disease response without subsequent therapy such as transplant. This approach also has promise as a salvage therapy for patients who relapse after allo-SCT with a low risk of GVHD. CTL019 therapy is associated with a significant CRS that responds rapidly to IL6-targeted anti-cytokine treatment. This therapy has received Breakthrough Therapy designation from the FDA, and phase II multicenter trials are underway and have been completed.

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Human pluripotent stem cell-based models of *MYCN*-amplified neuroblastoma

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While genetically engineered mouse models (GEMMs) represent valuable tools to study diseases, human pluripotent stem cell (hPSC)-based models offer several advantages over GEMMs including:
(1) Species identity with human patient tumors.
(2) Robust application to more quickly evaluate candidate genetic drivers.
(3) Synteny of chromosome segments enables modeling of chromosome copy number abnormalities.
Here, we describe hPSC-based models of neuroblastoma (NB). Normal hPSCs were differentiated toward trunk neural crest cells (NCC)[1]. We next introduced established/candidate drivers of NB, and implanted the resulting trunk NCCs orthotopically into renal capsules of mice. As proof-of-principle, we transduced trunk NCC with MYCN and/or mutant anaplastic lymphoma kinase (ALK F1174L), NB drivers described in human patients and demonstrated in GEMMs and zebrafish [2-11]. Within 3 months, 60% of mice developed tumors with ALK F1174L/MYCN, 10% with MYCN, and none with either empty vector or ALK F1174L alone. Tumors were transplantable and demonstrated small round blue cell histology characteristic of poorly differentiated tumors[12]. Further analysis revealed expression of markers typically found in NB, while lacking markers of the small round blue tumors rhabdomyosarcoma, lymphoma and Ewing sarcoma. We are currently evaluating candidate predisposition genetic events that may cooperate with MYCN, including loss of lncRNAs *CASC14, CASC15*, and deletion of chromosome 1p [13-16]. Our preliminary results suggest loss of the lncRNA or chromosome 1p combined with misexpression of MYCN promotes growth *in vitro* and/or *in vivo*. Thus, we present a hPSC-based model of NB that is useful to validate novel candidate genetic drivers and to evaluate personalized therapies.

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5

SOX11 acts as part of the MYCN regulatory protein complex implicated in neuroblastoma

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The high expression levels of the SRY-related HMG-box transcription factor 11 (SOX11) in neuroblastoma, frequent copy number gain and occasional focal amplification, and expression in developing sympathetic nervous system, suggested a role for SOX11 as lineage survival oncogene and prompted us to further investigate its role in MYCN driven neuroblastoma formation.

First, we observed a strong correlation with MYCN expression levels (R=0.576, p=1.9E-26) and high SOX11 expression was indicative for poor prognosis. *In vitro* SOX11 knock down experiments showed amongst others reduced colony formation capacity and G1-S phase cell growth arrest, in conjunction with decreased *MYCN* mRNA levels. Moreover, both in cell lines with *in vitro* MYCN induction and MYCN overexpressing mouse neuroblastoma models, SOX11 was significantly increased, suggesting the existence of

a SOX11 - MYCN regulatory loop. SOX11 and H3K27ac ChIP-sequencing revealed that more than half (62%) of the SOX11 binding sites were at enhancers while 19% of SOX11 targets contained an E-Box motif, typically known to be targeted by MYCN, in keeping with an observed overlap of 30% of SOX11 and MYCN binding targets. Next, we demonstrated close physical proximity/interaction between SOX11 and MYCN proteins using both proximity-ligation as well as co-immunoprecipitation assays.

To further evaluate *in vivo* effects of SOX11 overexpression in sympathetic progenitor cells, we established a zebrafish and mouse dbh-SOX11 model and are performing crossing with the previously established dbh-MYCN animal models to evaluate SOX11 cooperative accelerated tumour formation.

6

Aberrant activation of SHP2 cooperates with MYCN in neuroblastoma pathogenesis

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PTPN11, which encodes Src homology-2 domain-containing phosphatase 2 (SHP2), is the second most frequently mutated gene in high-risk neuroblastoma, and is often associated with amplified *MYCN*, suggesting that these two oncogenes act synergistically. To investigate this possibility, we programed aberrant Shp2 signaling using a transgenic approach to drive the expression of either mutationally activated *ptpn11*, wild-type *Gab2* (an upstream regulator of SHP2) or the *Gab2ΔP85* mutant (which activates SHP2 signaling but not the PI3K pathway) in the peripheral sympathetic nervous system. We found that aberrant activation of Shp2 significantly accelerates the rate of neuroblastoma induction induced by overexpression of MYCN and increases tumor penetrance. These effects were mediated by increased activation of the Ras-Erk pathway and enhanced proliferation and survival of MYCN-induced hyperplastic neuroblasts in the zebrafish analogue of the human adrenal gland. Overexpression of wild-type Gab2 activated the PI3K-Akt pathway as well, leading to enhanced synergism with MYCN in neuroblastoma induction. Our results not only identify a cellular mechanism for the interaction of overexpressed MYCN with mutationally activated SHP2 in neuroblastoma initiation, but also provide *in vivo* evidence that SHP2 signaling might be activated by overexpression of *GAB2* in neuroblastoma cases that lack *PTPN11* mutations.

7

The ALK-F1174L activating mutation mediates the upregulation of gene cluster located in the 15qD1 genomic region including the Myc locus in tumors derived from murine neural crest progenitor cells

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Introduction: The anaplastic lymphoma kinase (*ALK*) is overexpressed, mutated or amplified in most neuroblastoma (NB). The ALK-F1174L mutation mediates an enhanced activation of its downstream signaling cascade and an increased oncogenic potential relative to other ALK mutations. However, the precise molecular mechanisms remain unresolved.
Methods: To identify genes and pathways specifically activated by the ALK-F1174L mutation, human ALK-F1174L, ALK-R1275Q, or ALK-wt were stably expressed the murine neural crest progenitor cell (NCPC) line JoMa1. Transduced JoMa1 cells were injected orthotopically in adrenal gland of athymic Swiss nude mice and the transcriptomes of the resulting tumors were analyzed by Affymetrix microarrays.
Results: As expected, the ALK-F1174L activating mutation displayed a significantly enhanced oncogenic potential relative to ALK-R1275Q and ALK-wt in NCPC. Comparison of the whole gene expression profile of these tumors revealed 1179 or 645 differentially expressed probe sets (FC>2) between ALK-wt and ALK-F1174L, or ALK-wt and ALK-R1275Q tumors, respectively. Surprisingly, despite strong difference in tumor growth, ALK-F1174L and ALK-R1275Q-derived tumors displayed only 19 differentially expressed probe sets. Interestingly, among the 11 overexpressed genes in the ALK-F1174L group, 6 genes, including Pvt1, Mtss1, Nsmce2, are located on the chromosome 15qD1 region close to the Myc locus. Although Myc was not identified among the differentially expressed genes, overexpression of Myc, as well as Pvt1, Mtss1, and Nsmce2 was validated by real-time PCR in ALK-F1174L-expressing tumors relative to ALK-R1275Q and ALK-wt groups, as well as in tumor-derived cell lines. Moreover, we confirmed using the ALK inhibitor TAE684 that the upregulation of these genes is directly dependent on ALK-F1174L activity.
Conclusion: These results suggest that the strong oncogenic potential mediated by the ALK-F1174L activating mutation may be caused by the upregulation a cluster of genes located in the 15qD1 genomic region. Further work will allow us to elucidate the precise regulatory mechanisms and to identify the respective role of these genes on tumor initiation and growth.

8

Activated ALK signals through the ERK-ETV5-RET pathway to drive neuroblastoma oncogenesis

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Activating mutations of the ALK receptor occur in 8% of neuroblastoma tumors at diagnosis. The *RET* gene has been
previously identified as a target of activated ALK at the mRNA level in both human neuroblastoma cell lines and primary tumors as well as in murine tumors driven by mutated Alk and MYCN. We also reported that the Ret inhibitor vandetanib inhibited tumor growth in these murine neuroblastoma models.
In the present work, we sought to: (1) determine the mechanism of RET upregulation by activated ALK; (2) demonstrate the contribution of RET to ALK mutated dependent neuroblastoma oncogenesis by a genetic approach; (3) evaluate the therapeutic potential of a combination of vandetanib with the ALK inhibitor crizotinib.
Transcriptomic data of murine MYCN/Alk^{mut} tumors first identified Etv5 as a candidate transcription factor regulating *Ret* expression. We then showed that ETV5 is regulated both at the protein and mRNA levels upon ALK modulation in neuroblastoma cell lines and that ETV5 regulation precedes RET modulation. We further demonstrate that ETV5 and RET are regulated by ALK through the MEK/ERK pathway and show that RNA interference-mediated inhibition of ETV5 decreases RET expression. Then, to further determine the contribution of RET to ALK mutated dependent neuroblastoma oncogenesis *in vivo*, we bred knock-in Ret^{M919T} mice with TH-MYCN mice. We document an oncogenic cooperation between activated Ret and MYCN overexpression in neuroblastoma formation and show that MYCN/Ret^{M919T} tumors present with histological features and expression profiles close to MYCN/Alk^{mut} tumors. We finally demonstrate that the combination of vandetanib and crizotinib more efficiently reduces tumor growth than each single agent in our murine neuroblastoma models.
Altogether, these results define the ERK-ETV5-RET pathway as a critical axis driving neuroblastoma oncogenesis downstream of activated ALK and identify the crizotinib/vandetanib combination as an interesting approach for patients presenting with neuroblastoma harbouring ALK activation.

9

Reactivation of cAMP /PKA pathway is an early event that relieves EZH2-mediated epigenetic suppression in High-Risk Neuroblastoma(HR-NB)

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High-Risk Neuroblastoma(HR-NB) is characterized by dysregulation of sympathoadrenal progenitor cell's self-renewal and a failure to implement a differentiation program. Determining pathways that contribute to this state will lead to a better understanding of disease pathogenesis and potentially identify new targets. The re-programming of embryonic stem cells to a state primed for lineage specific differentiation first requires treatment with all-trans retinoic acid(ATRA). To gain insights into events important in reprogramming NB cells for differentiation, we used RNAseq analyses to assess early changes in the transcriptome of HR-NB cells(KCNR:MYCN-amplified) after 24hrs ATRA. We find a significant(FDR<0.01) up-regulation(760) and down-regulation(548) of genes. IPA analyses indicate cAMP signaling pathway is the most significantly up-regulated pathway. GSEA indicated that gene-sets associated with high MYCN expression are down-regulated and those enriched in PRC2(Polycomb Repressor Complex2) target genes are increased(p-value:0.001). Biochemical assays confirmed RA-mediated increases in cAMP levels and activation of PKA within 2hrs. By 6hrs, activation of PKA causes increases in phosphorylation of EZH2 at ser21(P-EZH2ser21) which inhibits EZH2 binding to H3, resulting in a decrease in the epigenetic gene suppression mark-H3K27me3. Such decreases in H3K27me3 at EZH2 target genes result in increased RNA transcription, consistent with the GSEA analysis of the RNAseq data. siRNAs against PRKACB, a catalytic subunit of PKA blocked the RA mediated activation of cAMP pathway in NB cells, prevented P-EZH2ser21 and induction of EZH2 target genes such as RARß. In primary NB tumors, microarray studies indicate that low levels of PRKACB are associated with poor prognosis. The 8-year survival in Stage 4 NB patients is only 20% for patients with low PRKACB levels in their tumors compared to 70% survival for patients whose tumors have relatively high levels of PRKACB (P=3.6e-05; R2database-Kocak). This study identifies PKA activation as an early event that may be necessary for re-programming HR-NB cells to differentiate.

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Whole exome sequencing of circulating free tumour DNA for study of spatial and temporal tumor heterogeneity: accumulation of new mutations at tumor progression of neuroblastoma

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Liquid biopsies are revolutionary tools to monitor tumour-specific genetic alterations. In neuroblastoma (NB), significant levels of circulating free tumor DNA (ctDNA) in the bloodstream enable the detection of tumour-specific markers including MYCN amplification or mutations. As clonal evolution plays a role in NB progression, monitoring of a single genetic marker will be insufficient for ctDNA-based disease follow-up.
To study NB clonal evolution, we isolated ctDNA from plasma at diagnosis (n=19) and during follow-up (final time-point: partial or complete remission (PR/CR), n=7; progressive disease (PD), n=9) for 19 NB patients for whom primary NB and matched germline DNA whole exome/whole genome sequencing data (WES/WGS) was available. CtDNA (7–100ng) was subjected to Illumina 100PE WES following modified library construction and capture approaches to account for small ctDNA molecules (target depth 100x). SNVs/mutations were called using GATK-UnifiedGenotyper, GATK-HaplotypeCaller and Samtools. Copy-number profiles were generated using Varscan and DNAcopy.
CtDNA WES yielded satisfactory depth in all cases. At diagnosis, SNVs common to the NB and corresponding diagnostic ctDNA of a given patient were observed (mean number of SNVs: 19; range 9-69), with few SNVs specific to the NB (mean: 6; range 0-18), and others specific to ctDNA (mean:22; range 9-69), suggesting spatial heterogeneity with different ctDNA amounts released by different clones. In PR or CR ctDNA, lower numbers of SNVs were detected (mean: 11, range 0-12). Interestingly, PD ctDNA samples harboured higher numbers of SNVs, with additional relapse-specific SNVs (mean: 22; range 0-55) targeting, amongst others, the protein kinase A signaling pathway. Analysis of additional ctDNA samples obtained between diagnosis and relapse (2-6 samples/patient) using deep sequencing techniques will determine the time of appearance of new driver clones.
In conclusion, CtDNA WES proves to be an extremely powerful tool to study spatial and temporal heterogeneity in NB, providing further proof of the importance of clonal evolution in NB progression. Full characterization of ctDNA, which might represent more aggressive clones, might orient targeted treatment approaches.

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Liquid biopsies reveal exosomal miRNA modulation in high-risk neuroblastoma patients after the induction therapy

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Introduction
Treatment after induction and prognosis of High-Risk (HR) neuroblastoma (NB) patients depends on their response to a two-month induction-chemotherapy. Unfortunately, there is no early predictive molecular indicator of sensitivity/resistance to treatment. Circulating exosomes are small vesicles which may represent the molecular bioprint of the tumor cells in liquid biopsies. We investigated whether exosomal miRNAs (Exo-miRs) could be an early biomarker of the response to induction-chemotherapy.
Methods
Exosomes were collected before and after the induction-therapy from plasma samples of 50 HR-NB-patients. Exo-miRs expression was measured by RTqPCR on a 381 miRNA panel. Data analyses included feature selection and pathway analysis.
Results
The expression of 24 exo-miRs was significantly modulated ($p<0.05$, fold-change>1.5) in response to chemotherapy, providing the first indication that exo-

miRs may serve as biomarker of the chemotherapic response. Cluster analysis demonstrated that the response to induction was heterogeneous and distinguished at least two groups of patients that may reflect distinct biological and clinical features. Pathway analysis determined whether chemotherapy affected the expression of miRNAs known to be involved in sensitivity/resistance to drugs commonly employed in the induction-chemotherapy. The analysis revealed that 42% of the 24 differentially expressed exo-miRs were associated with sensitivity/resistance to drug response. Mapping the results to individual patients, we clearly identified distinct groups of subjects totally or partially unresponsive/resistant to the induction drugs. The exo-miR profile in the middle phase of the induction therapy is under investigation to find miRNAs possible predictors of the response and that may allow a timely change in the induction protocol for those patients that do not respond to treatment.
Conclusions
We obtained the proof of principle that exo-miRs can represent biomarkers and indicators of sensitivity/resistance to specific drugs for patients with HR-NB. These results pave the way to a broad application of exo-miRs in liquid biopsies applied to neuroblastoma targeted treatment.

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Detection of copy number aberrations in cell free DNA from plasma of neuroblastoma patients using shallow massive parallel sequencing

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Neuroblastoma (NB) is a heterogeneous disease characterized by distinct clinical and biological features. The somatic mutation burden is low in these tumors, while copy number alterations are highly recurrent. Low stage tumors usually present with numerical chromosome aberrations, while high stage tumors are characterized by the presence of segmental aberrations, the latter being associated with poor prognosis. Presently, determination of DNA copy number profiles is mandatory in most treatment protocols and determined using FISH, MLPA, array-CGH or SNP-arrays. Such analysis can be precluded through the inability to biopsy the tumor or lack of sufficient tumor material.
In this study, we investigated whether cell free DNA (cfDNA) isolated from plasma samples of NB patients could offer an alternative to biopsies, and whether shallow massive parallel sequencing (MPS) could be used as a substitute for classical DNA copy number analyses.
CfDNA was isolated from plasma using the QiaAmp circulating nucleic acid kit (Qiagen). Samples were preprocessed with the Ion Plus Fragment Library kit, barcoded, pooled by six and sequenced on an Ion Proton (ThermoFisher) instrument. Data analysis for CNV detection was done using the QDNAseq algorithm implemented in the online genomic data visualisation tool Vivar. The minimal number of reads per sample was set at 10 million. Comparison of the MPS data with the array-CGH profiles of the tumors from the same patient showed that all structural and numerical chromosome aberrations including *MYCN* amplification could be readily detected with MPS using a very low input of cfDNA (5 ng). Interestingly, MPS data show a much better signal-to-noise ratio compared with array data.
In conclusion, MPS analysis of cfDNA isolated from plasma offers a cost-effective, non-invasive and rapid alternative for DNA copy number profiling in neuroblastoma.

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The transcriptomic landscape of bone marrow-derived disseminated tumor cells of high-risk neuroblastoma patients

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Background
Dissemination of tumor cells into the bone marrow (BM) is a frequent event in stage M neuroblastoma (NB) patients. So far, several studies have investigated gene expression signatures of primary NB tumors. Although BM aspirates are routinely obtained at different time points in current high risk neuroblastoma studies, e.g. HR-NBL1, the gene expression profiles of BM derived disseminated tumor cells (DTC) are poorly explored.
Methods
We established and validated a magnetic bead-based method for enrichment of DTCs from the BM suitable for subsequent RNA-Seq analysis of GD2 positive cells. RNAs of the enriched DTCs (n=43), the corresponding DTC-depleted BMs (n=43), the matched tumor samples (n=5) and the non-matched tumor samples (n=22) were sequenced (single-end, 50 bases reads). The data analysis was performed using a custom RNA-Seq pipeline built with Anduril.
Results
Our preliminary data clearly revealed distinct expression signatures separating DTCs and DTC-depleted BM cells with 2039 genes significantly differentially expressed ($q \leq 1x10^{-10}$; $|logFC| \geq 3$). Despite highly concordant expression profiles of DTCs and primary tumors, clear differences with 278 significantly differentially expressed genes ($q \leq 1x10-10$; $|logFC| \geq 3$) became apparent. While in primary tumors genes necessary for angiogenesis are up-regulated, the DTCs seem to adapt to the environment by down-regulation of angiogenesis specific genes and up-regulation of genes encoded by mitochondrial DNA. Even though the gene expression signature of DTCs at

diagnosis compares to a large extent to the signature of DTCs at relapse, we identified 89 differentially expressed genes ($q \leq 0.1$; $|logFC| \geq 2$). Interestingly, members of the MAGE gene family are found to be mainly expressed in non-*MYCN* amplified relapse samples.

Conclusion

BM-derived disseminated NB cells are characterized by an expression signature that most likely reflects their adaptation to the BM niche. Furthermore, we expect that the differential expression signature of DTCs at diagnosis versus relapse will help in deciphering the processes leading to relapse.

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A novel neuroblastoma metastatic mouse model identifies genes, pathways and drugs regulating metastasis

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Metastatic relapse is the major cause of death in Neuroblastoma (NB), yet there are no therapies to specifically target metastases. Most NB murine models do not recapitulate the bone, bone marrow and brain metastases seen in patients. We generated a NB metastatic mouse model using *in vivo* selection by intra-cardiac injection of NB cells expressing a GFP-Luciferase reporter. Cells from specific metastatic sites (bone, CNS) were isolated, GFP-sorted, and injected into a second cohort of mice. Bone and CNS metastases were confirmed by *ex vivo*bioluminescence, histology, microCT, and MRI. The metastatic subpopulations derived from bone and CNS exhibited enhanced metastatic burden and decreased latency *in vivo* as determined by bioluminescence monitoring. In comparison to the parental cells, these metastatic subpopulations showed increased migration, invasion, and chemoresistance *in vitro*, but not proliferation.

Gene expression profiling and unbiased clustering revealed two distinct subtypes, parental and metastatic, with differential regulation of 412 genes and multiple pathways including Hippo and integrin signaling. Using univariate Cox-regression, we identified a 28-gene "metastatic signature" that predicts NB patient outcome in multiple datasets. We validated candidate genes that were upregulated including *GJA1*, *SPHK1*, and *YAP/TAZ* and downregulated including *CADM1* in our metastatic subpopulations, and each independently predicted patient survival. Functional experiments with cells where GJA1, SPHK1, or YAP/TAZ expression was knocked down with shRNA or CRISPR, or CADM1 was overexpressed by lentiviral-mediated infection rescued metastatic phenotypes in culture and in some cases in mice. In addition, treatment with pharmacological inhibitors SKI II and Verteporfin that target SPHK1 and YAP/TAZ, respectively, inhibited NB metastasis *in vivo*. We have also identified drugs that selectively inhibit the growth of the metastatic subpopulations in culture.

Our metastatic NB model provides a platform to identify genes that regulate NB metastases and candidate drugs to target recurrent NB in the bone and brain.

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A native and immunocompetent *in vivo* model of chemorefractory, bone-marrow metastatic, "ultra-high risk" neuroblastoma.

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Introduction. Treatment of chemoresistant or refractory, bone-marrow metastatic neuroblastoma remains a major challenge. The substantial fraction of patients who fail induction chemotherapy can be considered ultra-high risk and require novel treatments targeted at the actionable genetic changes associated with development of chemorefractory disease and bone-marrow metastasis. The lack of any *in vivo* models that are immunocompetent and natively recapitulate ultra-high risk disease and spontaneous bone-marrow metastasis has impeded experimental focus in this critical area.

Results. We subjected the well-characterised Th-*MYCN* genetically-engineered mouse (GEM) model, which is largely chemosensitive and free of metastasis at tumour onset, to multicycle induction chemotherapy protocols. Th-*MYCN*^{R} mice develop primary tumours that are fully chemorefractory and metastatic to bone-marrow. Explants and primary cell cultures derived from Th-*MYCN*^{R} primary tumours retain treatment resistance in subcutaneous and intratibial re-implantation, indicating that the growth advantage attributed to chemoresistance is cell-intrinsic. Gene expression analysis of primary resistant tumours reveals changes in PI3K-AKT, MAPK and RAS pathways. Misregulation of "extracellular matrix receptor (ECM) interaction" and β-catenin pathways reveals a signature consistent with epithelial-mesenchymal transition (EMT). In support of this finding, immunohistochemical analysis showed increased staining of ECM and mesenchymal markers in relapsed tumours compared to untreated tumours.

Discussion. Here we report the first native, unmanipulated neuroblastoma model that recapitulates treatment induction failure and widespread disease metastasis in an immunocompetent setting. Th-*MYCN*^{R} reveals critical changes associated with development of treatment resistance and metastasis, such as altered expression of actionable pathways, and a potential EMT state change in metastasis. Th-*MYCN*^{R} will be a powerful

experimental tool for molecular dissection of clonal evolution that drives development of treatment resistance and metastasis in neuroblastoma.

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The prognostic and therapeutic relevance of *TERT* activation in neuroblastoma

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Background: We recently discovered that genomic rearrangements of *TERT* occur in one-quarter of high-risk neuroblastoma (NB), and that both *TERT*-rearrangements and *MYCN*-amplification lead to massive telomerase activation. We therefore investigated the potential prognostic and therapeutic relevance of *TERT* activation in NB.
Methods: Tumor samples of 284 NB patients were analyzed by whole-genome-sequencing or break-apart FISH analysis. The *in vitro* growth of seven NB cell lines (*TERT*-rearranged, n=2; *MYCN*-amplified, n=2; *Alternative Lengthening of Telomeres* (ALT), n=3) was assessed by viable cell counting upon treatment with the telomerase inhibitors BIBR1532, costunolide or 6-thio-2'-deoxyguanosine. Tumor growth of two cell lines treated intraperitoneally with 6-thio-2'-deoxyguanosine was investigated in nude mice.
Results: In the entire cohort, we detected 33 *TERT*-rearrangements (11.6%; 30 high-risk and 3 intermediate-risk patients). The clinical outcome of patients whose tumors harbored *TERT*-rearrangements was similar to that of patients with *MYCN*-amplified tumors, but significantly worse than that of high-risk patients without these alterations (event-free survival, p=0.023; overall survival, p=0.055). Treatment of NB cell lines with the nucleoside analogue 6-thio-2'-deoxyguanosine led to a significant growth inhibition in both *TERT*-rearranged and *MYCN*-amplified cells in a dose dependent manner (mean IC_{50}, 1.33 µM and 1.27 µM, respectively). By contrast, NB cells with ALT pathway activation were largely unaffected by 6-thio-2'-deoxyguanosine treatment (IC_{50}>10 µM each). Similar effects were observed for costunolide and BIBR1532, whereas etoposide impaired the growth of *TERT*-, *MYCN*- and ALT-positive cell lines at comparable concentrations (mean IC_{50}, 0.55, 0.39, and 0.41 µM, respectively), emphasizing the on-target specificity of telomerase inhibitors. In addition, 6-thio-2'-deoxyguanosine significantly impaired tumor growth of *TERT*- and *MYCN*-positive cells in mouse xenograft models.
Conclusion: We demonstrate that the presence of *TERT*-rearrangements is a strong prognostic marker for poor outcome in neuroblastoma. Furthermore, we suggest that activated telomerase may represent a therapeutic target in NB harboring *TERT*-rearrangements or *MYCN* amplification.

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Telomere biology in neuroblastoma: focusing on alteration of TERT promoter lesion

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Backgroud: We have reported telomere biology in NBL: telomerase activation and alternative lengthening of telomere (ALT) due to *ATRX/DTXX* mutations. Recently, genomic rearrangements at 5p15.33 proximal of the telomerase reverse transcriptase gene (TERT) were found in some group of unfavorable NBLs. Therefore, we analyzed alteration of TERT promoter lesion (PL) in our NBL series.
Methods: We analyzed the alterations of TERT-PL using SNP array or next generation sequencing in 146 NBL samples whose telomere length, telomerase activity, TERT expression, *ATRX/DTXX* mutations as well as other biological factors (*MYCN*, *ALK*, etc.) were already analyzed. Among them, 80 were diagnosed under 18 months and 34 were detected by mass-screening. INRGSS classification showed 60 L1, 30 L2, 47 M and 9 MS.
Results: In 11 ALT-activated tumors with elongated telomere (15 - 40 kb) and *ATRX/DAXX* alterations showed no alteration of TERT-PL. In the 135 remaining cases, telomere length were under 15 kb and high expression of telomerase/TERT was detected in 63 tumors consisting of 5 L1, 10 L2, 46 M and 2 MS tumors. All 28 *MYCN* amplified tumors showed high telomerase/TERT expression but alteration of TERT-PL were detected in only 4 of them. In *MYCN* non-amplified tumors, 29 of 35 cases with high telomerase/TERT expression and 9 of 72 cases without high telomerase/ TERT expression had alteration of TERT-PL (P <0.01). The outcome of the cases with high telomerase telomerase/ TERT expression showed significantly worse than others (P <0.01). Interestingly, 7 of 34 screening-detected tumors and 13 of 80 tumors diagnosed under 18 months had alteration of TERT-PL.
Conclusion: In NBLs, alteration of TERT-PL might activate TERT expression in unfavorable cases without *MYCN* amplification. As *MYCN* activates TERT expression, the stabilization of telomere by ALT or high telomerase activity might be directly correlated with unfavorable tumors. Thus, a better understanding of telomere biology and developing of therapeutic strategies for telomere-stable NBLs may help to improve the outcome of NBL patients.

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Constitutive activation of ATM kinase in neuroblastoma cell lines with the alternative lengthening of telomeres (ALT) phenotype induces resistance to DNA damaging agents

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Background: Prior studies suggest 10-20% of high-risk neuroblastomas (NB) use the alternative lengthening of telomeres (ALT) mechanism (associated with a poor prognosis) to maintain telomeres. We identified 3 ALT NB cell lines in screening a panel of 112 human NB cell lines for TERT expression and c-circle content (extrachromosomal telomeric DNA) , ALT was confirmed by TRAP assay and telomere FISH.
Methods: Cytotoxicity was assessed using DIMSCAN, gene expression and c-circle content by qPCR, DNA damage by immunostaining (53BP1 foci), telomere damage by IF-FISH, and ATM knockdown by lentiviral shRNA transduction.
Results: The 3 ALT NB cell lines had a higher mean IC 90 for DNA damaging drugs relative to 79 comparator telomerase+ NB cell lines and higher expression of multiple DNA damage response (DDR) genes (29 of 59 genes analyzed, $p<0.05$) when compared to 9 comparator multidrug resistant telomerase+ NB cell lines. Numerous baseline DNA damage foci were observed in the nuclei of the 3 ALT NB cell lines and greater than 90% of these DNA damage foci co-localized to telomeres, indicating spontaneous telomere damage. DNA damage foci were not observed in telomerase+ NB cell lines. We assessed activation of both ATM/ATR kinases (which are involved in DNA damage signaling at telomeres) and observed a marked increase in phosphorylation of ATM kinase and its downstream target CHK2 in ALT lines but not in telomerase+ lines. Knockdown of ATM in 2 ALT NB cell lines reduced DNA damage foci ($p<0.05$), reduced C-circle (ALT marker) content ($p<.05$), downregulated 13 of 25 DDR genes that are commonly induced by genotoxic stress ($p<0.05$), and sensitized ALT NB cells to melphalan, 4-hydroperoxy-cyclophosphamide, doxorubicin and irradiation ($p<0.05$).
Conclusions: ATM kinase is constitutively activated in ALT NB and may be necessary for the ALT phenotype. Our data suggest that activation of ATM kinase contributes to DNA damaging agent resistance by upregulating DDR expression. These data identify ATM kinase as a potential therapeutic target in ALT NB.

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A unique mechanism for the continual proliferation of high-risk neuroblastoma cells

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The end of chromosomes are characterised by a repetitive sequence known as the telomere. Normal somatic cell division results in telomere erosion, ultimately triggering senescence. Cancer cells bypass senescence via activation of a telomere lengthening mechanism (TLM), either telomerase or Alternative Lengthening of Telomeres (ALT). Identification of ALT relies on the observation of unique phenotypic characteristics including long and heterogeneous telomere lengths, presence of ALT associated PML bodies, and abundant c-circles (extrachromosomal telomeric circular DNA). A cohort of 149 high-risk neuroblastoma tumours was screened for ALT by assessing the amount of telomeric DNA and c-circles. Evidence of ALT (high telomere content and c-circles) was found in 24% of the tumours while another subset (11%) had high telomere content but were c-circle negative. This unique group had a similarly poor 5-year overall survival compared to c-circle positive and MYCN-amplified tumours (47 vs 31 vs 28%; P=0.83). Testing 35 neuroblastoma cell lines identified two neuroblastoma cell lines (unique by STF) with a similar phenotype to the unique tumours, specifically they were negative for telomerase and c-circles. The two cell lines have exceptionally long and heterogeneous telomere length (35kb) (demonstrated by telomere FISH and terminal restriction fragment analysis) and when cultured for >300 population doublings, telomere length shortened by 50 to 80 base pairs per population doubling, consistent with the lack of a TLM. Gene mutations associated with ALT (TP53, ATRX, DAXX, IDH1/2, H3.3) were not present in the two cell lines. All continuously growing cell lines to date have a TLM so this is an unprecedented finding. This is the first report of sustained proliferation of aggressive human cancer despite continuous telomere shortening; patients with tumours of the same phenotype have a poor outcome even though they lack a TLM. These discoveries have implications for the use of telomere maintenance inhibitors in the clinic and in understanding mechanisms of immortalization during carcinogenesis.

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Targeting a novel MYCN onco-factor, PA2G4, for the treatment of neuroblastoma

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MYCN oncogene amplification is found in one third of primary neuroblastoma at diagnosis, and correlates with poor prognosis. We have defined increased MYCN protein stability as a necessary feature during MYCN-related tumorigenesis, providing a completely novel therapeutic target (1-2).

For the first time, we have identified Proliferation-Associated protein 2G4 (PA2G4) as a direct protein-binding partner of MYCN which acts in a forward feedback expression loop with MYCN to drive neuroblastoma tumourigenesis. PA2G4 belongs to a family of DNA/RNA binding proteins already implicated in cell growth, apoptosis and differentiation. High PA2G4 expression correlated with high expression of MYCN in neuroblastoma cells and tumour tissues, and, strongly predicted poor neuroblastoma patient survival. Suppression of PA2G4 by siRNAs decreases neuroblastoma cell growth, cell migration and colony formation. Most significantly, our *in vivo* data showed stable overexpression of PA2G4 in a non-tumorigenic neuroblastoma cell line was able to induce tumour growth. Conversely, using nanoparticles to deliver siRNA targeting PA2G4, we were able to delay neuroblastoma tumour growth significantly in a mouse xenograft model. Furthermore, a small molecule known to bind PA2G4, WS6, significantly decreased PA2G4 levels in tumour tissues and tumorigenicity in TH-MYCN mice.

Collectively, our data suggest that PA2G4 acts as an onco-factor, binding and protecting MYCN from proteolysis, thus increasing MYCN levels. Our research provides strong evidence demonstrating PA2G4 is a driver of tumorigenicity and for the first time identifies PA2G4 as a novel therapeutic target for the treatment of neuroblastoma.

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Targeting tumor-promoting neuroblastoma microenvironment; Inhibition of tumor development and progression by therapy targeting mPGES-1 and prostaglandin E_2 expression in cancer associated fibroblasts

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Background: High-risk neuroblastomas present a tumor promoting microenvironment with infiltrating cancer associated fibroblasts (CAFs) expressing the mPGES-1 enzyme, essential for prostaglandin E2 (PGE_2) synthesis regulating tumor inflammation and immune suppression, angiogenesis, genetic instability, tumor progression and therapy resistance. We investigated the impact of novel therapy targeting the COX/mPGES-1/PGE_2 pathway.

Methods: Human neuroblastomas were investigated for immunosuppressive microenvironment and expression of the COX/mPGES-1/PGE_2/EP-receptor pathway. High-risk *in vivo* models, human 11q-deleted xenografts and transgenic MYCN-driven tumors, were treated with a novel specific mPGES-1 inhibitor. Tumor-fibroblast co-cultures examined cell migration. Inflammatory lipid mediators were analyzed by LC-MS/MS. Tumor tissues were analyzed by immunohistochemistry, immunofluorescence and FACS.

Results: Tumor microenvironment in human high-risk neuroblastomas and both 11q-deleted xenografts and MYCN-driven transgenic mice displayed mPGES-1 expression in PDGFRb+ cancer associated fibroblasts. MPGES-1 expression correlated with high-risk neuroblastoma prognosis and infiltration of tumor-promoting macrophages with M2-polarization markers CD163 and CD206. The inflammatory regulator STAT3 was active in mPGES-1 expressing CAFs. Expression of the inflammatory COX/mPGES-1/PGE_2/EP-receptor pathway in experimental tumors resembled high-risk primary human neuroblastomas.

Targeting mPGES-1 with a novel compound decreased PGE_2, induced M1 polarization of macrophages, decreased cancer associated fibroblasts and reduced angiogenesis significantly in treated tumors. Tumor development in the xenograft model was delayed 50% (median 38 vs. 25.5days) and growth of established xenografts and transgenic tumors was significantly decreased by non-toxic treatment *in vivo* when compared to neuroblastoma tumors in untreated animals. Tumor cell stimulated CAF migration and infiltration was inhibited by targeting mPGES-1.

Conclusions: Tumor-promoting inflammation and suppression of anti-tumor immunity in neuroblastoma is mediated through prostaglandin E_2 and STAT3

expression in cancer associated fibroblasts in the tumor microenvironment. Early targeting of mPGES-1 may inhibit CAF infiltration and tumor development. This novel tumor treatment targeting mPGES-1 decreases inflammatory mediators, modulates tumor-promoting microenvironment, decreases cancer cell migration and inhibits significantly aggressive tumor growth and progression. We conclude that treatment targeting non-malignant cells in the neuroblastoma microenvironment may constitute a novel clinical therapeutic approach.

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Targeting the LIN28B/let-7 axis by small molecules in neuroblastoma

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An important paradigm in current cancer therapeutics is the progressive shift from broad-spectrum chemotherapy to personalized, targeted treatment. Following intense research, a few proteins have been proposed as neuroblastoma-specific targets. The two LIN28 paralog RNA-binding proteins (RBPs) are well known oncogenic drivers in several types of tumors; they exert their effects mainly by preventing maturation of miRNAs of the let-7 family, which drives cell differentiation. In neuroblastoma LIN28B is of particular importance. The first genome-wide association study for familiar neuroblastoma unveiled a polymorphism closely associated with disease and survival influencing LIN28B expression. Moreover, LIN28B is extensively overexpressed in NB compared to several other tumor types, and its forced expression in the sympathoadrenergic lineage of mice reproduces the human disease.
We aimed at preclinically identifying small molecules able to interfere with the binding of LIN28B to the pre-let-7 miRNAs, therefore increasing expression of the let-7 miRNAs and promoting differentiation of neuroblastoma with potential therapeutic outcomes. To this aim, we exploited the amplified luminescent proximity homogeneous assay technology. A human LIN28B recombinant protein was produced and challenged with a 5'-biotinylated ssRNA corresponding to the pre-let-7g pre-miRNA. We demonstrated a sequence-specific, high-affinity interaction between the RNA and the LIN28B RBP, the coefficient of variation, the Z-factor value, and the signal-to-background ratio, that collectively indicated the robustness and reliability of the assay in a high-throughput format. Then we applied the assay to a collection of ~2000 small molecules with known bioactivity. From the primary screening we selected 80 hits, and after validation and counter-screening we obtained 30 molecules, some of which characterized by a common chemical scaffold.
Docking in silico experiments followed by medicinal chemistry allowed us to design a second, focused small molecule library, which was tested in soaking with LIN28B protein crystals. The selected molecules were finally subjected to RNA Electrophoretic Mobility Shift Assay and a number of phenotypic studies by high content analysis.

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A small molecule kinome inhibitor screen identifies the TGF-beta-activated kinase 1 (TAK1) as a target for combination therapy in MYC-driven neuroblastoma

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MYCN amplification (*MYCNA*) and/or high levels of c-MYC expression predict recurrence and poor outcome for loco-regional and metastatic neuroblastoma. Although recent studies have identified novel agents, including bromodomain and aurora kinase inhibitors, which demonstrate selective activity in *MYCNA* neuroblastoma, there is a need to identify agents to enhance the efficacy of these drugs in MYC-expressing relapsed neuroblastoma.
We performed a high throughput screen with a unique collection of 480 kinase inhibitors to identify alterations in signaling pathways that regulate survival and differentiation of neuroblastoma. Hits were defined as compounds (2umol/L) with >75% inhibition of metabolic activity relative to control. Top hits included novel drugs and inhibitors of kinases previously implicated in neuroblastoma, including PLK1, cdks/cyclins, Chk1/wee1, aurora A/B, and PI3K/Akt/mTOR. To prioritize candidates with efficacy we compared our results with published shRNA screens. The serine threonine MAP kinase kinase kinase (MAP3K7) family protein TAK1 emerged as a potential novel regulator of neuroblastoma survival. Two TAK1 inhibitors (AZ-TAK1 and 5z-7-oxozeanol) demonstrated in vitro efficacy across a panel of 11 cell lines representing a range of genetic profiles (including 2 primary *MYCNA* lines). For AZ-TAK1 the IC_{50} ranged from 0.28- >10uM. The lowest IC_{50} was detected in cells with *MYCNA* or high c-myc and sensitivity correlated with levels of induced MYCN in SHEP-tet cells. The level of phospho-TAK1 was highest in cells with low IC_{50} and was inhibited by treatment with AZ-TAK1. Pharmacologic inhibition or TAK1 knockdown led to apoptosis, and in certain cases differentiation. As a single agent AZ-TAK1 suppressed tumor growth in mice with neuroblastoma xenografts. In vitro combination studies also demonstrated additive and synergistic activities with other agents that suppress MYCN expression and signaling including JQ-1 and MLN8327. These results suggest TAK1 may be a relevant target for combination therapy in relapsed tumors with high MYC activity.

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Preclinical characterization of meta-[^{211}At]astatobenzylguanidine ([^{211}At]MABG) as an alpha particle emitting systemic targeted radiotherapeutic for neuroblastoma

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Background Neuroblastoma (NB) is a radiosensitive malignancy and NB cells express the norepinephrine transporter (NET) enabling uptake of NET ligands. [^{131}I]MIBG (meta-iodobenzylguanidine) is a highly effective β-particle emitting NET ligand for cytoreduction of bulk tumor but does not target microscopic residual disease due to the long path lengths of the beta emission. Targeted radiotherapy with [^{211}At]MABG (α-particle emitter with higher biological effectiveness) can address this critical problem due to the short path lengths that will kill microscopic tumor clusters. **Methods** ^{211}At was synthesized using a bismuth target via the ^{209}Bi(α,2n)^{211}At reaction and used for solid-phase radiosynthesis of [^{211}At]MABG. We determined NET (*SLC6A2*) mRNA and protein expression in 35 human NB cell lines and created isogenic pairs by overexpression of NET in 5 NB cell models. We performed uptake, cytotoxicity and biodistribution studies using these models with [^{131}I]MIBG and [^{211}At]MABG and extrapolated human dosimetry. Additionally, *in vivo* dose escalation studies with [^{211}At]MABG (n=10 at each dose, range 10-100 uCi) were performed to determine toxicity. Finally, therapeutic *in vivo* trials are ongoing. **Results** We synthesized [^{211}At]MABG (radiochemical yield of 50-70%, radiochemical purity >99%). NET-overexpressing cell lines showed 4-10 fold higher uptake of NET ligands than parental isogenic lines, and tumor-specific [^{211}At]MABG uptake (tumor-muscle ratios of 7.37). Estimated dosimetry confirmed the potential to deliver therapeutic doses to tumors. Both 10 and 25 uCi of [^{211}At]MABG were well tolerated except for transient thrombocytopenia (nadir at 6 weeks; p=0.001 and p=0.0005 respectively) while doses higher than 50 uCi caused significant weight loss. [^{211}At]MABG was a potent cytotoxic agent *in vitro* (EC50's ranged from 0.0006-0.1 uCi/ml compared to 0.25-46 uCi/ml with [^{131}I]MIBG). Significant tumor growth delay was seen in SKNSH NET transfected xenografts treated with 12 uCi of [^{211}At]MABG. **Conclusions** The biodistribution and uptake of [^{211}At]MABG is similar to [^{131}I]MIBG and there was no unanticipated toxicity. [^{211}At]MABG is more potent than [^{131}I]MIBG and could be an effective retrieval strategy for patients with disseminated NB

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A CD56 (NCAM1) targeting antibody-drug conjugate is potently effective in preclinical models of high-risk neuroblastoma

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Background: The GD2-targeting monoclonal antibody dinutuximab was the first FDA approved immunotherapy specifically for high-risk neuroblastoma. While dinutuximab therapy improved event-free survival rates, therapy is associated with severe pain caused by on-target activity on nerve cells, highlighting the need to consider other cell surface molecules as candidate targets for immunotherapy. CD56 is robustly over-expressed in many cancers including neuroblastoma.
Methods: We developed a fully human IgG1 targeting CD56 (m906-IgG) following phage-display-based isolation and optimization of CD56-specific binders. Using antibody glycan sites on m906-IgG1 for conjugation, we synthesized an antibody drug conjugate (ADC) with the DNA intercalating agent pyrrolobenzodiazepine dimer (m906-PBD) and tested for anti-neuroblastoma activity in both *in vitro* and *in vivo* preclinical models.
Results: Using immunofluorescence and flow cytometry, we showed that m906-IgG is rapidly internalized in a panel of 20 neuroblastoma cell lines. We next demonstrated potent cytotoxicity across this cell line panel (median IC_{50} = 1.48nM, range 10.5pM to 4.1nM) Somatic TP53 mutation was a biomarker for resistance to the ADC in the panel (p=0.03; median IC_{50} for cell line models with wild-type TP53 = 211pM with a range from 10.5pM to 1.5nM vs median IC_{50} for mutated lines = 260nM, range 2.7nM to 4.1uM). Antibody-competition studies confirmed the specificity of m906-PBD. In an initial dose finding experiment using the NB-1643 patient-derived xenograft model and a twice weekly for a total of 4 doses schedule, we showed significant tumor regression at 1 mg/kg/dose, with eventual tumor regrowth at a median of 40 days. At 3mg/kg/dose, there were sustained regressions without regrowth out to 110 days.
Conclusions: The CD56-targeting m906-PBD ADC shows potent cytotoxicity in models of high-risk neuroblastoma.

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GPC2 is a putative oncogene and candidate immunotherapeutic target in high-risk neuroblastoma

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Background: Immunotherapeutic strategies have improved outcomes in high-risk neuroblastoma; however a major challenge remains in identifying cell surface molecules that meet the criteria for modern immune-based therapies.
Methods: Potential cell surface immunotherapeutic targets were identified by comparing high-risk neuroblastoma (N=126 primary tumors) and normal tissue RNA sequencing data (GTEx; N=25 normal tissues, 1-313 replicates/tissue). Genes were filtered by cell surface prediction, absolute RNA expression, and tumor DNA copy number. Candidate gene protein expression and cellular localization were confirmed in neuroblastoma primary tumors, patient-derived xenografts (PDXs), and cell lines. Genes were further surveyed for MYCN-mediated transcriptional control with chromatin immunoprecipitation (ChIP) sequencing. Finally, neuroblastoma cell lines were subjected to candidate gene gain and loss of function studies.
Results: The transcriptome-based discovery identified 86 differentially expressed predicted cell surface molecules. We prioritized the GPI-anchored signaling co-receptor glypican-2 (GPC2) for validation given robust differential RNA expression (log-fold change tumor vs. normal tissue = 2.1-8.2; $p<3 \times 10^{-10}$), potential tumor-specific epitope expression, high-level absolute RNA expression (median FPKM=57) and frequent DNA copy number gain (43% of tumors; N=170) associated with higher *GPC2* expression ($p<0.001$). *MYCN* amplification was also correlated with higher *GPC2* expression ($p<0.01$), MYCN was found to bind the *GPC2* promoter by ChIP sequencing, and MYCN depletion resulted in significantly decreased GPC2 expression. Immunoblot, immunofluorescence, immunohistochemistry (IHC), and membrane extraction analysis of primary tumors, PDXs, and cell lines confirmed dense plasma membrane GPC2 expression. IHC analysis of 41 pediatric tissues confirmed very limited normal tissue GPC2 expression. GPC2 depletion in neuroblastoma cell lines (N=12) resulted in significant apoptosis and growth inhibition, and GPC2 forced overexpression significantly increased neuroblastoma cell proliferation. Finally, fully human antibodies specifically targeting neuroblastoma-associated GPC2 were created after identification of specific Fab binders from a phage display antibody library.
Conclusions: GPC2 is a neuroblastoma oncogene and candidate immunotherapeutic target

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Phase I study of anti-G_{D2} humanized 3F8 (hu3F8) monoclonal antibody (MAb) plus GM-CSF: High dosing and major responses in patients with resistant high-risk neuroblastoma (HR-NB)

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Background: Strategies to improve anti-G_{D2} immunotherapy include: avoiding sensitization (as can occur with murine and chimeric MAbs); augmenting affinity of MAb for Fc-receptor, thereby enhancing antibody-dependent cellular cytotoxicity (ADCC); and reducing pain associated with complement activation. Murine-3F8 anti-G_{D2} MAb is active against HR-NB but hu3F8 may be preferable because it has significantly greater ADCC and less complement activity *in vitro*. GM-CSF is well tolerated clinically and increases ADCC.
Methods: In a phase I study (NCT01757626) (opened in 12/2012), patients with resistant HR-NB receive cycles of hu3F8+GM-CSF monthly x4 in the absence of human antihuman antibody (HAHA) and then with 1-to-2-month intervals if clinically indicated. MAb dosing follows the standard 3+3 design, beginning at 0.9mg/cycle, to identify the maximum-tolerated dosage (MTD) (assessed in first cycle). MAb is infused intravenously (30") on Mon-Wed-Fri (i.e., 3 days/cycle). Daily GM-CSF is administered subcutaneously 5 days pre-hu3F8 through the last day of hu3F8. After cycle 2, hu3F8 can be increased to the highest dosage level that completed dose-limiting toxicity assessment.
Results: At study entry, the 51 patients enrolled to date were 2.4-31.3 (median 6.8) years old and 0.6-8.9 (median 3.2) years from HR-NB diagnosis. Pharmacokinetic studies showed dose-dependent increases in peak serum concentration; terminal half-life was >3 days. Treatment has been outpatient without unexpected acute, late, or cumulative toxicities, even in patients who received >10 cycles. MTD has not been reached. Current hu3F8 dosage is 9mg/kg/cycle. HAHA developed post-cycle 1 in only 1/9 patients who had no prior treatment with any anti-G_{D2}MAb. Major responses and prolonged progression-free survival have been observed at all dosage levels.
Conclusions: Modest toxicity allows high dosing of hu3F8 (>2.5x dosage of murine-3F8 and ch14.18) which, in addition to powerful *in vitro* cytotoxic features, can account for the notable anti-NB activity of hu3F8+GM-CSF. Pharmacokinetic studies support the alternate-day schedule.

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Integrative genome-scale analysis identifies epigenetic mechanisms of transcriptional deregulation in unfavorable neuroblastomas

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Background:
Neuroblastoma is a childhood tumor derived from sympathetic nervous system cells. Its broad clinical spectrum ranges from spontaneous regression to rapid progression despite intensive multimodal therapy. Known genetic aberrations only partially mirror this diversity, pointing towards epigenetic involvement in neuroblastoma pathogenesis.
Methods:
In an integrative approach, we analyzed methylomes, transcriptomes and copy number variations in 105 neuroblastomas, complemented by primary tumor- and cell line-derived global histone modification analyses and epigenetic drug treatment *in vitro*. Prognostic signatures were identified by investigating methylomes of 362 neuroblastomas via lasso penalized regression analysis.
Results:
DNA methylation patterns defined strongly divergent patient subgroups with respect to survival and clinicobiological variables including amplified *MYCN*. Transcriptome integration and histone modification-based definition of enhancer elements revealed intragenic enhancer methylation as a mechanism for high-risk-associated transcriptional deregulation. Further, we provide evidence for PRC2 activity and DNA methylation collaborating in ongoing repression of tumor suppressive pro-differentiation programs in high-risk neuroblastomas. Intriguingly, these programs can be effectively re-induced via combination treatment targeting the repressive effect of both PRC2 and DNA methylation in neuroblastoma cells. In addition, we identified a prognostic methylation signature that outperforms current risk stratification.
Conclusion:
Our data considerably extend the understanding of how epigenetic deregulation contributes to neuroblastoma pathogenesis and can inform novel prognostic and therapeutic development for children with unfavorable neuroblastoma.

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Dissecting neuroblastoma specific regulatory networks through epigenome mapping and transcriptional profiling of neuroblastoma and neural crest cell lines

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Neuroblastoma is a cancer of the peripheral nervous system, a structure that derives from the neural crest. The *MYCN* and *ALK* genes have been shown to be major drivers of neuroblastoma oncogenesis and more recently abnormalities of *ARID1A/ARID1B*, *ATRX* and *TERT* genes have been associated with a high-risk phenotype.
Here, we performed comprehensive epigenome mapping of 19 neuroblastoma and 2 human neural crest cell lines. Epigenome profiles for the chromatin marks H3K4me3 (active or poised promoters), H3K27me3 (PRC2-based repression) and H3K27ac (active promoters, enhancers and super-enhancers) were generated using ChIP-seq whereas transcriptional profiles were defined by RNA-seq analysis. The genomic alterations of the analyzed neuroblastoma cell lines have been previously characterized. The HMCan (Histone Modifications in Cancer) and Rose algorithms were used to identify distinct chromatin states and particularly super-enhancers.
Integrative bioinformatics analysis of the obtained profiles for neuroblastoma and neural crest samples and the use of epigenome profiles for other cancer types form the ENCODE project allowed us to identify neuroblastoma specific promoters, enhancers and super-enhancers. Unsupervised hierarchical clustering using neuroblastoma specific super-enhancers separated neuroblastoma cell lines from other cancer types and from neural crest cell lines. However, among neuroblastoma cell lines, no clear separation emerged according to the MYCN or ALK status. Our data revealed super-enhancers in key neuroblastoma oncogenes including two distinct super-enhancers within the ALK gene. The strength of super-enhancers correlated linearly with gene expression. Bivalent

promoters marked by H3K4me3 and H3K27me3 were also defined in both neuroblastoma and neural crest samples. Interestingly, whereas only the H3K4me3 mark was detected on the ALK promoter in neuroblastoma cell lines expressing this gene, the ALK promoter appeared to be bivalent in neural crest samples with no ALK expression detected by RNA-seq. Additional analysis is ongoing to further define the specific regulatory networks that drive neuroblastoma identity and oncogenesis.

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The histone chaperone CHAF1A promotes tumorigenesis and opposes neuroblastoma differentiation via metabolic reprogramming.

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Background. Neuroblastoma (NB) arises from embryonal neural crest secondary to a block in differentiation and long-term survival inversely correlates with the degree of differentiation. Inhibition of epigenetically controlled developmental programs is critical for NB de-differentiation and treatment with differentiation agents like retinoic acid (RA) has modestly improved survival. We have recently demonstrated a novel function for the histone chaperone CHAF1A (a subunit of the Chromatin Assembly Factor-1) in opposing NB differentiation.
Material and Methods. Loss-of-function studies were performed in NB RA-resistant cell lines upon CHAF1A knockdown. Conversely, gain-of-function studies were performed in NB RA-sensitive lines. Gene expression profiling (affymetrix u133+2 array) of knockdown and overexpressing lines was performed and quantitative-PCR assays used to validate most enriched metabolic gene sets. Inducible CHAF1A over-expressing cells were xenografted using a non-tumorigenic NB model.
Results. CHAF1A loss-of-function effectively drives neuronal differentiation in multiple NB RA-resistant lines. Conversely, CHAF1A overexpression in RA-sensitive lines induces colony formation and causes resistance to RA treatment. Moreover, GSEA and Q-PCR revealed that CHAF1A profoundly alters cell metabolism pathways (AA, glutamine, and glucose metabolism; $p<0.01$). Using conditional expression of CHAF1A (Tet-ON) in a NB line that has a poor engraftment rate, we demonstrate that CHAF1A overexpression significantly promotes tumor initiation in vivo with a much higher engraftment rate and tumor size (engraftment rate CHAF1A control: 2 out of 9, 22%; engraftment rate CHAF1A induced: 9 out of 12, 75%, $p< 0.03$).
Conclusion. These gain-of-function studies support our hypothesis that CHAF1A expression restricts neural crest differentiation and contributes to the resistance of NB tumors to RA therapy by altering glucose and glutamine metabolism. Importantly CHAF1A overexpression markedly enhances tumor initiation in vivo. Further understanding of the metabolic changes induced by CHAF1A will guide the development of novel differentiating therapies for high-risk neuroblastoma.

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The TBX2 super-enhancer marked transcription factor on 17q is overexpressed in neuroblastoma and infers poor prognosis

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Neuroblastoma (NB) is characterised by a low mutational burden while exhibiting highly recurrent large DNA copy number alterations including 17q gain in aggressive NBs. Together with the finding of gain of a large mouse chromosome 11 segment syntenic to human chromosome 17q in MYCN driven NB mouse models this strongly supports a crucial role for 17q gain in human NB formation and suggests the presence of oncogenic drivers at this genomic location. To prioritize 17q candidate genes we combined analysis of dynamic upregulation during MYCN driven tumour formation in mice with super-enhancer analysis based on H3K27 acetylation marks near candidate genes. We observed a significant higher number of 17q super-enhancers in MYCN amplified NB cell lines versus MYCN single copy cell lines suggesting that MYCN acts as amplifier to boost activation of super-enhancers on 17q leading to overexpression of target genes. Integration with expression profiling data allowed to prioritize the T-box2 transcription factor (TBX2) as strong candidate MYCN cooperative driver gene. In a panel of tumour cell lines TBX2 was most highly expressed in NB. Importantly, in primary NBs, high TBX2 expression was correlated with unfavorable prognosis. Moreover, TBX2 is upregulated in tumours of the LSL-MYCN mouse as compared to normal adrenal tissue and ChIP-sequencing data indicated binding of MYCN to the TBX2 super-enhancer and promotor region. TBX2 plays a central role during embryonal development and was implicated in bypassing senescence and inducing EMT in cancer. Knockdown was shown to impair growth in NB cells. Further functional assays are ongoing, zebrafish modeling has been initiated and TBX2 ChIP-sequencing and RNA-sequencing following TBX2 knockdown will allow to unravel the TBX2 regulome. In conclusion, we identified TBX2 as highly expressed 17q gene marked by super-enhancer activity in MYCN driven NB formation and propose TBX2 and TBX2 controlled signaling as novel drug target.

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The HBP1 tumor suppressor is a negative epigenetic regulator of MYCN driven neuroblastoma through interaction with the PRC2 complex.

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The MYCN oncogene signaling pathway is a key driver in initiation and progression of neuroblastoma (NB) and represents a major target for novel drug strategies. We previously identified the MYC repressor HBP1 as a robust mutant ALK regulated gene.
Pharmacological pathway analysis of mutant ALK NB cells allowed to identify HBP1 as a transcription factor downstream of the PI3K-AKT-FOXO3 signaling axis.
Here, we show that HBP1 upregulation represses MYCN signaling and activity and negatively impacts on cell growth. Acceleration of dbh-MYCN driven neuroblastoma formation was shown in HBP1 deficient zebrafish background, supporting the tumor suppressor role for HBP1. Next, we showed that MYCN represses HBP1 levels through the miR-17~92 cluster. Unraveling of the ALK-HBP1-PI3K-AKT-FOXO3 and the MYCN-miR-17~92-HBP1 axis suggested new opportunities for more precise combination treatments of neuroblastoma tumors with activated ALK and/or MYCN. Indeed, combining EGCG, a HBP1 upregulating tool compound, with the BET inhibitor JQ1 showed both in vitro and in vivo synergistic effects, while the PI3K/mTOR dual inhibitor BEZ-235 together with JQ1 also showed very strong synergistic effects. Further dissection of the HBP1 regulome was done using Gene Set Enrichment Analysis (GSEA) and iRegulon analysis (http://iregulon.aertslab.org), identifying SUZ12 as a central node in HBP1 regulated signaling, mainly through controlling the repression of MYCN regulated genes. As SUZ12 and EZH2 form the repressive PRC2 epigenetic regulatory protein complex, comparison of HBP1 and EZH2 regulated genes was performed, which revealed two interesting druggable targets: a novel candidate differentiation gene ARHGEF16 and the pro-apoptotic and HDAC inhibitor sensitizing CTSL2 gene. Based on these findings, further drugging efforts include combined EZH2/PI3K-AKT as well as EZH2/HDAC inhibition.
We conclude that HBP1 is a crucial component in MYCN controlled repression of gene activity through PRC2 interaction and demonstrate novel opportunities for precision drugging of MYCN overexpressing NB cells.

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Combination therapy with the bromodomain inhibitor JQ1 and the histone deacetylase inhibitor panobinostat synergistically reduce LIN28B gene and N-Myc protein expression and suppress neuroblastoma progression

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Patients with neuroblastoma associated with *MYCN* oncogene amplification or *LIN28B* gene over-expression experience a very poor prognosis. BET bromodomain inhibitors are emerging as one of the most promising novel classes of anticancer agents by blocking the BET bromodomain proteins BRD3 and BRD4 from activating transcription of oncogenes such as *MYC* and *MYCN*. However, treatment with BET bromodomain inhibitors alone does not result in cancer remission in many murine models. Here we show that BRD3 and BRD4 directly bound to the*LIN28B* gene promoter and activated *LIN28B* gene transcription, that treatment with the BET bromodomain inhibitor JQ1 reduced *LIN28B* gene expression, and that knocking down LIN28B expression reduced the expression of N-Myc protein, but not N-Myc mRNA. Combination therapy with JQ1 and the histone deacetylase inhibitor panobinostat synergistically induced growth inhibition and apoptosis in neuroblastoma cells, but not normal non-malignant cells. Genome-wide differential gene expression studies showed that JQ1 reduced oncogene expression, that panobinostat activated tumour suppressor gene expression, and that combination of JQ1 and panobinostat synergistically reduced the expression of a large set of genes, predominantly oncogenes including *LIN28B* but not *MYCN*. Importantly, JQ1 and panobinostat synergistically and considerably reduced the expression of both LIN28B and N-Myc oncoproteins. In neuroblastoma-bearing mice, JQ1 and panobinostat combination therapy synergistically and considerably reduced LIN28B and N-Myc protein expression in tumor tissues and blocked tumor progression. Our findings have therefore identified a novel strategy to simultaneously reduce LIN28B and N-Myc expression and a novel therapeutic approach for the treatment of aggressive neuroblastoma.

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High frequency of mutations in chromatin remodeling genes in neuroblastoma

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Background: Chromatin remodeling complexes including SWI/SNF are implicated in a wide variety of cellular processes including nuclear organization, chromosomal stability and gene expression, and mutations in SWI/SNF components play an important role in many cancer types. In neuroblastoma (NB), recent whole-genome/whole-exome sequencing efforts results have detected genetic alterations in chromatin remodeling genes such as ARID1A and ARID1B.
Methods: To explore the potential recurrence of genetic alterations in chromatin remodeling genes in a clinically representative cohort of NB patients (255 diagnostic samples), we designed a TruSeq Custom Amplicon panel (TSCA, Illumina) targeting 33 SWI/SNF genes (261.686bp). Libraries prepared from 50ng of genomic DNA were subjected to 150bp paired-end sequencing, with a high coverage (mean 2000X). After sequence alignment, two analyses were initiated. Clonal/sub-clonal mutations were detected by ACGT-base calling approach and statistical comparison between samples and controls. Structural variations will be searched for by gene dosage normalization within and between samples/controls.
Furthermore, a series of 31 NB cell lines and 6 germline controls were included in this study.
Results: A total of 96 clonal mutations (mutated allele fraction >20%) were detected. Overall, 35% of NB patients showed a mutation in at least one chromatin-remodeling gene; the most frequently mutated genes were ARID1A (10/255), ARID1B (3/255), BRD7 (3/255), MLL3 (10/255) and SMARCC2 (6/255) genes. Furthermore, 11 NB cell lines showed a clonal mutation in at least one of the studied genes. Mutations detected in NB cells lines were validated by RNA sequencing. Analyses to detect sub-clonal mutations and structural variations are ongoing.
No statistically significant differences in survival of patients with chromatin-remodeling genes wild-type versus chromatin-remodeling genes mutated at clonal level were observed.
Conclusions: The high frequency of clonal mutations highlights the dysregulation of chromatin remodeling in pediatric tumorigenesis and suggests potential new approaches for the management of patients with neuroblastoma.

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Identification of spliceosomal components as novel therapeutic targets for the treatment of high-risk, MYCN-driven neuroblastoma

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Background: Despite extensive research, the five-year survival rate for children with high-risk neuroblastoma (NB) is still as low as 40-50%; thus, better therapies are desperately needed. Amplification of the MYCN oncogene drives about half of the aggressive forms. Unfortunately, MYCN is considered undruggable. Based on the hypothesis that we can target MYCN through its protein-protein interactions/interactors (PPI), we aim to identify novel therapeutic targets to treat children with MYCN-amplified neuroblastoma.
Methods: We mapped the MYCN interactome across a panel of neuroblastoma cell lines with differential MYCN status and paediatric tumour samples by using label-free quantitative interaction proteomics. We inhibited selected MYCN PPIs by RNAi or drugs (splicing inhibitors); and measured cell viability as well as anchorage-independent growth of NB cells. We inferred the differential exon usage from mRNA-Seq data by using DexSeq; and analysed alternative splicing of selected genes in the presence of splicing inhibitors by qPCR. To further validate the effect of splicing inhibition on NB growth *in vivo*, we treated *Tg[dbh:MYCN-EGFP]* zebrafish (a generous gift from A. T. Look, Dana-Farber Cancer Institute) developing tumours in the fish-equivalent of adrenal medulla with splicing inhibitors.
Results: By comparing proteomics data obtained from MYCN-amplified tumours and NB cell lines, we shortlisted MYCN PPIs that are relevant in MYCN-amplified tumours. Ingenuity Pathway Analysis of MYCN PPIs revealed that pre-mRNA splicing is among the top altered pathways. Knocking down spliceosomal MYCN PPIs decreased cell viability of MYCN-amplified NB cells. Moreover, selected splicing inhibitors reduced transformation of MYCN-amplified NB cells in soft agar assays. Furthermore, we identified differentially spliced genes in MYCN-amplified NB; many of them are tumour suppressors, and regulate invasion and apoptosis. In addition, the splicing inhibitor reduced tumour size in the zebrafish model of MYCN-driven neuroblastoma.
Conclusions: We confirmed a novel strategy to target the pharmacologically undruggable MYCN. We pinpointed spliceosomal components as novel MYCN PPIs, and confirmed that we can inhibit MYCN-

amplified NB growth by altering mRNA splicing.

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The orally bioavailable small molecule CDK9 inhibitors CYC065 and CCT68127 are potent inhibitors of MYCN transcription

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Introduction: Amplification of the *MYCN* oncogene is a common and tumor-specific genomic event in aggressive tumours, but is not effectively targeted by any existing clinical drug. Attempts to target MYC transcription factors using direct approaches have failed. The recent discovery that the CDK7 inhibitor THZ1 targets superenhancer-driven expression of transcription factors such as MYC and MYCN generated much excitement, although this compound is not a clinically viable inhibitor. We explored the ICR med:chem compound library of CDK inhibitors for small-molecules with selective activity against MYCN-dependent neuroblastoma cells. The orally bioavailable, tri-substituted purine CDK inhibitors CCT68127 and CYC065, analogues of CYC202 (celiciclib, Cyclacel, Ltd), a clinical inhibitor of CDK2, exhibited an exquisite selectivity profile concomitant with enhanced potency and selectivity for CDK9, a component of PTEFb (CDK9:cyclinT1), and a rate-limiting regulator of MYC transcription. The aim of this study was to explore the sensitivity of neuroblastoma cells *in vitro* and *in vivo* to CCT68127 and CYC065.
Methods: Proliferation assays were used to assess the response of neuroblastoma cells *in vitro* to CCT68127 and CYC065. The efficacy of CYC065 was evaluated in subcutaneous xenograft models of both MYCN amplified (Kelly) and non-amplified neuroblastoma (SKNAS) and a Th-*MYCN* genetically-engineered murine model of neuroblastoma.
Results: Neuroblastoma cell lines were highly sensitive to both CCT68127 and CYC065 and the cellular increased sensitivity to the inhibitors correlated with *MYCN* amplification and expression levels. CCT68127 and CYC065 blocked neuroblastoma cell proliferation, induced apoptosis and depleted MYCN mRNA and protein in a time- and dose-dependent manner. Preclinical evaluation of CCT68127 and CYC065 in *MYCN*-dependent models of neuroblastoma, resulted in significantly reduced in tumour burdens and prolonged survival.
Discussion: We report that CYC065 which exhibits excellent pharmacokinetic characteristics, a low toxicity profile, and which is currently in early phase clinical evaluation for lung cancer, rapidly and selectively killing MYC- or MYCN dependent cancer cells is a powerful clinical tool for treatment of neuroblastoma and other MYC-dependent malignancies.

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Long term, continuous exposure to panobinostat induces terminal differentiation and long term survival in the TH-NMYC neuroblastoma mouse model

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Background/Aims: Deregulated acetylation of histones plays a key role in the pathogenesis of hematological as well as solid tumors by changing the chromatin structure and consequently altering transcription of genes involved in cell cycle control, differentiation and apoptosis. The aim of this study was to investigate the impact of panobinostat dosing on the TH-NMYC mouse model for neuroblastoma, with emphasis on apoptosis, NMYC expression and differentiation.
Methods: Homozygous TH-MYCN transgenic mice underwent abdominal ultrasounds (US) until neuroblastomas of 50mm^3- 200 mm^3 were detected. Mice were randomized to receive panobinostat, daily for 3 or 9 weeks, or vehicle. Tumour volume was monitored by serial US. Upon tumor development, or at 100 days following cessation of panobinostat, tumors were harvested for immunohistochemical (IHC) analysis of differentiation markers. For short-term experiments, tumors were harvested after 24hr for western blot and IHC analysis of markers of apoptosis and HDAC inhibition. RNA-seq analysis was performed on tumors harvested at baseline and at 24hr post panobinostat treatment. Gene set enrichment, and pathway analysis was then performed to investigate changes in the expression of genes associated with apoptosis, differentiation and NMYC expression.
Results: Treatment with panobinostat significantly improved survival with 3 weeks of treatment increasing mean survival from 7 to 68 days ($p<0.0001$), while 9 weeks of panobinostat treatment resulted in 88.9% of mice alive 100 days post withdrawal of drug ($p<0.0001$ compared to 3 week and vehicle groups). Panobinostat induced rapid tumour regression and significant caspase-dependent apoptosis. Panobinostat also induced differentiation of neuroblastomas into benign ganglioneuromas as evidenced by H&E and IHC analysis of the differentiation markers S100, NSE and SSTR2.

Correspondingly tumors had reduced expression of NMYC. RNA-seq confirmed tumors up-regulated gene pathways associated with apoptosis and differentiation, and down-regulation of NMYC associated pathways.
Conclusions: Treatment of TH-NMYC mice with panobinostat significantly improved survival and reduced tumour burden, supporting the further evaluation of panobinostat as a treatment option for high-risk neuroblastoma patients.

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A novel antibody-drug conjugate directed to the ALK receptor demonstrates efficacy in models of neuroblastoma

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Activated ALK by mutation or amplification is a validated therapeutic target in patients with high-risk neuroblastoma, and tyrosine kinase inhibitors are being developed to abrogate ALK signaling (Bresler et al. 2014). Native ALK is expressed on the surface of the majority of neuroblastoma tumors, but not on normal tissue, giving it properties of a tumor antigen. We hypothesized that ALK-targeted antibodies may be useful in neuroblastoma as a single-agent therapy or in combination with small-molecule ALK inhibitors – especially where mutations reduce kinase inhibitor sensitivity. In this work, an anti-ALK monoclonal antibody (anti-ALK2) was conjugated to a cytotoxic agent (ALK2-ADC) and its anti-tumor activity was investigated in a panel of neuroblastoma cell lines (n=10) harboring wild-type and mutant ALK. Cell surface ALK was quantified using flow cytometry and revealed differential antigen expression. Using *in vitro* growth inhibition assays, there was evidence for a dose-dependent cytotoxicity to neuroblastoma cells at subnanomolar concentrations that correlated with cell surface ALK expression and was independent of the underlying mutation status. In order to evaluate the therapeutic potential of ALK2-ADC *in vivo*, mice bearing Felix-patient-derived xenograft tumors, containing the third most common ALK mutation (R1245C), were used to demonstrate that ALK2-ADC led to significant reduction in tumor growth compared to unconjugated antibody and a control ADC ($p<0.0001$). Although ALK2-ADC binds to mouse ALK as well as human ALK, doses up to 10 mg/kg in the rodent model appeared to be well tolerated with no overt toxicity noted. Additional in vivo studies are ongoing to assess the anti-tumor potential of the ADC in a broader range of ALK-expressing neuroblastomas. Thus, targeting human ALK-expressing with an antibody-drug conjugate demonstrated favorable efficacy and tolerability supporting future development of this approach as a novel therapy for neuroblastoma patients.

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Oncolytic herpes Simplex-1 virotherapy augments chimeric antigen receptor T-Cell (CAR-T) therapy in Neuroblastomas

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While chimeric antigen receptor T-cell (CAR-T) therapies have demonstrated extraordinary clinical effectiveness in relapsed/refractory lymphoid leukemias, their efficacy against solid tumors has thus far lacked success, with modest migration to tumors and insufficient persistence. These challenges may be attributed to the characteristically immunosuppressive microenvironment found within solid tumors. Oncolytic virotherapy, a unique immune-based therapy, is an attractive potential adjunct to cellular therapies which may potentiate the competence of CAR-T within solid tumors. These attenuated viruses cause tumor-specific cell death not only through direct cell lysis, but also through the induction of pro-inflammatory responses. HSV1716 (trade name Seprehvir, Virttu Biologics, Ltd), an oncolytic Herpes Simplex-1 virus (oHSV) lacking the virulence factor ICP34.5 similar to the recently FDA-approved T-VEC, has established safety in multiple phase I clinical trials, including an ongoing trial for adolescents and young adults with refractory solid tumors initiated by our team (NCT00931931). We sought to determine whether Seprehvir might enhance CAR-T efficacy against neuroblastomas. We characterized oHSV-induced chemokine and cytokine gene expression in human neuroblastoma models, showing increases in T-cell attractant chemokines CXCL-10 and CCL-5 and T-cell activating cytokines IFN-gamma and TNF-alpha, but decreased inhibitory cytokine TGF-beta. Flow cytometry revealed variable tumoral cell GD2 expression, while third-generation (containing CD28, OX40, and CD3zeta signaling domains) GD2-directed human CAR-T displayed high CXCR-3 and CCR-5 expression, allowing for chemotactic signaling through CXCL-10 and CCL-5, respectively. We performed transwell assays and found increased CAR-T migration toward Seprehvir-infected neuroblastoma cells over non-infected cells. Neuroblastoma xenograft tumor-bearing athymic nude mice treated with combination therapy displayed prolonged survival (median 27, range 17-61 days) compared to mice treated with oHSV (median 19, range 17-21 days) or CAR-T monotherapies (median 10, range 10-12 days). These results indicate oHSV as a beneficial adjunct to CAR-T therapy for neuroblastoma and should be further explored in clinical trials.

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Prenatal stress increases NB tumorigenicity in TH-MYCN mice.

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Although genetic aberrations are essential in neuroblastoma (NB) development, its etiology and heterogeneity cannot be explained solely by genetics. NB arises due to defects in sympathetic neuron differentiation occurring during fetal development. Strikingly, the two factors promoting de-differentiation of NB cells, hypoxia and glucocorticoids, are elevated in the fetus during maternal stress, suggesting a role for prenatal stress in NB tumorigenesis. To test this hypothesis we used mice expressing MYCN under tyrosine hydroxylase promoter (TH-MYCN mice), which spontaneously develop NBs. To mimic stress, pregnant mothers carrying hemizygous TH-MYCN offspring were implanted with pellets containing either corticosterone or placebo at the time of neuroblast proliferation (E10-20). Tumor frequency was compared between these two experimental groups and TH-MYCN offspring from intact pregnancies. Surprisingly, in pregnant mothers from the placebo group, stress associated with experimental procedures alone was sufficient to elevate their corticosterone levels and increase tumorigenicity in their hemizygous TH-MYCN offspring from 32 to 64% ($p = 0.03$). A similar effect was observed in offspring of corticosterone-treated mothers with its levels comparable to mice eliciting a physiological stress response in the placebo group (<1200ng/ml). This stress-induced increase in tumorigenicity was associated with decreased tumor cell apoptosis, suggesting defects in neuroblast elimination as a potential mechanism underlying this effect. While all female mice presented with ovarian metastases, tumor-bearing prenatally stressed offspring had increased frequency of other distant metastases (0% vs 71% for intact control and stressed groups respectively, $p<0.05$). No increase in tumorigenicity was observed in offspring of corticosterone-treated mothers having corticosterone levels markedly exceeding those observed in the placebo group (>1200ng/ml), indicating that only physiologically relevant levels of stress mediators accurately recapitulate the stress response. These findings
support the role for prenatal stress in NB development, as well as implicate other pathologies associated with elevated levels of glucocorticoids and fetal hypoxia in its etiology.

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Identifying mechanisms of neuroblastoma tumorigenesis using single cell transcriptomics

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Neuroblastoma is a pediatric cancer of the sympathetic nervous system. MYCN oncogene amplification occurs in approximately 20% of patients and is associated with poor patient prognosis. MYCN overexpression has been confirmed to recapitulate neuroblastoma tumorigenesis in TH-MYCN transgenic mice. In this model, sympathetic ganglia exhibit hyperplasia of a premalignant neuroblast population, which are clonally selected prior to malignant transformation. The genetic determinants of clonal selection in this heterogenous population of premalignant cells is unknown.
To decipher early changes in gene expression during neuroblastoma tumor initiation, we conducted single cell transcriptomics on sympathetic ganglia derived from 10 day old pre-tumor TH-MYCN$^{+/+}$ mice using the Fluidigm C1 Single-Cell Auto Prep System. We successfully profiled the expression of 152 single cells according to 69 custom-derived genes (a 62 gene tumorigenesis signature, 5 lineage markers and 2 housekeeping genes). Hierarchical clustering of the profiled cells identified that hyperplastic neuroblasts (Phox2b^{+},Dbh^{+},PCNA^{High}) fell within two dominant clusters by gene expression, with only one of the clusters showing exclusive expression of a 11 member subgroup of genes involved in regulation of mitosis (ASPM, BUB1, BUB1B, NUF2, KIF23, PRR11, CCNB2, KIFC1, DEPDC1, SGOL1, CCNB1). Interrogation of a 649 patient neuroblastoma tumor dataset, showed that the same 11 mitotic genes were strongly correlated with MYCN target gene expression, stage 4 disease and MYCN amplification. Moreover in a panel of 30 neuroblastoma cell lines, high expression of the 11 mitotic genes sensitized cells to anti-mitotic compounds such as preclinical agents that target Aurora Kinase B, PLK1 and EG5, as well as clinically used microtubule inhibitors. Together this supports a hypothetical model: that deregulation of mitotic genes occurs as a secondary event to MYCN amplification in neuroblastoma tumor initiation, and high mitotic gene expression is associated with clonal selection, malignant transformation and tumor progression. These findings also support that mitotic deregulation may impart a therapeutic targeting vulnerability that could be exploited as a novel targeted strategy for MYCN-amplified neuroblastoma patients.

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ARID1A and *ARID1B* mutations in the Swi/Snf BAF chromatin remodeling complex drive poor outcome neuroblastoma

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Background: *ARID1A* and *ARID1B* are mutually exclusive subunits of the mSwi/Snf (BAF) complex implicated as a tumor suppressor in >20% of cancers. Programmed exchanges among subunits directs divergent cell fates, such as the transition between embryonic stem cell BAF composition (esBAF) to neural progenitor (npBAF) to neuron (nBAF). *ARID1A/B* mutations disrupt BAF structure and function and define an aggressive neuroblastoma subset.

Methods: Deep-genome sequencing identified BAF subunit mutations in neuroblastoma cell lines and primary tumors, and gene expression profiles (GEP) were correlated with BAF mutation status. BAF wild-type and mutant neuroblastomas were characterized by co-IP and MS/MS to define complex composition, ChIP-seq to identify genome binding sites, and synthetic-lethal screening to define specific vulnerabilities.

Results: Excluding common hemizygous deletion of *ARID1A* (1p35), mutually exclusive *ARID1A* (biallelic; n=6), *ARID1B* (n=9), *ARID2* (n=2) and *SMARCA4* (n=2) mutations were identified in 19/74 (26%) neuroblastoma cell lines. Target-capture sequencing of 476 primary neuroblastomas identified *ARID1A* or *ARID1B* mutation, excluding deletion (ongoing), in 2% (correlated with unfavorable features). mRNA expression of subunits unique to npBAF correlated directly with each other (r>0.3; p<0.001) and inversely with subunits unique to nBAF (r>-0.3; p<0.001). Similarly, BAF-driven neurogenesis genes correlated directly with nBAF and indirectly with npBAF, and high npBAF expression correlated with unfavorable outcome. Biochemical analyses of *ARID1A/1B* mutant complexes demonstrate a role for heterozygous and homozygous mutations on BAF complex abundancy and subunit and associated factor composition. ChIP-seq studies reveal differential BAF complex genomic localization and GEP in wild-type versus *ARID1A/B* mutant cell lines.

Conclusion: We identified BAF complex mutations (predominantly in *ARID1A* and *ARID1B*) in a large proportion of neuroblastoma cell lines and in aggressive primary tumors, underscoring their oncogenic relevance. Functional deregulation of BAF complexes may not be restricted to tumors with BAF-complex mutations as evidenced by strong correlations between npBAF subunit expression and poor outcome in neuroblastomas lacking *ARID1A/B* gene mutation.

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Identification of somatic mutations determining the neuroblastoma phenotype

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Background: The molecular mechanisms driving the contrasting clinical courses of neuroblastoma have remained largely enigmatic to date. We therefore aimed to determine the genetic alterations underlying the distinct neuroblastoma phenotypes by massively parallel sequencing.

Methods: Mutation patterns of 416 diagnostic neuroblastoma specimens were determined by massively parallel sequencing (whole-genome sequencing, n=56; whole-exome sequencing, n=162; targeted sequencing, n=198).

Results: We discovered genomic rearrangements of the *TERT* locus in 12/56 neuroblastomas by whole-genome sequencing. *TERT* rearrangements affected exclusively high-risk tumors, in mutually exclusive fashion with *MYCN* amplification and *ATRX* mutations. In an extended cohort (n=217), *TERT* rearrangements were detected in 24% of high-risk neuroblastomas, and were associated with particularly poor patient outcome. We demonstrated that such rearrangements led to massive induction of *TERT* expression and telomerase activation caused by translocation of strong enhancer elements. Similarly, we observed upregulation of *TERT* and telomerase in *MYCN*-amplified tumors, while activation of the *Alternative Lengthening of Telomeres* pathway was detected in high-risk tumors lacking these alterations. In addition, we identified mutations in *ALK* and other RAS/MAPK pathway-acting genes as well as p53/MDM2 pathway genes in 36/402 (9.0%), 25/402 (6.2%) and 18/402 (4.5%) neuroblastomas, respectively. Such mutations affected predominantly high-risk tumors (51/227 cases, 22.5%) but occurred also in non-high-risk tumors (22/173 cases, 12.7%). Notably, survival of patients whose tumors harbored mutations in these cancer genes was significantly worse than those without such mutations in the entire cohort, and inboth the high-risk and the non-high-risk subgroups (p<0.001 each).

Conclusion: Our data demonstrate that activation of

telomere maintenance mechanisms is a hallmark of high-risk neuroblastoma, while low-risk tumors lack such mechanisms, which may explain their capacity of spontaneous regression. Furthermore, the clinical course of the disease is strongly influenced by the presence of mutations in RAS/MAPK and p53/MDM2 pathway genes at diagnosis. Together, our data strongly suggest that the clinical phenotype of neuroblastoma is driven by specific patterns of somatic mutations.

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MYCN and HDAC5 transcriptionally repress *CD9* to trigger an invasion-metastasis cascade in neuroblastoma

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The systemic and resistant nature of metastatic neuroblastoma renders it largely incurable with current multimodal treatment. Clinical progression stems mainly from the increasing burden of metastatic colonization. Therapeutically inhibiting the migration-invasion-metastasis cascade would be of great benefit, but the mechanisms driving this cycle are as yet poorly understood. In-depth transcriptome analyses and ChIP-qPCR identified the cell surface glycoprotein, CD9, as a major downstream player and direct target of the recently described *GRHL1* tumor suppressor. CD9 is known to block or facilitate cancer cell motility and metastasis dependent upon entity. High-level *CD9* expression in primary neuroblastomas correlated with patient survival and established markers for favorable disease. Low-level *CD9* expression was an independent risk factor for adverse outcome. MYCN and HDAC5 colocalized to the *CD9* promoter and repressed transcription. *CD9* expression diminished with progressive tumor development in the *TH-MYCN* transgenic mouse model for neuroblastoma, and *CD9* expression in neuroblastic tumors was far below that in ganglia from wildtype mice. Primary neuroblastomas displayed differential *CD9* methylation in 450K methylation array analyses, and *CD9* hypermethylation was associated with reduced *CD9* expression, supporting epigenetic regulation. Inducing CD9 expression in a SH-EP cell model inhibited migration and invasion in Boyden chamber assays. Enforced CD9 expression in neuroblastoma cells transplanted onto chicken chorioallantoic membranes strongly reduced metastasis to embryonic bone marrow. Combined treatment of neuroblastoma cells with HDAC/DNA methyltransferase inhibitors synergistically induced CD9 expression despite hypoxic, metabolic or cytotoxic stress. Our results show CD9 is a critical and indirectly druggable mediator of neuroblastoma cell invasion and metastasis.

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MYCN amplified neuroblastomas require TEAD4 to orchestrate transcriptional programs, exposing a therapeutic vulnerability

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BACKGROUND: High-risk neuroblastomas (NBL) display substantial clinical and molecular heterogeneity. Despite progress understanding NBL genetics, little progress has been made in procuring personalized treatments. Here we sought to determine effector molecular mechanisms downstream of genetic alterations sustaining different tumor subtypes[1].
METHODS: We first defined molecular subtypes from the TARGET and NRC Consortium gene expression datasets by consensus clustering and identified specific master regulators (MRs) of transcriptional

programs using VIPER/ssMARINA algorithm[2] available as a bioconductor R package. We performed *in vitro* and *in vivo* validation of MRs by RNAi screening followed by experimental and computational assays to elucidate the interdependencies between top MRs and their biological role. Finally, we studied tissue microarrays (TMA) and applied multivariate Cox regression to understand the clinical relevance of our findings.
RESULTS: We identified MRs from three distinct molecular subtypes of high-risk NBL, conserved across cohorts. siRNA and shRNA screens identified a TEAD4-MYCN positive feed forward loop as a key NBL state maintenance of MYCN dysregulated tumors. RNA-seq upon MYCN and TEAD4 knockdown showed that MYCN controls cell growth and represses differentiation and that TEAD4 is essential for G1 to S transition and DNA repair. Specifically, TEAD4 transactivates AURKA, CDKs, Cyclin D, E2Fs, DNA replication factors, checkpoint kinases and ubiquitin ligases. Consistently, TEAD4 inhibition induced critical loss of NBL cell viability, both *in vitro* and *in vivo*. TMA staining shows increased TEAD4 protein levels in high risk tumors (P = 0.021); expression/activity is an independent predictor of survival in multivariable analysis (P = 0.0084 corrected for age, stage and MYCN status).
CONCLUSION: TEAD4 plays a critical role in maintaining the high proliferative state of undifferentiated NBLs with sustained growth promoted by MYCN dysregulation. The critical effect of disrupting TEAD4-MYCN module on cell viability and its clinical relevance brings forward TEAD4 as an excellent candidate target for therapeutic intervention.

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Clinical, biologic, and outcome differences according to MIBG avidity in children with neuroblastoma: A report from the Children's Oncology Group (COG)

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Background: Prior investigations suggest that neuroblastomas (NBL) that do not accumulate MIBG (MIBG non-avid) may have more favorable features compared to MIBG avid tumors. We compared clinical/biologic features and clinical outcomes between patients with MIBG non-avid vs. avid NBL.
Methods: A retrospective analysis was performed of patients with metastatic high- or intermediate-risk NBL treated on COG protocols A3973 or A3961, respectively. A3973 patients had baseline MIBG scans centrally reviewed (n=306). A3961 patients with bone metastasis at baseline were included (n=37). Comparisons of clinical/biological features according to MIBG avidity were made with chi-squared or Fisher exact tests. Event-free survival (EFS) was compared using log-rank tests and modeled using Cox models.
Results: Thirty of 343 patients (8.7%) in the analytic cohort had MIBG non-avid NBL (n=1 from A3961 and n=29 from A3973). Non-avid tumors were less likely to be adrenal primary tumors (34.5% vs. 57.2%; p = 0.02) or have bone metastases (36.7% vs. 61.7%; p = 0.008) and were more likely *MYCN* amplified (53.8% vs. 32.6%; p = 0.03). The rate of positivity for urine catecholamine levels (available from A3973 only) was lower in patients with non-avid NBL (66.7% vs. 91%; p < 0.001). Patients with MIBG non-avid NBL had a 5-year EFS of 50.0% compared to 38.7% for patients with MIBG avid NBL (p = 0.03). On backward-selected multivariate testing in high-risk patients, MIBG avidity was the sole adverse prognostic factor for EFS identified (hazard ratio 1.77; 95% confidence interval 1.04 – 2.99; p = 0.03); *MYCN* status, age, grade, MKI, and ploidy were not prognostic.
Conclusions: MIBG non-avid NBL represents a distinct clinical subgroup with lower rates of adrenal primary tumors, bone metastasis, and catecholamine secretion. Despite being more likely to have *MYCN* amplified tumors, patients with MIBG non-avid NBL have superior outcomes compared to patients with MIBG avid NBL.

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Discordance in ^{123}I-MIBG (MIBG) and ^{18}FDG positron emitting tomography (PET) scans after multimodality therapy for high-risk neuroblastoma: clinical implications

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Background: MIBG scans are considered the "gold standard" for detection and staging of neuroblastoma. However, at Memorial Sloan Kettering Cancer Center (MSKCC), over the last decade we have encountered increasing numbers of patients with high-risk neuroblastoma who are long-term progression-free survivors despite having persistently positive MIBG scans after multimodality treatment with chemotherapy, surgery, radiotherapy and anti-GD2 antibody-mediated immunotherapy.

We hypothesized that these patients could have mature or inactive disease and that PET scans could assist in identifying this cohort.
Methods: After obtaining permission from MSKCC Institutional Review Board, we retrospectively analyzed records of high-risk stage 4 neuroblastoma patients (>18 months at diagnosis) undergoing concurrent (within 30 days) MIBG and PET scans. We compared progression-free (PFS) and overall survival (OS) in patients with concordant (both MIBG and PET scans positive at ≥1 overlapping site) and discordant (positive MIBG but negative PET scans) using Kaplan-Maier and life table analyses and assessed variables by univariate analysis. "Positivity" was defined as ≥1 site of radio-isotope uptake.
Results: 47 consecutive patients (median age: 3.7±3.1years; 6 with MYCN-amplified disease) were identified between 2006-2013. 24 patients had primary refractory (PR) neuroblastoma and 23 secondary refractory (SR) disease after relapse. 14 patients had concordant and 33 discordant scans. 7 patients (2 with concordant and 5 with discordant scans) had bone marrow (BM) involvement with neuroblastoma at time of scans. The following did not have a significant impact on PFS and OS (p>0.05): MYCN status, paired scans being performed > or <12 months from diagnosis (PR group) or relapse (SR group), post-scan systemic therapy, BM involvement. However, MIBG-PET discordance was associated with significantly better prognosis for PFS (p<0.005; 5 yr PFS 73±8% vs 21±11%) and OS (p=0.06; 3 yr OS 94±4% vs 69±13%).
Conclusion: Negative PET scans after aggressive multimodality therapy for high-risk neuroblastoma might indicate a favorable outcome even if MIBG scans show uptake. Multivariate analysis on a larger cohort of patients is ongoing.

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Diagnostic FDG and FDOPA positron emission tomography scans distinguish the genomic type and treatment outcome of neuroblastoma

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Neuroblastoma (NB) is a heterogeneous childhood cancer that requires multiple imaging modalities for accurate staging and surveillances. The aim of this study is to investigate the utility of positron emission tomography (PET) with ^{18}F-fluorodeoxyglucose (FDG) and ^{18}F-fluoro-dihydroxyphenylalanine (FDOPA) in determining the prognosis of NB. During 2007–2014, NB patients undergoing paired FDG and FDOPA PET scans at diagnosis were evaluated for the maximum standardized uptake value (SUV_{max}) of FDG or FDOPA by the primary tumor, which values were correlated with clinical features, genomic types, treatment outcomes, and imaging-related gene expression. Among 42 NB patients (28 boys and 14 girls; median age, 2.0 years) who were eligible for analysis, patients with older age, advanced stages, or *MYCN* amplification showed higher FDG and lower FDOPA SUV_{max} (all $P < 0.02$). Receiver operating characteristics analysis identified FDG $SUV_{max} \geq 3.31$ and FDOPA $SUV_{max} < 4.12$ as an ultra-high-risk feature (PET-UHR) that distinguished the most unfavorable genomic types, *i.e.*segmental chromosomal alterations and/or *MYCN* amplification, at a sensitivity of 81.3% (54.4%–96.0%) and a specificity of 93.3% (68.1%–99.8%). Considering with age, stage, *MYCN* status, and anatomical image-defined risk factor, PET-UHR was an independent predictor of inferior event-free survival (multivariate hazard ratio, 4.9 [1.9–30.1]; $P = 0.012$). Meanwhile, the ratio between FDG and FDOPA SUV_{max} (G:D) correlated positively with hexokinase 2 (*HK2*; Spearman's $\rho = 0.86$, $P < 0.0001$) and negatively with dopa decarboxylase (*DDC*; Spearman's $\rho = -0.58$, $P = 0.02$) gene expression levels, which might suggest higher glycolytic activity and less catecholaminergic differentiation in NB tumors taking up higher FDG and lower FDOPA. In conclusion, the intensity of FDG and FDOPA uptake on diagnostic PET scans may predict the tumor behavior and complement the current risk stratification systems of NB.

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Towards a model for risk stratification of high-risk neuroblastoma. A report from the HR-NBL-1/SIOPEN study.

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Introduction: Neuroblastoma is commonly classified as low, intermediate or high-risk according to criteria reported by the International Neuroblastoma Risk Group (INRG) project. As yet there are no established methods for stratifying patients with high-risk disease at diagnosis. The aim of this project is to explore potential criteria in patients treated on the HR-NBL-1/SIOPEN study and to develop models for risk stratification.

Methods: Data were extracted from the HR-NBL-1 trial database. The analysis was limited to patients with metastatic disease, aged ≥18 months at diagnosis and treated on study prior to the introduction of immunotherapy. The primary endpoint was 5-year event-free survival (EFS). For the statistical evaluation, the pseudo-value regression model was used to focus on the primary endpoint whilst eliminating the impact of short-term survival effects. A score was developed based on the estimated log-cumulative hazard (cHR) for 5-year EFS.

Results: Multiple different models – incorporating tumour MYCN-amplification status, serum LDH and ferritin (cut-off twice upper limit of normal, ULN), patient age (5 year cut-off) and involvement of one or multiple metastatic compartments – were developed. One model (incorporating data from 847 patients) included age>5 years (cHR 1.62; 95% CI: 1.28-2.04; 2 points in final score); LDH>2xULN (cHR 1.27; 1.13-1.61; 1 point) and involvement of >1 metastatic compartment (cHR 1.66; 1.16-2.24; 2 points). Using this model, we were able to identify a group of patients (with a score of 5), representing 12% of the total population, with a 5-yr EFS of only 8±3%; in contrast to patients with a score 0–1 who had 5-yr EFS >44%.

Conclusion: The development of models for risk stratification based on scores for criteria present at diagnosis is potentially useful both for predicting outcome and for treatment stratification in future high-risk neuroblastoma protocols. The work is being further developed to explore additional models, undertake validation and ultimately to include criteria such as blood/bone marrow mRNA levels or other biological parameters at diagnosis.

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Revisions to the International Neuroblastoma Response Criteria: A consensus statement from the NCI-Clinical Trials Planning Meeting

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Purpose: More than two decades ago, an international working group established the International Neuroblastoma Response Criteria (INRC) to assess treatment response in children with neuroblastoma. However, this system required modification to incorporate modern imaging techniques and new methods for quantifying bone marrow disease that were not previously widely available. The National Cancer Institute sponsored a Clinical Trials Planning Meeting (CTPM) in 2012 to update and refine response criteria for patients with neuroblastoma.

Methods: Multi-disciplinary investigators from 13 countries reviewed data from published trials performed through cooperative groups, consortia and single institutions. Data from both prospective and retrospective trials were used to refine the INRC.

Monthly international conference calls were held from 2011 – 2015 and consensus was reached through review by working group leadership and the NCI CTPM leadership council.
Results: Overall response in the revised INRC will integrate response with respect to 3 components: primary tumor, soft tissue and bone metastases, and bone marrow metastases. Primary and metastatic soft tissue sites will be assessed using RECIST and ^{123}I-metaiodobenzylguanidine (^{123}I-MIBG) or ^{18}F-fluorodeoxyglucose (FDG)-Positive emission tomography (PET) scans if MIBG non-avid disease. ^{123}I-MIBG or FDG-PET scans (if MIBG non-avid) replace ^{99}Technetium (TC) bone scans to assess bone metastases. Bone marrow (BM) will be assessed by histology/immunohistology and immunocytology. BM with ≤5% tumor involvement will be classified as minimal BM disease. Urinary catecholamines will not be included in response assessment. Overall response categories will be defined as complete response, partial response, minor response, stable disease and progressive disease.
Conclusions: These revised criteria permit uniform application of consensus definitions of disease response, improve the interpretability of clinical trial results and facilitate collaborative trial designs.

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Risk factors for outcome after relapse or progression of localized Neuroblastoma

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Introduction:
Recent strategies aim to reduce cytotoxic treatment for patients with localized Neuroblastoma. This may lead to progressions manageable with limited treatment. On the other hand some patients experience multiple subsequent progressions with fatal outcome. We here aimed to determine risk factors for outcome of relapsed localized neuroblastoma.
Patients and methods:
Data of patient with localized non-amplified Neuroblastoma diagnosed between 1995 and 2004 were analysed. Events were classified as metastatic (to stage 4S or to stage 4) or locoregional, either with favorable course managed with one line of treatment or unfavorable course with multiple episodes of subsequent progressions.
Results:
Of 739 patients, 116 experienced relapse or progression (5-year-EFS 0.83±0.01; 5-year-OS 0.96±0.01). A metastatic pattern fitting the stage 4S definition was seen in 13 patients, progression to stage 4 in 17 patients. Of 86 patients with locoregional events, five patients died from complications or early progression, 53 patients were managed with one line of relapse treatment (surgery only in 22 patients), and 28 patients experienced multiple episodes of progression. Eight patients developed distant metastases in subsequent events, fifteen patients finally died.
Survival was excellent for patients with progression to stage 4S and with manageable locoregional events, but unsatisfactory for patients with multiple locoregional events (5-year-OS: 0.59±0.10) or progression to stage 4 (5-year-OS: 0.65±0.12). Risk factors for both subsequent progressions and for fatal outcome were age at diagnosis ($p<0.001$), INSS stage ($p=0.02$ resp. 0.001), histology ($p<0.001$), and chemotherapeutic treatment prior to relapse ($p<0.001$). Multivariable analysis revealed age (HR 15.2), histology (HR 3.02), and chemotherapeutic treatment (HR 2.43) as independent factors for subsequent progression and age (HR 8.07) as independent factor for survival.
Conclusion:
In a substantial portion of patients, locoregional progressions or progressions to stage 4S were manageable assuring an excellent prognosis. Established risk factors helped to define patients with unfavorable outcome.

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Altering the MYCN/MAX ratio in a drosophila MYCN model leads to homeotic transformation of the eye to wing through deregulation of specific HOX genes

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For children with high risk neuroblastoma characterized by amplification of the *MYCN* oncogene survival rates are particularly poor. MYCN is a transcription factor that heterodimerises with MAX to control a large set of genes. The process of amplification invariably leads to very high levels of MYCN protein, which alters the intracellular ratio between MYCN and MAX. This suggests that at least some of MYCN's oncogenic effects may be MAX-independent and that in turn the levels of MAX may be critical for normal MYCN function. To explore this hypothesis, we took advantage of a MYCN fly model in which it is possible to modulate the expression levels of drosophila Myc (dMyc), human MYCN or drosophila MAX (dMax) in the fly eye. Using this approach, we generated a range of drosophila lines with different combinations of dMYC, MYCN and/or dMAX expression levels. The results showed that MAX downregulation against a normal dMyc background reduced proliferation and organ growth but did not affect retina differentiation. Conversely, dMYC/MYCN overexpression with a normal dMAX background blocked eye cell differentiation with deformation of the eye structure. Surprisingly, flies with simultaneous high levels of dMYC or MYCN and concomitant silencing of dMAX showed an eye to wing homeotic transformation. Molecular and cellular investigation of

this phemomenon revealed that high levels of dMyc/MYCN protein in the eye primordium leads to ectopic activation of *Antennapedia*, the wing HOX-specific gene, most likely through repression of *Deformed*, the eye HOX-specific gene. Based on these results we propose that during development, very high MYCN levels may alter regulation of a specific set of HOX genes to alter/block cell differentiation, thus predisposing cells to neoplastic transformation.

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Identification and reprogramming of mesenchymal-type cells in neuroblastoma

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Background: Cellular heterogeneity within tumors is increasingly recognized as a source of therapeutic failure. However, the molecular mechanisms driving intratumor heterogeneity, drug-resistance and relapse in neuroblastoma remain ill-defined.
Results: Here we characterized neuroblastoma as a biphasic tumor. In new patient-derived cell lines, two phenotypically divergent cellular subtypes were detected, regardless of the shared genetic defects. One cell type is referred to as neuro-epithelial (NE) and expresses classic neuroblastoma markers from the peripheral sympathetic nervous system. In contrast, a newly characterized mesenchymal (MES) cell type lacks NE markers, instead expresses mesenchymal genes and is motile. Moreover, MES-type cells are resistant to a wide variety of chemotherapeutics used in clinical management of neuroblastoma.
The identification of a drug-resistant MES-type subpopulation of cells urged identification of the molecular pathways that control them. Analysis of mRNA gene expression and *cis*-regulatory elements of four MES-NE cell line pairs uncovered signaling routes (e.g. NOTCH, WNT) and a transcription factor module associated with the MES-state. The top transcription factors PRRX1 and NOTCH each efficiently converted NE-type cells to induced-MES (iMES) cells. iMES cells acquired many features of MES-cells including motility, mesenchymal gene expression and –histone modifications as well as chemo-resistance.
Immunohistochemical analysis of primary neuroblastoma detected a small fraction of PRRX1-positive MES-type cells. Importantly, MES-type cells enriched after chemotherapy treatment and in relapsed tumors, compared to the primary tumor.
Conclusions: Neuroblastoma is a biphasic tumor with classic NE-type cells and chemo-resistant MES-type cells. A subpopulation of MES-type cells is detected *in vivo* and can enrich after therapy and in relapses. Delineation of the core MES-regulatory network identified new pathways that induce the MES-state. A detailed understanding of MES-regulatory pathways will uncover new avenues for therapeutic intervention.

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Induction of a metabolic switch in neuroblastoma and in other human cancer types upon targeting MYC

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Neuroblastoma is one of the most aggressive solid tumors of early childhood. Amplification of the *MYCN* oncogene has been found in around 30% of neuroblastoma patients and is associated with rapid tumor progression and poor prognosis. As metabolic adaptations are crucial for cancer cell survival, identifying metabolic discrepancies of aggressive tumors may be central in order to find new treatment strategies. We have recently demonstrated 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 neuroblastoma cells. Importantly, we found that inhibition of MYCN results in changes in neuroblastoma cell metabolism including mitochondrial dysfunction leading to accumulation of intracellular lipid droplets (Zirath, PNAS 2013; Muller, PloS One, 2014).
We have now analyzed the effects of several small molecule MYC inhibitors including the structurally unrelated 10074-G5, the BET-domain inhibitor JQ1 and the Aurora A kinase inhibitor Alisertib on neuroblastoma cells. Our data show that treatment with all three compounds resulted in accumulation of cytoplasmic lipid droplets and in neural differentiation. Next we were interested in the extent of generality of this response and have therefore extended our study to a panel of different cancer cell types, including medulloblastoma, glioblastoma, melanoma, hepatocarcinoma, lung, ovary, breast, colon, prostate and cervical cancer. We found a cell type-dependent response to the treatments; some cells showing accumulation of neutral lipids while others not. Importantly, some of the treatments gave rise to morphological changes resembling cellular differentiation into neural or glial lineages. We are now performing functional assays using Seahorse Flux Analyzer to study the metabolic changes as well as analyzing expression of neural and glial differentiation markers in response to treatment in neuroblastoma and medulloblastoma cells. Our goals are to identify the types of cancers that are sensitive to MYC-inhibiting strategies and to analyze the mechanism of action of MYC inhibitors for development of future tailored therapies.

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The histone methyltransferase DOT1L induces neuroblastoma progression by regulating gene transcription

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Myc oncoproteins exert oncogenic effects by regulating the expression of target oncogenes. Histone H3 lysine 79 (H3K79) methylation at Myc-responsive elements of target gene promoters is a strict prerequisites for Myc-induced transcriptional activation. DOT1L is the only known histone methyltransferase that catalyses H3K79 methylation. Here, we showed that N-Myc up-regulated DOT1L mRNA and protein expression by binding to an E-box at the DOT1L gene promoter. Knocking-down DOT1L reduced the mRNA and protein expression of the N-Myc target genes, ODC1 and E2F2. DOT1L and N-Myc formed a protein complex, and knocking-down DOT1L reduced histone H3K79 di-methylation and N-Myc protein binding at the ODC1 and E2F2 promoters, reduced neuroblastoma cell proliferation *in vitro* and tumour progression in neuroblastoma-bearing mice. In addition, high levels of *DOT1L* gene expression in neuroblastoma tissues correlated with high levels of *MYCN*, *ODC1* and *E2F2* gene expression, and independently correlated with poor patient survival. Taken together, our data identify DOT1L as a novel co-factor in N-Myc-mediated gene transcriptional activation and neuroblastoma oncogenesis, and as a novel target for the therapy of neuroblastoma.

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Frequency of high and low level clonal *ALK* mutations in high risk neuroblastoma patients. A SIOPEN study

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Background: In neuroblastoma (NB), activating *ALK* receptor tyrosine kinase point mutations are detected in 8-10% of patients at diagnosis using conventional sequencing. To determine the occurrence and the prognostic impact of both high and low level clonal *ALK* mutations in high-risk NB patients we studied *ALK* variation frequencies using targeted deep sequencing in samples of patients enrolled in the HR-NBL1/SIOPEN trial.

Methods: Diagnostic NB samples from 524 high-risk NB patients were analyzed, focusing on the exons 23, 24 and 25 containing the F1174, F1245 and R1275 hotspots respectively. DNA was amplified via a two-step PCR approach, the second step consisting of addition of sample-specific barcodes for targeted resequencing in a single experiment. Amplicon sequencing (Illumina HiSeq2500) achieved an extremely high depth of coverage (80,000X). The background base variability (error rate) in 32 control samples was 0.017%+/-0.010; thus a base frequency >0.06% was significantly different from background noise (Fisher's exact test). Mutated allele fractions (MAF) <20% were defined as low level clonal.

Results: At the F1174 hotspot, mutations were observed in 28/524 samples, 15 being high (MAF was >20%) and 13 low level clonal (MAF: 0.123%-11.688%). At the R1275 hotspot, mutations were observed in 31/524 samples, 18 high and 13 low level clonal. Finally, at the F1245 hotspot, mutations were observed in 3/524 samples, 2 high and 1 low level clonal. All clonal mutations were validated by Sanger sequencing. Validation of all other events and analysis of additional samples from collaborating groups is ongoing.

A correlation between ALK mutations and MYCN amplification was detected (chi-square, p=0.0124).

No statistically significant difference in survival of patients with *ALK* wild-type versus *ALK*-mutated NB was observed.

Conclusion: Our study documents a high frequency of both high level (6.6%) and low level clonal *ALK* mutations (4.9%) in high-risk NB patients. These findings are of clinical importance given the potential role of *ALK* mutations in clonal evolution, and the possibility of *ALK*-targeted therapy.

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I-131-meta-iodobenzylguanidine therapy improves survival in high-risk neuroblastoma patients with mIBG positive residual metastatic disease

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Background: Randomized trials on the first-line treatment of high-risk neuroblastoma are limited. Therefore, we analyzed the national data base on the impact of I-131-meta-iodobenzylguanidine (mIBG) therapy, local radiotherapy, and single agent ch14.18 immunotherapy in the first-line therapy of high-risk neuroblastoma.

Methods: Patients of two consecutive national neuroblastoma trials were included if they met all key criteria: (1) stage 4 neuroblastoma, (2) age at diagnosis 18 months or older, (3) N5/N6 induction chemotherapy, (4) non-progressing residual mIBG positive metastatic disease after induction chemotherapy prior to myeloablative chemotherapy and autologous stem cell transplantation, (5) diagnosis between 1997 and 2012. mIBG therapy was scheduled for non-progressing MIBG positive lesions. Local radiotherapy 36-40 Gy was scheduled for mIBG positive residual at the primary site. Single agent ch14.18 was stratified according to open trials.

Results: A total of 232 patients were analyzed. The median observation time was 8.8 years. mIBG positive residual metastases were present prior to MAT in 92 patients. The median interval between mIBG therapy and stem cell transplantation was 21 days. The median mIBG activity applied was 7.4 GBq. The event-free survival was very similar between patients who underwent mIBG therapy (5yEFS 32.2+/-7.1%) compared to no mIBG therapy (5yEFS 27.9+/-6.9%, p=0.553). In contrast, a trend for better overall survival was found after mIBG therapy compared to no mIBG therapy (5yOS 58.1+/-7.6% vs. 38.9+/-7.5%, p=0.086). Multivariable analysis including MYCN, mIBG therapy, local radiotherapy, and immunotherapy with ch14.18 revealed an independent impact of MYCN amplification (p=0.028, hr 1.987) and ch14.18 treatment (p=0.004, hr 0.441) on EFS, and MYCN amplification (p<0.001, hr 3.476), ch14.18 treatment (p=0.008, hr 0.560), and mIBG therapy (p=0.047, hr 0.426) on OS.

Conclusions: mIBG therapy can improve survival in patients with incomplete metastatic response to induction chemotherapy. These results warrant a prospective multicenter trial on mIBG therapy. Moreover, this analysis confirmed the efficacy of immunotherapy with ch14.18.

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Did we improve results in infants with MYCN Amplified Neuroblastoma? Comparison of treatment strategy and outcomes in INES 99.4 and HR-NBL1/SIOPEN. A SIOPEN Study

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Background/Introduction:

Infants with MYCN amplified (MYCN-A) neuroblastoma treated in INES 99.4 (Canete et al, JCO 2009) showed a very dismal prognosis. Therefore, SIOPEN decided to submit these infants to the HR-NBL1/SIOPEN intensified treatment.

Methods and population:
94 MYCN-A infants (58 males, 36 females) were considered eligible, (75% stage 4; 13% stage 2-3, 12% 4s) with a median age of 8.5 months. All received induction chemotherapy (IC: Rapid-Cojec ± TVD) followed by BuMel/SCT, primary site surgery, radiation (21Gy) and 13-cisRA as maintenance. 35 received antiGD2 ch14.18/CHO treatment since 2010. Median follow up time is 3.9 years. In 99.4 trial, 35 patients (20 males, 15 females; median age 7,6 months) were analyzed (69% stage 4, 28% stage 4s and 3% stage 3). Treatment consisted in standard IC (carboplatin&etoposide-CADO), surgery, BuMel/SCT, local radiotherapy, 13-cisRA maintenance (JCO 2009).

Results: For HR-NBL1, 85% of evaluable cases (n=80) achieved CR/VGPR/PR following IC. Six infants went off treatment, one had PD whilst five had less than PR. The EFS and OS rates are 0.44±0.06 and 0.48±0.06 for all 94 MYCN-A infants. The 5 yr EFS and OS rates were 0.4 and 0.45 for stage 4; 0.51 and 0.51 for stage 2&3, 0.58 and 0.65 for stage 4s (NS). The main cause of death remained progression/relapse, while only 4 cases were due to toxicity. For 99.4 population, 30% of patients progressed/did not respond to IC, with median time to progression of 4 months. The 2y-OS was 28% (SD: 0.15), 2y-EFS 24% (SD:0,11) , 12 months median survival time.

Conclusions: Rapid-Cojec resulted in less disease progressions and better response rate than 99.4 IC. Although OS and EFS improved in HR-NBL1/ SIOPEN as compared to 99.4, it is below 50% with lack of disease control being the main cause of death. The number of infants treated with ch14.18/CHO is too small to conclude currently and will be further addressed.

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Myeloablative busulfan/melphalan (BuMel) consolidation following induction chemotherapy for patients with high-risk neuroblastoma. A Children's Oncology Group (COG) study.

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Background: The COG conducted a groupwide study of a Busulfan/Melphalan (BuMel) myeloablative regimen in patients with newly diagnosed, high-risk neuroblastoma (ANBL12P1). Previously used in SIOPEN studies, this is the first trial using BuMel following a COG induction platform. The primary objective was regimen-related toxicity, with a specific focus on pulmonary and hepatic events.
Methods: Five cycles of induction were administered, followed by intravenous busulfan (daily, days -6 to -3), melphalan (140mg/m2, day -1) and stem cell rescue. Age and weight based dosing were used for busulfan administration. First dose busulfan pharmacokinetics were mandated and adjustments made to target an AUC
Results: Between 4/2013 and 4/2015, 150 patients were enrolled. One hundred thirteen patients were evaluable for end-induction response assessment, with 27 (25%) CR, 27 (24%) VGPR and 39 (35%) PR, for an overall response rate of 82%. At the time of consolidation, 101 patients are evaluable for toxicity. The incidence of unacceptable pulmonary toxicity was 3.0% (n = 3), SOS 5.9% (n = 6), and combined hepato-pulmonary toxicity 8.9% (N = 9) during consolidation (days 0–28). There were 0 toxic deaths during consolidation. For all subjects (n=98), the median busulfan AUC was 3554 (range: 2360-4555) micromole/liter*minute, with a median AUC of 4558 (range: 3462-5189) micromole/liter*minute for those developing SOS (n =6) and 3232 (range: 3010-5037) micromole/liter*minute for those developing severe pulmonary toxicity (n= 3).
Conclusion: BuMel following COG induction regimen is well tolerated with acceptable pulmonary and hepatic toxicity in high-risk neuroblastoma.

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DFMO maintains remission and increases overall survival in high risk neuroblastoma: results of a phase II prevention trial

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Background: High Risk Neuroblastoma (HRNB) remains a challenge in pediatric oncology, accounting for 15% of all pediatric cancer deaths. While most patients are able to attain remission, the natural history of HRNB is well documented with approximately half of patients relapsing within 5 years after completion of immunotherapy. This study evaluated the effectiveness of the ODC inhibitor

difluoromethylornithine (DFMO), which targets cancer stem cell pathways in HRNB, as a maintenance therapy to prevent relapse in HRNB patients who were in complete remission at the completion of standard therapy.
Methods: This study was an open label, single agent, multicenter study. Enrollment began in June 2012 and ended in February 2016. Subjects received 27 4-week cycles of oral DFMO at a dose of 500-1000 mg/m^2 twice daily. Event free survival (EFS) and overall survival (OS) were determined on an intention-to-treat (ITT) basis.
Results: A total of 94 subjects received DFMO, 91 were eligible for the intention to treat (ITT) population. For all ITT subjects, EFS was 91% (± 4%) and OS 98% (+/-2%) at 2 years. For the subgroup of subjects (n=74) who were previously enrolled on the ANBL0032 study, the 2 year EFS was 95% (± 3%) and OS 98% (+/-2%). This is a significant improvement in comparison to ANBL0032 study which showed a conservative EFS of 76% 2 years post antibody therapy (p <0.01) and OS of 89% (p
Conclusions: Administration of DFMO at 500-1000mg/m^2 BID is an effective and safe dose. Following the completion of standard therapy for high risk neuroblastoma DFMO treatment was associated with improved EFS and OS decreasing the high rate of relapse in children with HRNB. An additional prospective trial is ongoing to confirm these results.

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The benefit of myeloablative chemotherapy with autologous stem cell transplantation in high-risk neuroblastoma patients is stable during long term follow-up. Results of the NB97 trial.

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Background: Randomized trials have demonstrated improved survival of high-risk neuroblastoma patients after myeloablative chemotherapy (MAT) with autologous stem cell transplantation (ASCT) compared to oral continuation chemotherapy (CC). This effect, however, could either be due to better cure rates or to delay of relapses. Therefore, we have re-analyzed the patient cohort of the randomized trial NB97 in order to find whether the survival benefit of MAT and ASCT is stable during long term follow-up.
Methods: Patients with stage 4 neuroblastoma older than 12 months and all patients with MYCN amplification were eligible. Treatment consisted of six-cycle induction chemotherapy, primary site tumor resection, consolidation either with MAT/ASCT or CC, MIBG therapy for MIBG avid residual disease, radiation therapy for active local disease present after operation, and post-consolidation therapy either with single drug anti-GD2 antibody ch14.18 or with 13-cis-retinoic acid. Outcome was analyzed by logrank test and Cox regression analysis.
Results: A total of 295 patients were randomized. The median observation time was 11.6 years. The 10 year event-free survival (10yEFS) was 40.0+/-4.0% in 149 patients randomized for MAT and 29.4+/-3.8% in 146 patients randomized for CC (p=0.027). The 10 year overall survival was 55.2+/-4.1% and 44.6+/-4.2% in patients randomized for MAT and CC, respectively (p=0.077). The last relapse occurred 12.7 years after diagnosis, so far. In the subgroup of stage 4 patients >18 months at diagnosis randomization for MAT was associated with better EFS (p=0.023) and a trend for better OS (p=0.098). Multivariable analysis identified stage, MYCN status, age, MAT, and treatment with ch14.18 as independent prognostic factors for EFS and also for OS.
Conclusion: Intensive multimodality treatments can achieve survival rates of 50% in high-risk neuroblastoma patients. The benefit of MAT with ASCT is due to improved cure rates and not to delay of relapses.

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Catecholamine metabolites: novel diagnostic insight, correlations with biological features and prediction of clinical outcome in patients with neuroblastoma

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Introduction: neuroblastoma accounts for 10% of pediatric malignancies and is responsible for 15% of pediatric cancer-related deaths. Vanillylmandelic acid (VMA) and homovanillic acid (HVA) are commonly analyzed in urine of neuroblastoma patients, however, their diagnostic sensitivity is quite low and their correlation with clinical outcome is still controversial. Other catecholamines metabolites have hardly been studied and for diagnostic purposes, analysis of a panel of catecholamine metabolites might be more accurate. Therefore, we performed in-depth analysis of the diagnostic sensitivity of catecholamine metabolites at time of diagnosis and their correlation with clinical outcome.
Patients and methods: retrospective study of urinary metabolites (VMA, HVA, 3-methoxytyramine, dopamine, epinephrine, metanephrine, norepinephrine and normetanephrine) from 301 neuroblastoma patients at diagnosis.
Results: normetanephrine was the most sensitive diagnostic metabolite with sensitivity of 89%, improving to 95% when all 8 metabolites were combined. Especially 3-methoxytyramine and

dopamine correlated with clinical and biological neuroblastoma features such as INSS stage and *MYCN* amplification. HVA and 3-methoxytyramine were significant independent risk factors for event free survival and overall survival. Patients with elevated 3-methoxytyramine had worse 5-years event free survival (29.2% vs. 76.3%, $p < 0.001$) and overall survival (35.5% vs. 84.1%, $p < 0.001$). Elevated 3-methoxytyramine also correlated with outcome within clinical subgroups such as patients with stage 4 disease (5-year OS: 25.0% and 58.3%, $p < 0.001$). In addition, patients with elevated 3-methoxytyramine had a worse prognosis regardless of their HVA status. **Conclusions:** our study demonstrates that analysis of a panel of urinary catecholamine metabolites panel, comprising 8 markers, during diagnostic work-up ensures the highest diagnostic sensitivity and can assist in estimating the clinical outcome.

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High frequency of Cytosine to Adenine mutations in neuroblastoma correlates with genomic aberrations in 8-Oxo-Guanine repair pathway

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Background
Somatic mutations can be grouped into 96 tri-nucleotide patterns, termed mutational signatures. These signatures can provide insights into underlying processes that fuel the evolution of tumor cells, and may expose therapeutic intervention avenues. Here we investigated the mutational signature of 84 whole genome sequenced neuroblastoma cases.
Results
Mutational signature analysis revealed an extreme bias towards Cytosine to Adenine (CtoA) mutations in a subset of tumors. This seems to be a unique feature of neuroblastoma. CtoA mutations are primarily found in high stage tumors and are therefore associated with a poor prognosis. Oxidative stress is known to be a source for such mutations, which can accumulate due to defects in the 8-Oxo-Guanine repair pathway genes OGG1, MUTYH and MTH1. We could show a strong correlation between CtoA mutations and chromosomal loss of OGG1, MUTYH. Besides copy number losses, sequencing also revealed a tumor with a homozygous inactivation of OGG1 and this tumor showed a very strong bias towards CtoA mutations. We could show that neuroblastoma cell lines with loss of either OGG1, MUTYH, or both, had higher 8-Oxo-Guanine content than cell lines without these losses. 8-Oxo-Guanine content is also correlated with CtoA mutation frequency in short term cultured neuroblastoma organoids. Overexpression of OGG1 in neuroblastoma cell lines with losses of OGG1 leads to rescue of the phenotype and a decrease in the amount of 8-Oxo-Guanine in the DNA. We are currently testing the efficacy of compounds that further interfere in this specific branch of DNA damage repair including the newly developed MTH1 inhibitors.
Conclusion
We identified a subset of neuroblastoma tumors with a high CtoA mutation frequency, which correlates with losses of glycosylases involved in the repair of CtoA mutations. 8-Oxo-guanine levels are elevated in cell lines with loss of OGG1, which can be rescued by overexpression of OGG1.

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The FOXM1 target gene BIRC5 (survivin) is a top ranked dosage sensitive gene located on the common large copy number 17q gained segment in neuroblastoma.

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Neuroblastoma can be regarded as a copy number disease. Mutation burden is low while both focal and large copy number imbalances are observed in nearly all cases. Amongst these, 17q gain is the most frequent in high stage tumors. In the TH-MYCN mouse model we observed dynamic upregulation of multiple 17q genes as measured in superior cervical and celiac ganglia and and/or full blown tumors which were harvested from TH-MYCN and wild type mice 1, 2 and 6 weeks after birth. Finally, for several of these genes we also observed strong negative correlation between expression levels and survival. These genes included TOP2A, BIRC5 and BRIP1 ranked as 3rd, 5th and 8th highest upregulated 17q genes, all of which are *bona fide* FOXM1 target genes. FOXM1 was recently identified as a major predictor of adverse outcomes across several human cancer types while we found FOXM1 as part of an embryonal stem cell signature marking patients with ultra high risk neuroblastoma. In keeping with this, the FOXM1 target gene BIRC5, implicated in cell division and apoptosis, is highly expressed during embryonic development as well as in several cancer types and correlated with poor survival in neuroblastoma patients. To establish the *in vivo* role of BIRC5, we created a BIRC5 stable zebrafish line by injection of *dβh-BIRC5* in wild type zebrafish. While stable BIRC5 overexpressing lines did not develop tumors, our first results of injection of *dβh-BIRC5* overexpression constructs in the offspring of dβh-MYCN zebrafish are indicative for accelerated neuroblastoma development. Also, BIRC5 inhibitors are already in clinical trials and currently we are testing for synergistic effects with TOP2A and BRIP1 inhibitors *in vitro* and *in vivo*. In conclusion, we provide evidence for dosage effects for several 17q genes as candidate

cooperative drivers during neuroblastoma development and provide a novel *dβh-BIRC5* overexpressing zebrafish model for future studies towards unraveling the enigmatic role of 17q gain in neuroblastoma biology and clinical behaviour.

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Identification of novel small molecule compounds to restore sensitivity to trophic factor withdrawal in MYCN-initiated death resistant cells

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Embryonal cancer arises from postnatally persistent embryonal remnant or "rest" cells that are uniquely characterised by the absence of p53 mutations (1). We have shown that perinatal neuroblastoma precursor cells exhibit a transiently diminished p53 response to MYCN oncoprotein stress and resistance to trophic factor withdrawal, in the TH-MYCN transgenic mouse model of neuroblastoma (2).

We performed high-throughput screening to identify novel compounds which restore the death response in MYCN-expressing precursor B lymphocytes, which demonstrates trophic factor Interleukin 7 (IL-7) independent growth *in vitro* and tumour growth a xenograft animal model. From an initial library of 40,000 compounds screening, we generated a short-list of 56 compounds which displayed cytotoxic activity against MYCN-expressing pre-B cells in the absence of IL-7, but had minimal effect on cells in the presence of trophic factor. Dose response curves were used to further refine the list to 5 lead hit compounds in the absence of IL-7 (IC50: 2.70 to 8.41μM), and more than 20μM for all controls including MYCN-expressing pre-B cells, normal pre-B cells in IL-7, and two normal human fibroblast cell lines. Two top compounds demonstrated marked differences in cytotoxic effect, with PB-798 causing cell cycle arrest and PB-370 inducing cell death. We have generated 14 PB-370 analogues for further investigation as PB-370 displayed the greatest difference in activities between the MYCN-expressing pre-B cells in the presence and absence of IL-7. Analysis of apoptotic genes from a PCR array and immunoblots of MYCN-expressing pre-B cells, in the presence and absence of IL-7 treated with PB370, revealed in increase expression of TP53 and Caspase 3/7/8, and decrease of MYCN protein expression. The mechanisms of cell death initiated by PB370 provide valuable insight into chemical inhibition of abnormally death persisting cells and a rationale for investigation into drug efficacy in MYCN-driven neuroblastoma mouse models.

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Metronomic Topotecan impedes tumor growth of *MYCN*-amplified neuroblastoma cells *in vitro* and *in vivo* by therapy induced senescence

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Background: Poor prognosis and frequent relapses are major challenges for patients with high-risk neuroblastoma (NB), especially when tumors show *MYCN* amplification. High-dose chemotherapy triggers apoptosis, necrosis and senescence, a cellular stress response leading to permanent proliferative arrest and a typical senescence-associated secretome (SASP). SASP components reinforce growth-arrest and act immune-stimulatory, while others are tumor-promoting. We evaluated whether metronomic, i.e. long-term, repetitive low-dose, drug treatment induces senescence *in vitro* and *in vivo*. And importantly, by using the secretome as a discriminator for beneficial versus adverse effects of senescence, drugs with a tumor-inhibiting SASP were identified.
Methods and Results: We demonstrate that metronomic application of chemotherapeutic drugs induces therapy-induced senescence, characterized by cell cycle arrest, $p21^{WAF/CIP1}$ up-regulation and DNA double-strand breaks selectively in *MYCN*-amplified NB. Low-dose topotecan (TPT) was identified as an inducer of a favorable SASP by protein array, while lacking NFKB1/p50 activation. In contrast, Bromo-deoxy-uridine induced senescent NB-cells secret a tumor-promoting SASP and this was dependent on NFKB1/p50 activation as shown by siRNA mediated knock-down and pharmacological inhibition. Importantly, TPT-treated senescent tumor cells act growth-inhibitory in a dose-dependent manner on non-senescent tumor cells and MYCN expression is significantly reduced *in vitro* and *in vivo*. Furthermore, in a mouse xenotransplant-model for *MYCN*-amplified NB metronomic TPT leads to senescence selectively in tumor cells, complete or partial remission, prolonged survival and a favorable SASP.
Conclusion: This new mode-of-action of metronomic TPT treatment, i.e. promoting a tumor-inhibiting type of senescence, is clinically relevant as metronomic regimens are increasingly implemented in therapy protocols of various cancer entities and are considered as a feasible maintenance treatment option with moderate adverse event profiles.

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High MYCN, low MYC, low CERS4, and low anti-apoptotic BCL2 gene family expression are associated with sensitivity to fenretinide in neuroblastoma cell lines and PDXs

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Introduction: Fenretinide (4-HPR) is a cytotoxic retinoid that acts via induction of reactive oxygen species (ROS) and dihydroceramide synthesis. 4-HPR-LXS oral powder has achieved durable complete responses in recurrent neuroblastoma. We sought to identify molecular mechanisms of 4-HPR sensitivity and resistance in neuroblastoma.

Methods: Fenretinide cytotoxicity profiles were assessed *in vitro* in 150 STR-validated, tyrosine hydroxylase-positive, EBV-negative continuous NB cell lines (CL) and *in vivo* using 6 subcutaneous xenograft models (3 PDXs, 3 CL-xenografts) in nu/nu mice treated with 4-HPR-LXS oral powder + ketoconazole. Highly sensitive, intermediate, and resistant CL sub-groups were categorized via an algorithm (SENS-RES) employing maximum observed cytotoxicity and calculated IC_{50} values from DIMSCAN cytotoxicity assays. ROS was by flow cytometry and RNA expression by RT-qPCR.

Results: Of the 150 CLs, 23 were classified as sensitive and 19 resistant to 4-HPR by SENS-RES. Even in the most resistant CLs, 4-HPR demonstrated >1-log cytotoxicity at clinically achievable 30μM. CLs with high multi-drug resistance to DNA damaging agents were not cross-resistant with 4-HPR. Age, INSS stage, clinically documented MYCN status, prior therapy, p53-function, MDR1/MRP1 expression, or ROS induction by 5μM 4-HPR were not correlated with sensitivity status, although multi-fold increased ROS was observed in several sensitive CLs. 4-HPR-sensitive CLs demonstrated higher MYCN ($p=0.043$) and lower MYC ($p=0.021$) expression. Comparison of the most highly sensitive (3CLs, 1PDX) and resistant (3CLs, 1PDX) models also demonstrated lower ceramide synthase 4 (CERS4) ($p<0.0001$) and lower MYC ($p<0.05$) expression in the most sensitive models. Ceramide-related genes CERS2, SPHK1, or SPHK2 expression levels were not different between the groups while BCL2 ($p=0.011$), MCL1 ($p=0.021$), and BCLxL/S ($p<0.0001$) expression was lower in the sensitive group.

Conclusion: High MYCN and low MYC, CERS4, BCL2, MCL1, and BCLxL/S expression are characteristics of neuroblastoma CLs and PDXs that are highly sensitive to 4-HPR.

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Preclinical assessment of CD171-directed CAR T cell adoptive therapy for childhood neuroblastoma: CE7 epitope target safety and product manufacturing feasibility

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Background: The identification and vetting of cell surface tumor restricted epitopes for chimeric antigen receptor (CAR) redirected T cell immunotherapy is the subject of intensive investigation. We have focused on CD171 (L1-CAM), an abundant cell surface molecule on neuroblastomas, and, specifically, on the glycosylation dependent tumor-specific epitope recognized by the CE7 monoclonal antibody.
Methods: CD171 expression was assessed by IHC using CE7 mAb in tumor microarrays of primary, metastatic, and recurrent neuroblastoma, as well as human and rhesus macaque tissue arrays. The safety of targeting the CE7 epitope of CD171 with CE7-CAR T cells was evaluated in a pre-clinical rhesus macaque trial on the basis of CD171 homology and CE7 cross reactivity. The feasibility of generating bioactive CAR T cells from heavily pretreated pediatric patients with recurrent/refractory disease was assessed.
Results: CD171 is uniformly and abundantly expressed by neuroblastoma tumor specimens obtained at diagnoses and relapse independent of patient clinical risk group. CD171 expression in normal tissues is similar in humans and rhesus macaques. Infusion of up to $100x10^6$ $CE7\text{-}CAR^+$ CTLs in rhesus macaques revealed no signs of specific on-target off-tumor toxicity. Manufacturing of lentivirally transduced $CD4^+$ and $CD8^+$ CE7-CAR T cell products under GMP was successful in 4 out of 5 consecutively enrolled neuroblastoma patients in a phase I study. All four CE7-CAR T cell products demonstrated *in vitro* and *in vivo* anti-tumor activity.
Conclusion: Our preclinical assessment of the CE7 epitope on CD171 supports it's utility and safety as a CAR T cell target for neuroblastoma immunotherapy.

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Phase II clinical trial with long-term infusion of anti-GD_2 antibody ch14.18/CHO in combination with interleukin-2 (IL2) showed clinical efficacy and improved toxicity in patients with high risk neuroblastoma.

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Background: A new delivery method of anti-GD_2 antibody ch14.18/CHO by long term infusion (LTI) may improve the toxicity profile but maintain effective immune modulation and clinical activity in patients (pts) with high risk relapsed/refractory neuroblastoma (NB).
Methods: 124 pts were enrolled into an open label Phase II clinical trial (EudraCT 2009-018077-31) to receive up to 5 cycles of $6x10^6$ IU/m^2 sc IL2 (d1-5; 8-12), LTI of 100 mg/m^2 ch14.18/CHO (d8-17) and 160 mg/m^2 oral 13-cis-RA (d19-32)(APN311-202). Primary efficacy endpoints were antibody (>1µg/ml) and NK-cell levels. Toxicity endpoint was i.v. morphine free antibody delivery after 5 days of cycle 1 in >80% patients. Secondary endpoints were increased ADCC levels over baseline, Fc gamma receptor (FCGR) polymorphisms, objective clinical responses and progression as well as overall survival.
Results:
All per protocol treated patients met primary efficacy endpoints. This translated into an increase in ADCC in all evaluable patients. Toxicity observed in this trial was improved compared to standard delivery methods of anti-GD_2 antibodies. Objective clinical response rate observed in this trial was 40%. The survival update of the cohort revealed a 2-y OS of 64±6% (mean OS 2.7± 0.2 y, median EFS 3.7 y (95% CI: 2.0-3.7 y)) and a 2-y EFS of 53 ± 6% (mean EFS 2.0 ± 0.2 y, median EFS 2.3 y (95% CI: 1.2-3.3 y)). This result is clearly superior to historical controls not treated with ch14.18/CHO (p<0.001), indicating clinical efficacy of the treatment. In this cohort, we found 63/124 pts with low affinity FCGR alleles (FCGR2A-H131R/R and/or FCGRA3A-V158 F/F). These patients showed lower EFS and OS rates compared to 59/124 patients with high affinity FCGR polymorphisms (p<0.05). These findings underline FCGR mediated ADCC as mechanism of action of this treatment modality.
Conclusion: Results of this Phase II study show efficacy, clinical activity and improved toxicity profile of a new delivery method of ch14.18/CHO and underline ADCC as the mechanism of action.

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Humanized anti-gd2 antibody (hu14.18k322a) with Chemotherapy +/- parental natural killer (nk) cells in children with recurrent/ refractory Neuroblastoma

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Purpose: Preclinical studies demonstrate that anti-GD2 antibodies, acting via antibody-dependent cell-mediated cytotoxicity (ADCC), enhance the effects of chemotherapy. We conducted a safety trial of a fixed dose of hu14.18K322A and 3 different chemotherapy combinations with parental NK cells to enhance ADCC.
Methods: Children with recurrent/refractory neuroblastoma received up to six courses of hu14.18K322A (40mg/m^2/dose, days 2-5) with GMCSF and low-dose IL-2 in combination with chemotherapy: cyclophosphamide/topotecan (courses 1,2), irinotecan/temozolomide (courses 3,4) and ifosfamide/carboplatin/etoposide (courses 5,6). Parental NK cells were administered with courses 2, 4 and 6. Serial serum hu14.18K322A pharmacokinetic studies and soluble IL-2 receptor (sIL2R) levels were obtained during each course.
Results: Thirteen heavily pretreated patients (5 males; median age 5.4 yrs; range 1.9-1 - 3.5yrs), including 9 with prior anti-GD2 treatment, completed 65 courses. One patient developed an unacceptable toxicity (grade 4 thrombocytopenia >35 days). Four patients discontinued treatment for adverse events (hu14.18K322A allergic reaction, viral infection, surgical death and myelodysplastic syndrome). Common toxicities included grade 3/4 myelosupression (13/13 patients) and grade 1/2 pain (13/13 patients). Eleven patients received 29 NK cell infusions. The median number of NK cells infused per dose was $15.5x10^6$/kg (range, $4.7x10^6$/kg - $59.5x10^6$/kg). All patients had an increase in sIL2R levels, indicative of immune activation. The overall response rate was 61.5%: 4 complete responses, 1 very good partial response, 3 partial responses and 5 with stable disease. The median time to progression

was 256 days (range 33 - 553days); one-year overall survival was 75.5% (95% C.I., 50.9 - 100%). Median hu14.18K322A α (initial phase) half-life was ~27hours.
Conclusions: Administration of chemotherapy with Hu14.18K322A and parental NK cells is safe, feasible and resulted in clinically meaningful responses in patients with refractory/recurrent neuroblastoma. Further studies are warranted of this approach in newly diagnosed patients.

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Tumor-associated macrophage polarization state and the dynamic nature of PDL1 expression in neuroblastomas

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Background: Tumor-associated macrophages (TAM) have prognostic significance in tumor microenvironment (TME) of neuroblastomas lacking MYCN amplification (MYCN-NA). However, little is known about the regulation of immune checkpoint expression or polarization state of macrophages in neuroblastomas.
Methods: Neuroblastomas microarrays (n=160) were analyzed using CIBERSORT to estimate relative proportion of immune cells in TME. Multiplex-immunofluorescence of CD163 (macrophages) and PDL1 (immune-checkpoint) was performed on tumor tissues (n=45). Polarized human macrophages (M1, M2a, or M2c) were characterized alone and in co-cultures with neuroblastoma cell-lines. PDL1 and B7H3 were evaluated at baseline for each cell type, post 24-hour incubation with IFNγ, and after 24-hour of co-culture.
Results: A serum-free *in vitro* macrophage polarization platform was used to create and characterize distinct macrophage phenotypes: M1 ($CD163^{dim}/CD206^{dim}/B7H3^{mod}/PD1^{high}$), M2a ($CD163^{dim}/CD206^{high}/B7H3^{high}/PDL1^{mod}$), and M2c ($CD163^{high}/CD206^{low}/B7H3^{low}/PDL1^{low}$). IFNγ exposure caused upregulation of PDL1 in M2a and M2c macrophages without altering polarization markers. Neuroblastoma cell-lines, which at baseline have $PDL1^{low}/B7H3^{mod}$ phenotype, also upregulated their PDL1 expression in response to IFNγ exposure. In co-culture experiments, neuroblastoma cell-lines upregulated PDL1 in presence of the IFNγ-induced M1 macrophages. Microarray CIBERSORT analyses showed high proportion of M2 macrophages, but not M0 or M1, in MYCN-NA tumors; this correlated highly with CD163 expression (r=0.59), but not PDL1 expression (r=0.2). Multiplex-immunofluorescence analyses of human tissues confirmed presence of $CD163^{+}$ TAM, but no evidence of PDL1/CD163 co-localization was identified.
Conclusions: While PDL1 positivity was observed in subset of neuroblastomas, TAM did not express PDL1. The high CD163, low PDL1 pattern suggests TAM have M2c phenotype. M2c macrophages and neuroblastoma cells, however, can robustly upregulate PDL1 in face of anti-tumor immune response, as mimicked by IFNγ exposure. Our results suggest that lack of PDL1 expression on tumors may not be a reliable estimate of response to anti-PD1 therapies and alternate biomarkers should be developed.

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Engineered Neuroblastoma Cellular Immunotherapy (ENCIT)-01: A phase 1 study of autologous T-cells lentivirally transduced to express CD171-specific Chimeric Antigen Receptors (CAR) for recurrent/refractory high-risk neuroblastoma (HR-NB)

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Background. Anti-ganglioside (GD2) antibody immunotherapy improves outcome for HR-NB but 35% of patients recur and off-tumor targeting of neuronal tissue may limit use of GD2 for novel immunotherapy such as CAR T cell therapy. L1-CAM (CD171) is expressed by neuroblastoma and mCE7 recognizes a tumor-specific glycosylated epitope of L1CAM, offering an alternative immunotherapy target for HR-NB.
Methods. Pts with recurrent HR-NB were enrolled to examine the safety and feasibility of administering autologous, peripheral blood (PB)-derived T cells genetically modified using a SIN lentiviral vector to express either scFv-IgG4hinge-CD28tm-4-1BB-zeta (second generation (2G)) or CD171-specific scFv-IgG4hinge-CD28tm/cyto-4-1BB-zeta) (third generation (3G)) CD171 (L1CAM)-specific CAR and the selection/suicide construct EGFRt. Patients received lymphodepleting chemotherapy followed by cryopreserved $CD4/EGFRt^{+}$ and $CD8/EGFRt^{+}$ CARs administered at a 1:1 ratio at each dose level. The MTD will be determined for each construct.
Results. From 11/2014 through 1/2016, 14 patients enrolled with CAR manufacturing successful in 12 patients. Seven patients (8.3 years at enrollment; range 7.1-18.7) were treated at dose level 1 ($1 X 10^6$ CAR T cells/kg, n=3-2G and n=1-3G); dose level 2 ($5 x 10^6$ CAR T cells/kg, n=1-2G) and dose level 3 ($1 x 10^7$CAR T cells/kg, n=1-2G). No CAR-associated toxicities have occurred. CAR T cell persistence was not documented by multi-parameter flow evaluation at Dose Level 1. Minimal PB persistence (< 0.5%) was detected at Dose Level 2 while persistence testing is underway at Dose Level 3. No objective responses were observed, 3 pts had stable disease (range 12 – 18 weeks).
Conclusions. It is feasible to harvest and manufacture CAR T cells from heavily pre-treated pts with HR-NB. In contrast to experience with the use of CD19-CAR T cell therapy, the initially planned T cell infusion doses

were insufficient to generate persistence in patients with solid tumors who do not have high burden for circulating antigen to stimulate T cell expansion. Higher cell dose cohorts and novel strategies to stimulate CAR expansion are underway.

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Phase II study of alisertib, irinotecan, and temozolomide in children with relapsed and refractory neuroblastoma: A report from the New Approaches to Neuroblastoma Therapy (NANT) consortium

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Background: In phase I testing in 22 patients with neuroblastoma, the maximum tolerated dose of alisertib tablets with irinotecan/temozolomide was 60 mg/m^2/dose. This combination showed significant activity [31.8% response rate; 52.4% 2-year PFS]. This study sought to: confirm the activity of this regimen; evaluate an alisertib oral solution (OS); and identify predictors of response and toxicity.
Methods: We conducted a two-stage phase II trial of alisertib tablets (60 mg/m^2/dose x 7 days), irinotecan (50 mg/m^2/dose IV x 5 days), and temozolomide (100 mg/m^2/dose orally x 5 days) in patients 1-30 years of age with relapsed/refractory neuroblastoma. The primary endpoint was best response. Secondary endpoints were toxicity, PFS, and pharmacokinetics. A cohort was treated with alisertib OS at 45 mg/m^2, a dose anticipated to be bioequivalent to 60 mg/m^2 tablets. Exploratory analyses evaluated predictors of response and toxicity using data from phase I, II, and OS cohorts.
Results: Twenty and 12 patients were treated in phase II and OS cohorts, respectively. Hematologic toxicities were the most common adverse events. In phase II, 4 responses (20%) were observed as well as 4 patients with minor responses. The 1-year PFS was 64.5%. In the OS cohort, 3 patients (25%) had first course dose-limiting toxicity (DLT). Two of 12 patients had objective responses (16.6%) and 1 had a minor response. The alisertib OS at 45 mg/m^2 had higher median Cmax (8.7 vs. 3.9 mM) and exposure (58 vs. 45 mM·hr) compared to tablets at 60 mg/m^2. Across 54 patients in all cohorts, there were trends to suggest that *MYCN* amplification and prior irinotecan treatment were associated with lower response rates. Alisertib exposure and trough concentrations were associated with higher rates of first course DLT.
Conclusions: Alisertib with irinotecan/temozolomide is an active combination. Alisertib oral solution at 45 mg/m^2 is tolerable in this context. Additional studies of biomarkers of response and toxicity are ongoing.

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Overview of neuroblastoma epidemiology and genetic predisposition

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Not available at time of printing

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Actionable genomic mutations

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Not available at time of printing

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Updates on the International Neuroblastoma Risk Group (INRG). Classification System and Interactive INRG Database (iINRGdb)

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The INRG Classification System, which was based on the analysis of over 8,800 patients, was established to advance treatment for children with neuroblastoma diagnosed around the world through international collaboration. The INRG Task Force recognized the potential value of this large patient cohort, and a process was developed to make the data available to the neuroblastoma research community. More than 20 research studies have been conducted, including a number of seminal studies analyzing rare cohorts, never before possible. In an effort to integrate genomic data, we have recently we transformed the original flat-file database into a queryable web-based format that is able to link to other databases (ie, the iINRGdb). We have also substantially expanded the clinical cohort in the iINRGdb, which now contains data on more than 17,800 patients. In addition, a cohort discovery tool has been built and is available to the public (http://inrgdb.org). Governance of data contribution and access is being refined, and an international effort is ongoing to catalogue and link existing neuroblastoma genomic. Germline genotype data generated in the Maris laboratory at the Children's Hospital of Philadelphia and array comparative genomic hybridization (CGH) data generated from patients enrolled on the SIOPEN infant trial are currently linked to the clinical cohort and are available to investigators for analysis. Genomic data generated through the Therapeutically Applicable Research to Generate Effective Treatments (TARGET) project will be linked in the near future. The iINRGdb will provide an unparralled resource to enable a deeper our understanding of the epidemiology of

neuroblastoma, expand our knowledge regarding the pathways that drive malignant growth and treatment resistance, and may ultimately lead to more effective, individualized therapies.

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Overview of treatment for low- and intermediate-risk patients

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For low and intermediate risk neuroblastoma, treatment strategies aim at delivering the minimum of therapy necessary in order to maintain an excellent survival, while diminishing treatment toxicity. For these patients, which comprise approximately one half of all newly diagnosed neuroblastoma, treatment intensity and length is adapted according to the clinical parameters stage, age, clinical symptoms, as well as tumor genetic features, with treatment ranging from observation only to more intensive treatment regimens with several courses of chemotherapy, surgical resection, radiotherapy and maintenance therapy. Fine-tuning of these treatment approaches will be based on further integration of genomic data, such as genomic copy number profiles or expression signatures. However with the discovery of more and more prognostic biomarkers, only large-scale integrative efforts will enable to combine this information to develop the clinically most meaningful and practicable approaches. It is likely that combining genomic prognostic and predictive biomarkers will lead to more precise treatment strategies.

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Whole exome and deep targeted sequencing of clinically aggressive neuroblastomas reveal recurrent somatic mutations in pathways involved in cancer progression

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The complete spectrum of somatic mutations of the most aggressive forms of neuroblastoma is still to be defined. Here we sought to identify additional potential cancer drivers in high-risk and ultra-high-risk (high-risk patients with any adverse event within 36 months from diagnosis) neuroblastoma.
Whole exome sequencing was performed for 17 ultra-high-risk germline and tumor pairs to identify somatic mutations and deep targeted sequencing of 134 genes selected from the initial screening in an additional set of 48 germline and tumor pairs (62.5% were ultra-high-risk and high-risk), 17 ultra-high-risk tumors and 17 human-derived neuroblastoma cell lines.
Combining both cohorts we found 22 significantly mutated genes, many of which implicated in cancer progression processes. Of these, fifteen (68.2%) were highly expressed in neuroblastoma supporting the biological rationale for their involvement in this malignancy. CHD9, annotated as cancer driver in public databases, was the most significantly altered gene (4.0% of cases) after ALK. Other genes (PTK2, NAV3, NAV1, LRRC17, PXDN, FZD1, ARHGEF10L and ATRX) expressed in neuroblastoma and involved in cell invasiveness and migration were mutated at frequencies between 4% and 2%. Pathways implicated in cell survival, proliferation and motility (focal adhesion and regulation of actin cytoskeleton) were the most frequently disrupted affecting 14.1% of cases, suggesting potential novel therapeutic strategies to prevent disease progression. Rare potentially pathogenic germline variants were significantly enriched in BARD1, CHEK2 and AXIN2.
To conclude, the combination of whole exome and deep targeted sequencing in a discovery and validation cohort experimental design, identified novel cancer genes in clinically aggressive neuroblastomas. Our analyses demonstrate that infrequently mutated genes may have pathway-level implications in leading tumor progression and suggests possible novel strategies for therapeutic interventions in aggressive forms of neuroblastoma.

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Identification of germline mutations in 776 children with neuroblastoma

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BACKGROUND. Genetic susceptibility to neuroblastoma is complex, and the contribution of rare germline mutations beyond ALK and PHOX2B is not known. Here, we report on our efforts to identify rare deleterious germline variants influencing tumorigenesis in sporadic neuroblastoma (NB).
METHODS. We conducted Complete Genomics whole-genome sequencing (106 high-risk tumor-germline) and Illumina exome sequencing (222 high-risk tumor-germline; 52 overlapping with whole-genome). A custom-capture 500-gene panel was sequenced in a representative validation cohort of 500 NB patients (germline-only) and compared to 1,001 cancer-free controls sequenced/analyzed by the same platform/pipeline. Rare variants were annotated using the database for non-synonymous functional predictions (dbNSFP), aggregated, and tested for enrichment compared to Exome Aggregation Consortium (ExAC). Indels were called by bam2MPG and Platypus in the 222 exome cohort and in 1000

Genomes as a control. We initially focused on known cancer predisposition genes and genes underlying syndromes associated with NB.
RESULTS. SNV calls showed high concordance in overlapping exome and whole-genome samples (average 90.4% per sample), and 119/119 (100%) of SNVs tested from non-overlapping cases verified by Sanger sequencing. We observed 131 potentially deleterious rare variants/mutations in known cancer predisposition genes that were either loss of function, or had a CADD score > 20 and predicted to be damaging by multiple algorithms. Considering these, 25/276 cases (9%) harbored known pathogenic mutations, many present in genes previously reported to be mutated in
NB: ALK (n=2), APC (n=3), BARD1 (n=3), BRCA1 (n=1), BRCA2 (n=2), CHEK2 (n=3), PALB2 (n=2), PHOX2B (n=1), PINK1 (n=1), NF1 (n=1), SDHB (n=1) and TP53 (n=1). In addition, we identified pathogenic variants in genes underlying syndromes associated with NB, including HRAS (n=1/276, Costello) and EZH2 (1/276, Weaver).
CONCLUSION. In this case series, we conservatively estimate ~10% of children with NB harbor a known pathogenic germline mutation in a cancer susceptibility gene. Validation sequencing is underway in the 500-sample cohort and evaluation of additional mutations, including non-coding, is ongoing.

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Fusion-transcripts are associated with an unfavorable phenotype in neuroblastoma

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Background: We aimed to systematically determine the spectrum of fusion-transcripts and their potential relevance for neuroblastoma pathogenesis.
Methods: We analyzed 498 primary tumors by RNA-sequencing. Fusion-transcripts were validated by dideoxynucleotide sequencing. Genomic rearrangements were determined in eleven samples by whole-genome-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 genes. In 16/20 cases, genomic rearrangements corresponding to the fusion-transcripts were identified, indicating that they mostly represent fusion-genes. We found that chromosomes 2, 12 and 17 were significantly enriched for genes involved in fusion-transcripts ($p<0.05$). Fifty-two and 45 fusion-transcripts were predicted to be potentially protein-coding and truncating, respectively. We observed that the 3'-partners of the fusion-genes had significantly higher expression levels in the index cases than their wild-type counterparts in the remaining cohort, suggesting that transcriptional dysregulation is a major effect of the underlying genomic event. Similar to SNV patterns in neuroblastoma, the spectrum of fusion-transcripts was heterogeneous, with most fusion-genes being private events. We noted, however, that fusion-transcripts were significantly enriched for genes annotated in the COSMIC database ($p<0.001$), and comprised genes previously associated with neuroblastoma (e.g., *FOXR1*, *HACE1* and *TERT*), pointing towards their potential mechanistic relevance. As an example, 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 patient subgroups, such as *MYCN*-amplified or high-risk patients.
Conclusions: Our data suggest that fusion-transcripts resulting from genomic rearrangements contribute to an unfavorable neuroblastoma phenotype.

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Clonal reconstruction in neuroblastoma shows enrichment of mutations in cell survival and DNA-repair pathways at relapse

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Neuroblastoma genomes show few recurrent somatic alterations except *MYCN* amplification and *ALK* mutations, this suggests that signaling pathways rather than specific genes are altered in neuroblastoma and participate in oncogenic clone expansion.

To investigate this hypothesis, for 22 neuroblastoma patients we applied whole genome sequencing to triplet libraries (germline DNA, diagnosis and relapse tumors; 15 data sets published previously). We characterized putative driver mutations as mutations present in genes from the cancer census list or from the pathways of the Atlas of Cancer Signaling Networks (ACSN); we restricted ASCN pathways to those enriched in mutations. Then we applied our method QuantumClone (https://cran.r-project.org/web/packages/QuantumClone/) to predict subclonal structure for the 22 triplet samples and assign somatic driver mutations to distinct subclones. In contrast to other existing approaches, QuantumClone uses copy number information when evaluating mutation cellular prevalence and assigning mutations to corresponding subclones. Therefore, on datasets with complex clonal architecture or medium read coverage, QuantumClone shows superior performance compared to other published algorithms. Application of QuantumClone to our data identified up to ten different clones in the diagnosis-relapse tumor pairs. Assignment of putative driver mutations to the reconstructed clones showed an enrichment of driver mutations in the relapse specific clones (p-value=0.0479, Fisher test). In particular, mutations in the apoptosis, cell survival and DNA repair pathways have been shown to be enriched at the relapse (Figure 1). Mutations in the MAPK pathway were enriched in the founding clone both at diagnosis and at relapse. Our analysis suggests evolution of alteration in distinct signaling pathways during neuroblastoma progression.

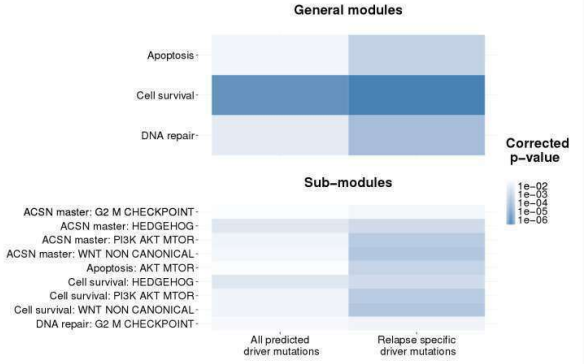


Figure 1: ACSN signaling pathways enriched for relapse-specific driver mutations. Color intensity corresponds to the p-value of the Fisher test of pathway enrichment; multiple test correction is done using the FDR method. Only modules with lower p-values for driver mutations in the relapse samples are shown.

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Chromosomal rearrangements juxtapose active enhancer elements to oncogenes in high-risk neuroblastoma

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Background: Exome sequencing studies revealed that neuroblastomas (NBs) harbor a low overall mutation rate with only few recurrently mutated genes leaving the molecular etiology of a large proportion of NBs elusive. In the present study, we applied a whole genome sequencing (WGS) approach to uncover structural rearrangements in noncoding regions that could potentially drive NB.

Methods: WGS was applied to search for structural rearrangements in NB tumors and cell lines. A protocol for chromatin immunoprecipitation sequencing (ChIP-seq) of tumors was established and used to identify active enhancer elements in NB. Circular chromatin conformation capture sequencing (4C-seq) was used to assay for physical promoter-enhancer interactions. Genomic excision of enhancer elements was performed by means of CRISPR/Cas9-techniques.

Results: WGS analyses revealed that chromosomal rearrangements are common in NB with the most frequent event encompassing the telomerase gene (*TERT*) which is rearranged in 25% of high-risk cases. ChIP-seq analyses confirmed that rearrangements

commonly juxtapose active enhancer elements to *TERT* and other oncogenes involved in translocation events in NB tumors and cell lines. 4C-seq analyses provided evidence for physical interactions of translocated enhancer elements with the promoters of the respective oncogenes. This is in line with elevated expression of the affected genes in rearranged cases. Currently, we test the functional relevance of enhancer translocations by CRISPR/Cas9-mediated excision of the enhancer elements in translocated NB cells. Furthermore, we test therapeutic concepts targeting enhancer-mediated gene deregulation with CDK7- or BET- inhibitors.

Conclusions: The study reveals that structural rearrangements in high-risk NB frequently juxtapose strong enhancers to oncogenes, leading to *de novo* physical promoter-enhancer interactions and overexpression of the oncogenes in rearranged cases. This mechanism of oncogene activation by enhancer hijacking may open a therapeutic window for epigenetic drugs, including BET inhibitors or CDK7 inhibition, in high-risk NBs.

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Molecular risk assessment of neuroblastoma patients eliminates the necessity of clinical prognostic markers

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Purpose: Current risk stratification systems for neuroblastoma patients consider clinical, histopathological and simple genetic variables. Recent studies, however, suggested that gene expression-based predictors may add significant value for prognostic classification. We here aimed to improve neuroblastoma risk assessment by developing a risk score that integrates hazard ratios of established prognostic variables and multigene expression-based predictors.

Patients and methods: We analyzed 695 neuroblastoma patients. The entire cohort was divided into training set I (n=75) for generation of multigene predictors, training set II (n=411) for risk score development, and a validation set (n=209). Prognostic variables were selected in a multivariable Cox regression analysis based on event-free survival (EFS) using the LASSO method, followed by subsequent backward selection. Selected variables and their hazard ratios were included into a prognostic index, which was then used for development of the final risk score.

Results: The variables stage, age, *MYCN* status and the multigene predictors NB_th24 and NB_th44 were independent prognostic markers in the LASSO analysis. In the subsequent backward selection procedure, only the two multigene predictors were retained in the final model. Integration of the two predictors in a risk scoring system identified three patient subgroups that differed significantly in their outcome both in the training (5-year-EFS, 83.2±2.6 vs. 64.8±9.0 vs. 32.0±4.0; p<0.001) and the validation cohort (5-year-EFS, 84.9±3.4 vs. 63.6±14.5 vs. 31.0±5.4; p<0.001). Multivariable analysis of risk groups defined by the current German NB2004 stratification system and the newly developed molecular risk score revealed only the molecular risk score as independent predictor for EFS.

Conclusion: We here propose a strategy for the development of neuroblastoma risk assessment that integrates prognostic variables based on hazard ratios. The final risk score considered only two multigene predictors, supporting the notion that clinical courses of neuroblastoma are precisely reflected by the molecular properties of the tumor cells.

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Genetic variants in *BARD1* and *KIF15* are associated with *MYCN*-amplification in neuroblastoma

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Background: Genome-wide association studies (GWAS) have identified validated neuroblastoma susceptibility loci associated with different tumor subtypes. Approximately 20% of neuroblastomas are *MYCN*-amplified, but it is unknown whether patients with *MYCN*-amplified tumors have different genetic predispositions than other patients. To investigate this, we performed a multi-ethnic GWAS on 763 *MYCN*-amplified cases and 3440 *MYCN*-nonamplified cases as controls.

Methods: Neuroblastoma patients with clinical and genomic information were obtained from the Children's Oncology Group. Genotypes from three cohorts typed on different Illumina platforms were subjected to quality control and imputation, and analyzed separately using SNPTEST. Meta-analysis was performed using a fixed-effects model in METAL.

Results: By meta-analysis, we identified two loci significantly associated with *MYCN*-amplification. The first was at the previously identified high-risk *BARD1* locus indexed by rs2070096 (P_{meta}=3.51×10^{-13}). The second was a novel locus at 3p21 indexed by rs112645395 (P_{meta}=8.83×10^{-13}). In GTEx, three variants in tight linkage (r^2>0.75) with rs112645395 are *cis*-eQTLs in tibial nerve for *KIF15* (P≤2.6×10^{-5}), a critical protein in mitotic pole separation, with the risk allele associated with decreased gene expression. We then used a subset of 1662 patients with recorded risk status to test if previously identified high-risk susceptibility loci differed by *MYCN* status. Variants in *BARD1* and *CASC15-S* were enriched in *MYCN*-amplified neuroblastoma but not *MYCN*-nonamplified

high-risk neuroblastoma (*BARD1*: $P_{rs2070096/MYCN\text{-}amp}=1.54\times10^{-5}$ vs $P_{rs2070096/MYCN\text{-}nonamp}=0.34$, *CASC15-S*: $P_{rs1543310/MYCN\text{-}amp}=7.90\times10^{-5}$ vs $P_{rs1543310/MYCN\text{-}nonamp}=0.10$). Conversely, *LMO1* was significant only in patients with *MYCN*-nonamplified high-risk tumors ($P_{rs110419/MYCN\text{-}amp}=0.38$ vs $P_{rs110419/MYCN\text{-}nonamp}=3.44\times10^{-5}$).

Conclusions: We demonstrate *MYCN*-amplified and *MYCN*-nonamplified high-risk neuroblastoma arise under the influence of distinct germline susceptibility alleles. These results also suggest the etiology of *MYCN*-amplified neuroblastoma may result from attenuated DNA repair and/or impaired mitotic chromosome segregation. Further functional analysis of *BARD1*, *CASC15-S*, and *KIF15* may provide insight into the pathogenesis of *MYCN*-amplification and identify potential therapeutic targets.

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Pharmacogenetics of treatment response in patients with high-risk neuroblastoma, a Children's Oncology Group study

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Background: While outcomes for patients with high-risk neuroblastoma have improved over time, largely through intensification of therapy, biologic factors predictive of high-risk treatment failure remain elusive. Furthermore, the role of germline genetic variation in treatment failure has not been extensively investigated.

Methods: Single nucleotide polymorphism (SNP) genotyping was performed on selected genes in the pharmacokinetic pathway of cyclophosphamide (CY) for children with high-risk neuroblastoma enrolled on the Children's Oncology Group high-risk neuroblastoma protocol, ANBL0532. Response after two cycles of topotecan/CY (ranging from complete response to progressive disease) was used as a continuous outcome and with dominant model scoring of the SNP genotype as the independent variable for the association studies, adjusting for age at diagnosis, race (as determined by 30 genotyped SNPs serving as ancestry informative markers), stage, *MYCN* status and histology.

Results: Of the 652 patients enrolled on ANBL0532, 303 participated in the pharmacogenetics aim. We tested 110 SNPs (in 37 genes) that were either tagging or had associations of interest previously reported. *ABBC1* and *ABCC4* had SNPs with nominal p-values < 0.01. After correction for multiple testing, two intronic SNPs in *ABBC1*, rs4148353 and rs17287570, were significantly associated with response to two cycles of topotecan/CY, p

Conclusions: *ABCC1*, is a key regulator of chemotherapeutic cellular efflux, including CY. Genetic variants in *ABCC1* which may alter the function of transporter impact outcome in children with high-risk neuroblastoma. Pediatric validation is underway in patients with rhabdomyosarcoma also treated with cyclophosphamide-containing regimens.

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Predicting "early" relapse/progression/death in children with INRGSS Stage M neuroblastoma using clinical and biologic factors: An INRG database analysis

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Introduction

Most high-risk neuroblastoma patients who relapse or progress quickly after starting frontline therapy will ultimately die. Identification of these patients upfront would allow early treatment with novel therapies. Our objective was to identify clinical and biologic factors prognostic of early relapse/progression/death ("early event").

Patients and Methods

From the INRG database, patients ≥18 months old diagnosed between 1998-2014 with INRGSS stage M disease were included. Patients were divided at random into a test set and a validation set to identify a cut-off to categorize patients as early event versus "later/no event". To define "early event" multiple cut-offs were tested at 30-day intervals from 30 to 547 days . Logistic regression identified clinical and biologic factors prognostic of an early event.

Results

1820 patients met inclusion criteria: 23% ≥5 years old, 35% *MYCN* amplified, 48% diploid, 39% 11q LOH, 39% 1p LOH, 11% LDH≥1400 U/L, 22% ferritin≥30 ng/mL, 33% high MKI, 3% Stage 4N (metastases exclusive to distant lymph nodes) and 9% liver metastases. Regardless of the cut-off tested, the risk of death was high (hazard ratio [HR]>5) for patients with an early event compared to later/no event. A 182-day cut-off (identified 8% of the cohort as "early events") was implemented. 5-year OS was 10±3% for patients with early events (n=150) and 46±1% for patients with later/no events (n=1670) (p<0.0001, HR=5.1 [95%CI: 4.2-6.1]). Factors prognostic of an early event were: *MYCN* amplification (p=0.03; odds ratio [OR]=1.5), LDH ≥1400U/L (p=0.04; OR=1.7) and liver metastases (p=0.02; OR=3.1).

Conclusions

MYCN, LDH, and liver metastases were determined to be strongly associated with early events in this INRG cohort. However, to precisely identify patients who

may benefit from alternative treatment approaches, a deeper understanding of the genomic alterations that drive rapidly progressive disease will be needed.

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Revised Children's Oncology Group (COG) risk stratification incorporating the international neuroblastoma risk group staging system

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Background: The COG risk classification system previously used the International Neuroblastoma Staging System (INSS). Because the INRG staging system (INRGSS) has been adopted for clinical trials we integrated INRG stage with biological and clinical prognostic factors to map patient categories, evaluate outcomes and develop a revised risk classification system. **Methods:** 4,255 newly diagnosed neuroblastoma patients were enrolled on COG Neuroblastoma Biology Study ANBL00B1 between 2006-2014. Staging per the INSS and INRG (using detection of Image Defined Risk Factor (IDRF)) was determined. Tumor biological and histologic features assessed in the centralized COG Neuroblastoma Reference lab included *MYCN* status, ploidy, INPC histology, and 1p and 11q LOH. Survival analyses were performed to identify independent prognostic factors and to calculate event-free and overall survival (EFS, OS) for combinations of variables used to determine risk group assignments according to both COG and INRG classification templates. **Results:** Using the COG risk classification 1,309 low-, 1007 intermediate- and 1,849 high-risk patients were identified. Concordance between INSS and INRG staging systems was higher for metastatic (4/M) as compared to loco-regional patients. 1,122 (67%) of loco-regional tumors had no IDRF (L1) and 545 (33%) had >1 IDRF (L2). Of the L1 patients 87% were INSS 1,2 while 61% of L2 patients were INSS 3. Subsets of L2 patients had sub-optimal outcomes (Table): **Discussion:** The COG revised risk classification will designate L2 patients >18mo with *MYCNA*, unfavorable INPC histology, and/or segmental chromosome aberrations as high risk. Efforts to identify additional prognostic biomarkers may enable further refinement of risk groups.

Stage	Age	MYC	SCA 1p or 11q LOH	Ploidy	INPC	3-yr EFS	3-yr OS	N
L1		Any	Any			92.0 ± 1.2	98.5 ± 0.5	1122
L1		Amp	Any			86.8 ± 6.9	97.4 ± 3.2	45
L2	Any	No	Known and No both	>1	FH	86.7 ± 3.5	100.0 ± 0.0	169
L2	<18 m	No	Any	Any	Any	86.2 ± 2.9	99.3 ± 0.7	276
L2	<18 m	No	Any	Any	UH	90.0 ± 14.2	90.0 ± 14.2	10
L2	<18 m	No	Any	1	Any	77.3 ± 16.5	93.8 ± 9.6	16
L2	<18 m	No	SCA	Any	Any	76.9 ± 10.7	100.0 ± 0.0	20
L2	Any	Amp	Any	Any	Any	73.5 ± 7.9	79.0 ± 7.1	61
L2	>18 m	No	SCA	Any	Any	65.5 ± 17.2	85.7 ± 11.5	16
L2	>18 m	No	Any	Any	UH	75.3 ± 7.2	83.2 ± 5.9	83
L2	>18 m	No	Any	1	Any	86.2 ± 8.3	90.7 ± 6.5	67

Amp- MYCN amplified; SCA- segmental chromosome aberrations; FH, UH- favorable, unfavorable histology

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High-dose ^{131}I-MIBG treatment incorporated into tandem HDCT/auto-SCT for high-risk neuroblastoma: Results of SMC NB-2009 study

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Background: The strategy using tandem HDCT/auto-SCT for high-risk neuroblastoma in which TBI was incorporated into the second HDCT/auto-SCT (SMC NB-2004 study) demonstrated a very encouraging survival rate. However, most survivors experienced significant short- and long-term toxicities associated with tandem HDCT/auto-SCT, particularly TBI. Therefore, we incorporated high-dose ^{131}I-MIBG treatment into the second HDCT/auto-SCT instead of TBI from 2009 (SMC NB-2009 study).
Methods: From 2009 to 2013, 54 patients were assigned to receive tandem HDCT/auto-SCT after 9 cycles of induction chemotherapy. CEC (carboplatin + etoposide + cyclophosphamide) and TM (thiotepa + melphalan) with (for stage 4) or without (for stage 3) high-dose ^{131}I-MIBG treatment were used as the first and second HDCT regimen, respectively. High-dose ^{131}I-MIBG was infused on day -21 of the second HDCT/auto-SCT. Local radiotherapy, 13-cis-retinoid acid, and IL-2 were given after tandem HDCT/auto-SCT. Acute toxicities during the second HDCT/auto-SCT, late effects, and survival rates were compared between NB-2004 and NB-2009 studies.
Results: All but 2 patients who experienced progression during induction treatment underwent the first HDCT/auto-SCT and 47 patients completed tandem HDCT/auto-SCT. Five patients died from toxicities during the first HDCT/auto-SCT. There was no significant immediate toxicity during ^{131}I-MIBG infusion and no toxic death during the second HDCT/auto-SCT. The duration of high fever was shorter (P<0.001) and frequencies of grade 3/4 stomatitis, diarrhea, and liver enzyme elevation during the second HDCT/auto-SCT were lower in NB-2009 study than in NB-2004 study (P=0.005, 0.054, and 0.028, respectively). Late effects evaluated at 3 years after the second HDCT/auto-SCT were less significant in 2009 study than in 2004 study (less GH deficiency, SNHL, cataract, and glomerulopathy). There was no difference in 5-yr EFS (67.4 ± 6.7% vs. 64.9 ± 6.8%, P=0.833).
Conclusions: High-dose ^{131}I-MIBG treatment incorporated into tandem HDCT/auto-SCT was feasible and could reduce acute and chronic toxicities without jeopardizing survival rate.

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Incidence and risk factors for secondary malignancy in patients with neuroblastoma after treatment with ^{131}I-metaiodobenzylguanidine

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Intro: ^{131}I-MIBG is a highly active form of tumor-targeted radiotherapy in patients with relapsed neuroblastoma, with response rates of 30% to 40%. Several reports of second malignant neoplasm (SMN) in patients after treatment with ^{131}I-MIBG suggest the possibility of increased risk of secondary malignancy, particularly myelodysplasia and leukemia. Incidence of and risk factors for SMN after ^{131}I-MIBG have not been defined.

Methods: This is a multi-institutional retrospective review of patients with neuroblastoma treated with ^{131}I-MIBG therapy at four institutions between March 1, 1984 and March 1, 2014. A competing risks approach was used to calculate the cumulative incidence of SMN from time of first exposure to ^{131}I-MIBG. Competing risks regression was used to identify potential risk factors for secondary malignancy.

Results: The analytical cohort included 644 patients treated with ^{131}I-MIBG. The cumulative incidence of SMN was 7.6% (90% CI 4.8-11.9%) and 14.3% (90% CI 9.1-22.1%) at five and ten years from first ^{131}I-MIBG, respectively. No increase in SMN risk was found with increased number of ^{131}I-MIBG treatments or higher cumulative activity per kilogram of ^{131}I-MIBG received. An increased risk of SMN was found in patients who had bone disease at the time of first ^{131}I-MIBG therapy. In a multivariate analysis, patients with relapsed/progressive disease had significantly lower risk of SMN (Subdistribution Hazard Ratio 0.3, 95% CI, 0.1-0.8, p=0.023) compared to patients with persistent/refractory.

Conclusion: The cumulative risk of SMN after ^{131}I-MIBG therapy for patients with relapsed or refractory neuroblastoma is similar to the published incidence for high-risk neuroblastoma overall. We found no dose-dependent increase in SMN risk. As the number of patients treated with ^{131}I-MIBG earlier after diagnosis, and length of follow up time from ^{131}I-MIBG therapy increase, it will be important to reassess this risk.

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Central nervous system relapses in patients with high-risk neuroblastoma: the SIOPEN experience

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Introduction: Incidence of central nervous system (CNS) relapses in high-risk neuroblastoma patients has not been clearly defined yet. In this study, we aimed to identify the incidence and the risk factors of CNS recurrences at first relapse in patients with high-risk neuroblastoma.

Methods: Patients with stage 4 > 12 months and/or MYCN amplified tumors treated in the high-risk neuroblastoma HR-NBL1/SIOPEN trial were studied. Characteristics at diagnosis, treatment and pattern of first relapse were analyzed. Central review of imaging was performed to confirm CNS metastases. Cumulative incidences of CNS-relapses were estimated taking into account the completing risks of Non-CNS relapses and Death. The statistical comparison was done with Gray test.

Results: Among the 2209 patients included into the HR-NBL/SIOPEN trial, 1323 patients relapsed and 64/1323 had CNS metastases. Five-year incidence of CNS recurrence was 3%±0% for the whole population, and 5%±1% of all relapses. CNS was the only site of relapse in 37/64 patients. Time to relapse was 1.03 and 1.2 years for CNS recurrences and other recurrences, respectively (p=0.117). Post-relapse overall survival (OS) was worse (p=0.057) in children with CNS relapse. Age, gender, stage, MYCN status and bone marrow, bone and lung involvement at diagnosis had no impact on the risk of CNS relapses; only liver metastases (p=0.001) and the involvement of several metastatic compartments at diagnosis (p<0.001) correlated to CNS relapse. The HDC regimen (Busulfan-Melphalan (Bu-Mel) vs Carboplatin-Etoposide-Melphalan (CEM)) had no impact on the incidence of the CNS recurrences (p= 0.649).

Conclusions: The risk of CNS recurrence in patients with HR neuroblastoma is 3% at 5 years after diagnosis. Since the CNS evaluation is not routinely performed in the current HR-NBL1/SIOPEN protocol, the real incidence of CNS relapses might be higher and should be prospectively evaluated in order to establish the need of a prophylactic strategy.

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Autologous T cells expressing a GD2 specific chimeric antigen receptor with CD28 and OX40 costimulatory endodomains for children with neuroblastoma

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Infusion of T cells expressing a GD2 specific chimeric antigen receptor (CAR) in patients with neuroblastoma can induce sustained complete remissions. Introducing costimulatory endodomains into the CAR may improve the antitumor potential of T cells; therefore, we conducted a Phase I study with adaptive trial design of T cells expressing a GD2 CAR with both CD28 and OX40 costimulatory endodomains. We tested the safety, persistence and antitumor efficacy of the CAR T in 11 patients with neuroblastoma, in 3 cohorts. The 1st cohort received escalating doses of thawed GD2 CAR T cells that had been grown with interleukin-2 (IL2). The 2nd cohort received lymphodepletion with fludarabine and cyclophosphamide followed by escalating doses of fresh cells grown with interleukin-7 and -15 (IL7/15). The 3rd cohort received 2 doses of the PD1 inhibitor, pembrolizumab following infusion of CAR T as per cohort 2. CAR expression was similar in cells irrespective of cohort (median 65%; range 43%-83%). In vivo CAR T cell expansion was greater in cohorts 2 and 3 than in cohort 1 at week 1 post-infusion ($p<0.01$), as was persistence at week 4 ($p<0.02$), although CAR T cell numbers in all patients declined after their peak at +7-14 days. The treatments were tolerated well and no dose limiting toxicities were observed. Antitumor responses were modest: at 6 weeks 1 patient had mixed response, 4 had stable disease and 6 patients progressed. Thus, T cells expressing a GD2 CAR with CD28 and OX40 costimulatory endodomains are safe, expand and are detectable for >4 weeks in vivo but early antitumor responses were disappointing even after lymphodepletion and co-administration of PD1 Ab.

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Killer-cell Ig-like receptor (KIR) haplotypes and Fcγ-receptor polymorphisms correlate with antibody-dependent cell-mediated cytotoxicity levels and survival of high-risk relapsed/refractory neuroblastoma patients treated by long-term infusion of anti-GD_2 antibody ch14.18/CHO in combination with interleukin-2 (IL-2).

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Background: Killer-cell Ig-like receptors expressed on natural killer (NK) cells and their ligands on target cells may predict outcome in patients (pts) with high-risk relapsed/refractory neuroblastoma (NB) treated by LTI of ch14.18/CHO.

Methods: 53 pts received $6x10^6$ IU/m^2 sc IL-2 (d1-5; 8-12), LTI of 100 mg/m^2 ch14.18/CHO (d8-17) and 160 mg/m^2 oral 13-cis-RA (d19-32) in a closed single center program (53 pts) (APN311-303).
Polymorphisms in Fcγ-receptor genes 2A (H131R), -3A (V158F) and -3B (NA1/NA2), and KIR- as well as KIR ligand expression (HLA-C1; HLA-C2; HLA-Bw4+) were determined by real-time PCR.

Results: A best response rate of 40.5% translated into a 5-y OS of 56.4±7.1% (mean OS 4.35y [0.3-6.2y]) and a 5-y PFS of 29.1±6.3% (mean PFS 2.4y [0.1 - 5.9y]). Median TTP/PFS was 1.35 y (95% CI: 0.21-2.48 y). This result is clearly superior to historical controls not treated with ch14.18/CHO with a 5-y OS of 14.8±6.8% ($p<0.005$), indicating clinical efficacy of the treatment.
We identified 21/53 pts with the NK- stimulatory KIR haplotype (2DS2+) who had superior OS- and PFS-rates compared to 16/53 pts with an inhibitory haplotype and KIR/KIR-ligand match (2DS2-, 3DL1+, Bw4+) ($p<0.02$). Similarly, we found 33/53 pts with low affinity FCGR alleles (FCGR2A-H131R/R and/or FCGRA3A-V158 F/F). These patients showed lower PFS rates compared to 19/53 patients with high affinity FCGR polymorphisms ($p<0.01$).
Importantly, ADCC analysis on day 15 of cycle 1 showed higher levels in the pt group with NK-stimulatory versus NK- inhibitory KIR/KIR ligand haplotypes (27±5 % vs. 12±3%, $p< 0.01$). A similar effect was observed in pts with high affinity FCGR polymorphisms with an ADCC increase of 21±7% compared to 11±2% in the low affinity FCGR control.

Conclusion: KIR-haplotypes and FCGR-polymorphisms correlated with the functional immune parameter ADCC and clinical outcome, and may therefore be useful biomarkers for LTI with ch14.18/CHO.

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A Phase I Study of Lenalidomide in Combination with ch14.18 and isotretinoin in Patients with Refractory/Recurrent Neuroblastoma (RR-NB): New Approaches to Neuroblastoma Therapy (NANT) Consortium Trial

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Background: Ch14.18(dinutuximab) increases event free and overall survival in patients with high-risk neuroblastoma given with GM-CSF/IL-2. However, this therapy has significant toxicities and 40% of patients still relapse. Lenalidomide has shown immunomodulatory effects in pediatric solid-tumor patients and is well tolerated. Combining lenalidomide with ch14.18 was supported by preclinical data demonstrating immunomodulatory/anti-tumor effects in neuroblastoma. We are conducting a phase 1 trial to determine the tolerability of lenalidomide in combination with ch14.18 and istotretinoin in patients with RR-NB.

Methods: Lenalidomide dose escalation is following a 3+3 design(25, 50, 75 and 100mg /m^2/day. The administration schedule is lenalidomide days 1-21, ch14.18(17.5mg/m^2/day) days 8-11 and isotretinoin(160mg/m^2/day) days 15-28(DL2-5).

Results: Twenty-one patients have been enrolled, median age 8.3 years (range: 3.7-20.8), of whom 17 were evaluable for dose escalation. The median number of courses was 2 (range 1–12). Dose limiting toxicity (DLT) of grade 3 diarrhea occurred in 2 patients at DL1, one of whom also experienced neutrophil count recovery delay. Since diarrhea was transient, the protocol was amended to allow this toxicity. Six additional evaluable patients were enrolled on DL1 with no DLT's (or diarrhea). No grade ¾capillary leak or hypotension were reported and one patient had grade 3 fever. Grade 3/4 hematological toxicities observed did not delay subsequent course start except in one patient.. Immunomodulation was seen with increases in frequency of effector NK cells and increased antibody dependent cytotoxicity (ADCC) in patients pre/post therapy on DL1. Pharmacokinetics, ADCC, flow cytometry of NK cells, T cells and monocytes and cytokine analyses will be discussed for all dose levels.

Conclusion: Lenalidomide doses up to 75mg/m^2/day for 21 days is tolerable in combination with ch14.18 and isotretinoin with acceptable clinical toxicities, and demonstrated immunomodulatory effects. DL5 evaluation is ongoing to determine the optimal dosage of lenalidomide in this regimen.

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Overview of treatment for high-risk patients

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Not available at time of printing

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Overview of treatment for relapsed disease

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Not available at time of printing

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Long-term effect of treatment

Lisa Diller

Not available at time of printing

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Impact of bone marrow-derived disseminated neuroblastoma cells on the identification of the relapse seeding clone

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Introduction: Poor survival of stage 4 neuroblastomas is largely due to a high relapse rate. We tested whether genomic analysis of disseminated tumor cells (DTCs) from the bone marrow helps to better understand the tumor evolution and to characterize the relapse-seeding clone.

Methods: Seven samples from different regions of the primary tumor, DTCs at diagnosis, metastatic tumor and DTCs at relapse were analyzed by SNP array in a stage 4 patient. Relapse-associated chromosomal aberrations found in this case were tested in a validation cohort of 122 DTC and 81 tumor samples from 154 stage 4 patients.

Results: In the case study, independent tumor evolution in the primary tumor and metastatic cells resulted in exclusive aberrations in each sample. DTCs at diagnosis, DTCs at relapse and the metastatic tumor displayed a unique aberration, i.e. 1q sub-terminal deletion, which was not found in any of the primary tumor samples. Thus, the relapse clone most likely originated from a clone presented in the DTCs at diagnosis.

In the validation cohort, 1q sub-terminal deletions/imbalances were found in 27.5% of the DTCs at relapse, 17.8% of the diagnostic DTCs and 11.1% of the primary tumors. Patients with 1q deletions/imbalances in the diagnostic DTCs had a higher chance of relapse or early death (5-years EFS: 18.8% vs 36%). Additionally, 19q deletion, significantly associated with 1q deletion/imbalance, was found in 27.5%, 15.1% and 12.3% of relapse DTCs, diagnostic DTCs and primary tumors, respectively. 19q deletion in the diagnostic DTCs was associated with decreased EFS (5-years EFS: 13.3% vs 42%) and OS (5-years EFS: 25.9% vs 38.9%).

Discussion: We provide evidence of branched clonal evolution and parallel progression of the primary tumor and metastatic cells. 1q sub-terminal deletions/imbalances were highly associated with relapse and were more frequently found in the DTCs at diagnosis compared to the primary tumors. Thus, analyzing DTCs at diagnosis may provide a higher probability for detecting the relapse founder clone compared to tumor biopsy.

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Analyzing risk factors for stem-cell collection failure in patients on the High-Risk Neuroblastoma 1 trial (HR-NBL1/SIOPEN)

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Background

The current HR-NBL1/SIOPEN strategy permits stem-cell leukopheresis only after completion of induction therapy due to the rapid 10-day schedule of COJEC cycles. Our aim was to define risk factors for insufficient stem cell collection of fewer than $3x10^6$ CD34+ cells/kg body weight (BW) in this population.

Patients and Methods

Patient eligibility in this analysis was completed induction therapy (COJEC ±TVD), accrual between 2002-2015 and at least one documented peripheral stem cell collection (PSSC) attempt. A total of 1024 patients were included, of which 896 (88%) had stage 4 disease and the remaining 128 MYCN-amplified (MNA) localized or 4S disease. Median age at diagnosis was 2.7 years (range 22d – 19.8y). Median follow-up time is 5.4yrs. The minimum successful total stem cell yield was $3x10^6$ CD34+ cells/kgBW as mandated by the trial protocol.

Results

689/1024 patients (67.3%) had a successful collection with one collection attempt while in 270 (26.5%) ≥2 collection cycles were undertaken, of whom 46/270 did not achieve the threshold of

$3x10^6$ CD34+ cells/kgBW.
In 111/1024 children (11%) ≤$3x10^6$/kg CD34+ were collected (range 0.2-2.9x10^6/kg, median 2.4). Failure of PBSC collection >$3x10^6$/kg was more frequent in patients without MNA disease (13.7% vs 8.4%, p=0.01 Fisher exact) and in patients aged ≥5 years at diagnosis (16.3% vs 9.8%, p=0.015). Persistent bone marrow (BM) involvement or other metastatic sites had no apparent impact on collection yield.
35/111 patients underwent additional BM harvest (0.2-14.1x10^6/kg CD34+ cells, median 2.6). 31/35 reached the threshold and received high-dose chemotherapy; 2/35 were transplanted with <$3x10^6$/kg CD34+ cells and 2 patients were not transplanted.
Among patients with <$3x10^6$/kg CD34+ cells collected by PSSC 90.8% (n=99) underwent high-dose chemotherapy, compared to 97.4% (n=832) in patients above threshold.

Conclusion

Unsuccessful PSSC was more common in patients aged >5 years and without MNA disease; there was no statistical correlation with metastatic site involvement at diagnosis. Overall, >$3x10^6$/kg CD34+ were collected in 92% of patients.

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Quantification of bone marrow disease in high risk neuroblastoma patients by anti-GD2 immunocytochemistry – impact on survival. A SIOPEN High Risk Study.

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Background: Bone marrow (BM) infiltration frequently occurs in patients with metastatic neuroblastoma and is routinely analyzed with trephine biopsies and conventional cytomorphology (CCM) on BM smears. The SIOPEN High Risk Study includes a prospective analysis of tumor cell infiltration using anti-GD2 immunocytochemistry (ICC) at diagnosis, after induction chemotherapy, prior to myeloablative therapy and at the end of treatment.

Objective: (1) to evaluate the prognostic impact of the number of infiltrating tumor cells at diagnosis and after induction chemotherapy and (2) to compare the results of anti-GD2 ICC with those of CCM and trephines.
Methods: Mononuclear cells from 878 bilateral BM aspirates pulled from 289 patients were stained with anti-GD2 and evaluated according to international consensus guidelines (Beiske et al, BJC, 2009). Numbers of GD2-positive tumor cells and numbers of investigated BM cells were recorded. Time to progression (EFS) and time to death (overall survival OS) were analyzed using the Kaplan-Meier method. ROC analysis was applied to evaluate possible cut-offs for continuous predictors.
Results: ROC analyses of the sensitivity and specificity of selected cut-off values and various numbers of investigated tumor cells to predict EFS and OS revealed that only BM samples containing >2E+06 BM cells provided prognostic information. At diagnosis, the number of GD2-positive tumor cells was prognostic of EFS (p=0.005) and OS (p=0.003) as it was for BM trephines (EFS p<0.0001, OS p=0.001). In contrast, CCM predicted only EFS (p=0.034) but not OS. After induction, ICC alone was prognostic of EFS (p=0.008) and OS (p=0.007) while cytomorphology and trephines were not.
Conclusion: At diagnosis, both ICC and trephine biopsies were associated with OS and EFS. After induction chemotherapy, only ICC remains informative. It is mandatory to analyze >2E+06 bone marrow cells in order to obtain a prognostically informative result. The ICC assay may contribute to identify patients at ultra-high risk (UHR).

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Central imaging review in the SIOPEN high-risk neuroblastoma trial: preliminary data on central nervous system recurrences

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Introduction: Central nervous system (CNS) recurrences in patients with high-risk neuroblastoma (HR-NBL) are rare, and their radiological characteristics are not well defined.
Methods: A secure web-based system has been developed by the Austrian Institute of Technology (AIT) within the frame of SIOPEN to perform central imaging review in selected populations of the current HR-NBL1/SIOPEN trial. Of 64 patients with CNS

relapse identified in the SIOPEN-R-NET database, brain MRI/CT and MIBG scans were requested to 45 centers from 12 different countries. Imaging gathering and uploading was centralized in 6 centers. The review was performed by two independent pediatric radiologists and two nuclear medicine experts. Preliminary results are presented.

Results: Brain MRI/CT scans of 26 patients (41%) belonging to 18 centers from 8 countries could be retrieved and uploaded. Images were evaluable for 22/26 patients (85%). In 2 patients images at diagnosis only were provided, and in 2 patients the images were not from the time point of relapse. CNS relapse was confirmed in 21/22 (95%; 95% CI: 78%-99%) patients. The CNS lesions were parenchymal in 19/21, mainly located in the parietal lobe (9/19) and cerebellum (3/19), and as unique lesion in 11/19 patients. Leptomeningeal disease was found in 5/16 evaluable images (non-contrast CT scan in 5 patients). Edema around parenchymal lesion was observed in 14 patients, intratumoral hemorrhage in 11 patients, midline shift and hydrocephalus in 6 and 3 patients, respectively. Only 1/6 patients with brain MRI/CT scan performed at diagnosis had previous CNS involvement. CNS recurrence could be identified also by MIBG scan in 6/10 patients.

Conclusions: This work shows that central imaging review is feasible within a phase 3 academic international trial. It is of major help to better understand radiological features especially in rare subpopulations such as CNS recurrences, and should be prospectively considered in next trials

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Characteristics and risk factors of 517 patients with first recurrence from stage 4 neuroblastoma over 18 months

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Objective
To study the impact of presentation features and treatment modalities on the outcome in a large cohort of patients
Methods
Patients were included in this retrospective analysis with (i) stage 4 neuroblastoma at initial diagnosis ≥18 months and <21years, (ii) enrolment in first line trials between 01.01.1990 and 31.12.2010, and (iii) first recurrence or progression. Excluded were patients with continuous progression from initial diagnosis, with refusal of curative front-line therapy, with second malignancy, or insufficient medical information.
Results
517 patients met the inclusion and exclusion criteria. 15% had recurrence at primary only, 57% at metastatic sites only and 28% at primary and metastatic sites. Involved sites were osteomedullary (66%), primary site (43%), CNS (14%), lymph nodes (9%), and liver (9%). 36% of patients received palliative or no treatment for recurrence, 46% chemotherapy (± other modalities except myeloablative therapy), and 18% chemo- and myeloablative therapy. The median time from 1st to 2nd recurrence was 3.8 months (secEFS) and from 1st recurrence to death 7.0 months (secOS). Children under palliative care had a median secEFS time of 1.9 months, under chemotherapy (± others) of 5.4 months and under chemo- and myeloablative therapy of 15.0 months (p<0.001). The 5 year secEFS for *all* patients including the palliative care group was 6.1±1.1%, the 5 year secOS 9.8±1.4%. By multivariate analysis most significant diagnostic factors for secEFS were time to 1st recurrence <18 months (HR 2.204), MYCN amplification (HR 1.424), liver metastases at inital diagnosis (HR 1.830), lung/pleural metastasis at recurrence (HR 1.830) and >1 recurrent site (HR 1.435). In non-palliatively treated patients, the most significant therapeutic impact had myeloablative therapy (HR 0.544) and radiotherapy (HR 0.611).
Conclusion
This study describes risk factors which may help to tailor recurrence treatment in high risk neuroblastoma patient groups.

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Detection of PHOX2B and TH mRNA by RTqPCR in peripheral blood stem cell harvests may identify children with stage 4 neuroblastoma that have an increased risk of an event post reinfusion: a SIOPEN study

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Purpose: To evaluate the prognostic power of paired-like homeobox 2b (PHOX2B) and tyrosine hydroxylase (TH) mRNAs detected by reverse transcriptase quantitative polymerase chain reaction (RTqPCR) in peripheral blood stem cell (PBSC) harvests from stage 4 children entered into the European trial HR-NBL-1/SIOPEN.

Methods: Samples were collected and RTqPCR performed for PHOX2B and TH mRNA in PBSC (n=189) and paired blood taken at diagnosis (n=166) as previously described (Viprey et al, 2007,EJC,43;341-50; Viprey et al, 2014,JCO,32;1074-83).

Results: When considered as binary or continuous variables there was no significant relationship between

TH or PHOX2B mRNAs in PBSC harvests, the time of reinfusion and event-free survival (EFS). Additionally no prognostic cut-point could be identified. Consistent with previous observations, high levels of TH (log_{10} >0.8) or PHOX2B (log_{10}>0.28) mRNA in blood at diagnosis identified a group of children with an increased risk of an event, with 5 year EFS of 16% compared to 43% ((HR (95% CI) =2.44 (1.58, 3.75), p<0.0001). In the children with a more favourable outcome (n=117), the presence of TH or PHOX2B mRNAs in PBSC harvest predicted an increased risk of an event post reinfusion. This effect appeared greatest when both TH and PHOX2B mRNA were detected (HR (95% CI) =3.55 (1.30, 9.73), p=0.01); this requires validation. There was no correlation between the level of PHOX2B or TH mRNA in PBSC harvest and the time of harvest or surgery.
Conclusion: PHOX2B and TH mRNAs in PBSC harvests do not predict EFS from the time of reinfusion in all children with stage 4 neuroblastoma. However, they may identify a sub-group of children who have an increased risk of an event post reinfusion of the PBSC harvest.

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Genome-wide analysis of liquid biopsies reveals a novel layer of tumor heterogeneity in neuroblastoma

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Purpose: Recent data show that genomic changes are frequently subclonal in neuroblastoma and may occur in an undetectable fraction in a primary tumor. Alternative analytic strategies need to be evaluated to obtain a more complete picture of the genomic landscape and to circumvent an often insufficient amount of tumor tissue. Isolating cell-free tumor DNA from more easily accessible patient biomaterial could surmount these challenges. We investigated the feasibility of this approach to use cell-free peripheral blood (PB) and bone marrow (BM) plasma for neuroblastoma tumor genome analyses.
Experimental Design: Cell-free DNA (cfDNA) isolated either from BM or PB or both from 14 stage M neuroblastoma patients were analyzed by ultra-high-density SNP arrays (23) and by low-coverage whole genome sequencing (WGS) on an Illumina HiSeq2500 (6/23) and compared with genomic data from the corresponding tumor or GD2-enriched disseminated tumor cells from BM (BM-DTCs).
Results: Genomic aberrations were unambiguously detectable in cfDNA samples using SNP array analysis (17/23) and WGS (6/6) and included typical segmental chromosomal aberrations (SCA) and *MYCN* amplifications as well as atypical SCAs, amplicons and micro-deletions. A high concordance between genomic aberrations found in patient-matched cfDNAs from BM plasma and BM-DTCs (3/3) was found. However, discordances were detected between BM and PB cfDNA samples obtained at the same time point (3/6).
Conclusions: Cell-free tumor DNA can serve as an additional source for tumor genome analysis allowing the identification of genetically distinct tumor clones which are otherwise difficult to detect in the primary tumor. Our findings contradict the widely accepted view that all bodily fluids present an identical picture of tumor genetics. Comparing datasets from different intra-patient locations will produce a more complete representation of tumor heterogeneity, thus improving our understanding of tumor dynamics and progression in individual patients.

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Detection of tumor *ALK* Status in neuroblastoma patients using peripheral blood

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New protocols based on ALK-targeted therapy by crizotinib or other ALK targeting molecules have opened for the treatment of patients with neuroblastoma (NB) if their tumors showed mutation and/or amplification of the *ALK* gene. However, tumor samples are not always available for analysis of *ALK* mutational status in particular at relapse. Here, we evaluated the *ALK* mutational status of NB by analysis of circulating DNA, using the droplet digital PCR (ddPCR) system. ddPCR assays were developed for the detection of *ALK* mutations at F1174 and R1275 hotspots found in NB tumors and was applied for the analysis of circulating DNA obtained from 200µl of serum or plasma samples collected from 114 patients. The majority of samples were obtained from patients with high risk NB (stage 4 (n=97), stage 2/3 with MYCN amplification (n=7) and stage 3 without MYCN amplification (n=10)). *ALK* mutations were found in circulating DNA of 24 cases (22%). The mutations F1174L (exon23 position 3520, T>C and position 3522, C>A) and the mutation R1275Q (exon 25 position 3824, G>A) were detected in circulating DNA of 2, 11 and 15 patients respectively. Concurrent distinct mutations were observed in 4 cases, indicating

tumour heterogeneity. A perfect concordance for the mutation F1174L (position 3520, T>C) was observed when we compared the circulating DNA and tumor DNA samples. For the mutations F1174L (position 3522, C>A) and R1275Q (position 3824, G>A), our test showed a good sensitivity (85% and 92%, respectively) and a high specificity (91% and 98%, respectively). In conclusion, these ddPCR assays offer reliable, noninvasive blood tests to assess*ALK* mutational status at F1174 and R1275 hotspots and should help clinicians to identify patients showing an *ALK* mutation in particular when no tumor tissue is available. Moreover, the analysis of *ALK* mutation in circulating DNA may help to capture tumor heterogeneity.

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Amplification of *CDK4* and *MDM2* is associated with atypical clinical features in high risk neuroblastoma patients

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MYCN-amplification and 11q-deletion are important, although incomplete, markers of high-risk neuroblastoma. Thus, characterization of additional genomic alterations that can be used as prognostic and/or predictive markers is of clinical importance in order to provide best treatment possible.
By SNP-microarrays we identified a group of neuroblastomas with high grade amplification of one or multiple loci on 12q, commonly involving the potential oncogenic target genes *CKD4* (12q13-14) and/or *MDM2* (12q15). The *CDK4* and *MDM2* regions were co-amplified in 13/16 samples, two tumors had *CDK4*-amplification in absence of *MDM2*-amplification while one tumor had *MDM2*-amplification without *CDK4*-amplification. Exome sequencing was performed on seven tumors and whole genome sequencing (WGS) was performed on tumor and constitutional DNA from one patient. No novel protein altering SNVs were detected within the amplified regions except a rare *INHBE* mutation in one patient. The tumor examined with WGS show extensive structural rearrangements on chromosome 12 leading to high grade amplification of *CDK4* and *MDM2*. This tumor displayed 21 somatic protein changing alterations although not in any gene with known function in chromatin stability or DNA repair.
Interestingly, the majority of the 12q-amplified neuroblastomas were of abdominal origin, some with renal location with initial suspicion of Wilms' tumor. Atypical metastatic pattern were also seen in this patient group showing low degree of bone marrow involvement favoring other metastatic sites such as lung.
The consistent co-amplification of two separate chromosome 12 regions in this subset of neuroblastoma suggests that there are one or more genes with importance in tumor development/progression. Our study indicates that *CDK4* appears as main target in this 12q-amplified neuroblastoma subgroup although other genes such as *MDM2* and *FRS2* also could provide proliferative advantages. The 12q-amplified neuroblastomas exhibit distinct clinical features and may benefit from targeted therapy using a small molecule CDK4/CDK6 inhibitor such as LEE011 (Novartis).

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A comparison of ^{123}I-mIBG planar imaging and SPECT/CT with ^{68}Ga-DOTATATE PET/CT for staging and response assessment of high-risk neuroblastoma

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Aims

Standard neuroblastoma staging involves ^{123}I-mIBG planar scintigraphy. Neuroblastoma can also be imaged with radiolabelled somatostatin analogues. The aims were (1) to compare planar ^{123}I-mIBG imaging (mIBG) with maximum intensity projection ^{68}Ga-DOTATE PET (GaDO), and (2) to assess the additional information that may be acquired through the use of mIBG SPECT/CT data and the CT component of GaDO imaging.

Methods

Paired mIBG and GaDO scans were analysed. Uptake patterns on GaDO PET/CT maximum-intensity projection and mIBG planar scintigraphy were compared and evaluated by semi-quantitative scoring. Additional mIBG SPECT/CT data were compared with the cross sectional CT component of GaDO imaging.

Results

42 patients, 22 male, 20 female, median age 8 years (range 2 - 51 years) were imaged: 3 at diagnosis, 13 had refractory disease and 26 had relapsed disease. The mean time between scans was 24 days.
GaDO was positive in all 42 patients; mIBG was positive in 40 patients. Bone lesions were identified 36 patients. GaDO identified bone lesions in 35, and mIBG in only 29 patients. GaDO identified soft tissue lesions 33 patients, whereas ^{123}I-mIBG identified these in only 29 patients. Overall, GaDO revealed more lesions than mIBG, and semi-quantitative scores were significantly higher with GaDO. In 61% of patients GaDO revealed

more extensive disease, in 5% mIBG revealed more. In 28%, both modalities gave the same results, and in 6% images were discordant as different lesions demonstrated different tracer uptake patterns. The use of SPECT/CT in addition to planar scans increased the accuracy of mIBG imaging.

Conclusions

Planar scintigraphy alone with mIBG is insufficient to map disease extent accurately. SPECT/CT increases diagnostic accuracy. GaDO imaging often shows more extensive disease, and discordant uptake in some patients has implications for molecular radiotherapy. Both modalities should be used for optimal imaging.

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Development of a targeted sequencing panel for detection of subclonal mutations in neuroblastoma at diagnosis

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Background: Relapsed neuroblastoma tumors harbor increased mutational burdens and we recently reported that relapse-specific somatic mutations are enriched in *ALK* and/or activation of the RAS-MAPK pathway. Importantly, many of these mutations were subclonal or not detectable in the matched primary tumor sample. We therefore hypothesized that stringent design and optimization of a custom amplicon sequencing panel will allow prospective detection of prevalent relapse-specific subclonal mutations in primary tumors. This could allow for the integration of targeted therapy earlier, preventing the emergence of resistant subclones.

Methods: We used our previously published relapse sequencing data and analyzed available FoundationOne targeted sequencing data from primary (N=78) and relapse (N=67) samples to design a 28-gene panel. The most frequently mutated genes were *ALK, PTPN11, ATRX, HRAS, KRAS, NRAS, TP53* and *MYCN*. Our panel contains roughly 200 amplicons covering 55 unique mutations, plus the coding regions of *TP53* and *NF1*. The panel was designed with Illumina's dual-strand design in order to detect and correct for DNA deamination events occurring during formalin fixation. High-risk primary neuroblastoma tumors (N=299) were sequenced across a total of ~12,000bp. To increase sensitivity and likelihood of detection, we used 100 ng of genomic DNA as input for TruSeq Low Input library construction, added unique molecular identifiers to identify PCR duplicates, and sequenced to an average depth of coverage of 100,000X using NextSeq500 high output flowcells.

Results: Control blood samples (N=3) were used to determine the background variability and normalize base calling prior to variant detection. Relapse tumors (N=3) harboring known mutations were used as positive controls and a Horizon Tru-Q standard, genomic DNA containing verified low-level variants (1.3%) in *ALK, IDH2, KRAS,* and *NRAS,* was used to determine the limit of detection and assess variant calling accuracy at subclonal levels.

Conclusions: Data are currently being generated and will be presented at the meeting. We plan to use this panel to improve patient outcomes prospectively by coupling targeted therapy to chemotherapy.

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Survival tree analysis of an independent cohort reveals risk factors as proposed in the INRG system

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Background:
In 2009 Cohn et al. proposed the International Neuroblastoma Risk Group (INRG) classification system based on a risk factor analysis for event free survival (EFS) of a large international cohort. The aim of our work was to evaluate this classification system in an independent representative national cohort with respect to event free survival and overall survival (OS).
Methods:
Risk factors of 1,063 neuroblastoma patients (age 0-21 years) registered in two consecutive national neuroblastoma trials between 01.01.2003 and 31.12.2010, were compared to the published INRG data. Finally, the impact on outcome was analysed by survival tree regression analyses for the endpoints EFS and OS.
Results:
Compared to the INRG cohort, patients of the national cohort were more often diagnosed in infancy (p=0.003) and with localized stages (p=0.004), showed less often elevated LDH (p<0.001), normal serum ferritin (p<0.001), differentiated tumors (p<0.001) and tumors with low/intermediate MKI (p<0.001). Regarding genetic factors, fewer patients had 1p (p<0.001) and 17q aberrations (p= 0.026). In contrast no differences were seen for INPC diagnostic category, 11q and MYCN status.
As previously published for the INRG cohort, survival tree analysis revealed INSS stage as the most important prognostic risk factor in the national cohort for EFS but also for OS. MYCN, age and serum ferritin were further prognostic risk factors for EFS, whereas for OS MYCN, age and LDH were of prognostic relevance.
Conclusion:
Application of survival tree analysis on an independent and differently composed representative neuroblastoma cohort resulted in similar selection and ranking of risk factor as in the published INRG survival tree.

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An 18-gene Myc activity signature predicts poor clinical outcome in multiple Myc-associated cancer types

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Background
Myc transcriptional activity is frequently deregulated in human cancers, but a Myc activity signature with predictive ability and clinical utility in multiple tumour types remains to be developed.
Methods
18 Myc-regulated genes were selected from published studies of Myc family targets based on analysis of gene expression and correlation with clinical outcome in epithelial ovarian cancer (EOC) and neuroblastoma. A Myc family activity score, derived from the expression of 18 genes using qPCR assays arrayed in Taqman low density format, was first correlated to *MYC/MYCN/MYCL1* expression in a panel of 35 cancer cell lines. The prognostic utility of this signature was evaluated in neuroblastoma (n=649), medulloblastoma (n=130), diffuse large B-cell lymphoma (DLBCL; n=122) and EOC (n=540) microarray gene expression datasets using Kaplan-Meier and multivariate Cox regression analyses, and was further validated in 42 primary neuroblastomas using qPCR.
Results
Cell lines with high *MYC*, *MYCN* and/or *MYCL1* gene expression exhibited elevated expression of the signature genes. Survival analysis showed that high signature score was significantly associated with poor outcome in the overall cohort ($p<0.001$) and a subset of tumours lacking *MYCN* amplification ($p<0.001$) in neuroblastoma. Moreover, high signature score was associated with poor prognosis independently of well-defined prognostic factors in neuroblastoma, breast cancer, DLBCL and medulloblastoma. In EOC, the 18-gene Myc activity signature was capable of identifying a group of patients with poor prognosis in a "high-*MYCN*" molecular subtype but not in the overall cohort. The predictive ability of this signature was reproduced using qPCR analysis of an independent cohort of neuroblastomas, including a subset of tumours without *MYCN* amplification.
Conclusion
An 18-gene Myc activity signature is highly predictive of clinical outcome in diverse Myc-associated malignancies, independent of Myc amplification. This suggests its potential clinical application in the identification of Myc-mediated tumours that might be treated with Myc-targeted therapies.

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Opsoclonus myoclonus syndrome in children with neuroblastoma

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Introduction
Opsoclonus myoclonus syndrome (OMS) is a rare paraneoplastic syndrome associated with neuroblastoma (NB) in 50-80% cases. The aim of the study was to analyze characteristics of neuroblastoma associated with OMS treated in the multicenter study in Russia.
Methods
285 patients with sympathetic nervous system tumors were included for the period 01.2012-06.2015 (42 months). 19 (6.7%) patients had tumor associated with OMS. The diagnosis has been established on the basis of international criteria of OMS. The diagnosis of NB has been confirmed by histological examination in all cases. Patients were stratified and treated according to the German NB2004 protocol.
Results
Male: female ratio was - 0.35:1. The median age at the diagnosis of OMS and NB was 24.7 months (range 14.9-54.0). Paravertebral location was noted in 15/19 (78.9%) cases. Most tumors were small (median volume - 6.5 ml (range 0.4-80.9 ml). In 11/19 (58.0%) patients the tumor was visualized only by CT/MRI. Increased neuron specific enolase was observed in 1/19 (5.3%) case. Scintigraphy with metaiodobenzylguanidine (MIBG) was positive in only 9/17 (53.0%) cases. Segmental aberrations were observed in 1 patient (11q deletion). All but 1 patient were stratified to the observation group. Comparing with non-OMS cases NB associated with OMS showed female preponderance ($p=0.005$), older age at tumor diagnosis (24.7 versus 11.2 months, $p=0.02$), non-adrenal primary tumor ($p=0.0008$), more differentiated histology ($p=0.0001$), lack of MYCN amplification ($p=0.057$) and 1p deletion ($p=0.04$), favorable stage ($p=0.003$) and risk group distribution ($p=0.003$). 3-year EFS was 82.6% in OMS group and 61.6% in non-OMS group ($p=0.22$), 3-year OS - 100.0% and 72.7% ($p=0.2$).
Conclusion
NB associated with OMS showed more favorable biologic characteristics. CT and/or MRI are the most informative diagnostic methods to detect tumors in patients with OMS given the small size, location and low metabolic activity of NB.

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Prognostic and predictive significance of *p53, MDM2* and miRNAs gene expression in patients with neuroblastoma.

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Neuroblastoma (NB) is *TP53*-wild type malignant childhood tumor. In addition to MDM2, many genetic and epigenetic regulators of p53 activity and its signaling pathway are known. Currently are not well known changes in p53 pathway its deregulation arising in NB. We have investigated changes of p53, *MDM2* and miRNA miR-34 a/b/c, miR-137, miR-380-5p, miR-885-5p gene expression in NB and their impact in NB progression and survival.
We have analyzed tumor tissue samples of 61 patients with NB. *MDM2* and *p53* expression levels (EL) was detected with real-time PCR using TaqMan primers and microRNAs with TaqMan MicroRNA Assay, Applied Biosystems (USA). Results were normalized to relevant controls. FISH method was used for *MYCN* amplification (MNA) detection.
In this study we have found that higher *MDM2* EL was associated with unfavorable clinical NB features (MNA ($p<0.03$), disease stage ($p<0.001$), indicating a link between *MDM2* overexpression and the high-risk phenotype. *MDM2* overexpression was associated with a significant decrease in event-free survival of NB patients (F-Cox criterion: 3.22, $p < 0.001$).
We have established that miR-34 a/b/c and miR-380-5p don't have significant independent effect on the clinical behavior of NB, whereas low expression of miR-885-5p and miR-137 EL is associated with unfavorable disease course. Thus, low miR-885-5p and miR-137 EL in NB tumor cells correlated with MNA ($p<0.05$ and $p<0.001$), *MDM2* overexpression ($p<0.02$ and $p<0.001$) and was significantly more common in patients with advanced NB stages ($p<0.04$). In addition, low miR-137 expression was associated with chemotherapy resistance ($p<0.05$). We have established significant decrease of event-free survival rates of patients with low miR-885-5p and miR-137 expression (F-Cox criterion: 3.54, $p < 0.001$ and F-Cox criterion: 2.89, $p<0.02$).
Deregulation of 53-mediated pathway due to alterations of its regulators genes and microRNAs expression is associated with an unfavorable outcome in NB. Alterations in aforementioned genes and microRNAs can be used as predictive and prognostic markers in NB.

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Microarray CGH analysis of genomic imbalances in neuroblastoma FFPE specimens – pilot study.

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BACKGROUDN: Genomic imbalances especially structural chromosomal aberrations are associated with a poor prognosis and a malignancy in neuroblastoma (NB). Microarray Comparative Genomic Hybridization (aCGH) permits the detection of genome-wide copy number alterations with high resolution. However, the use of this technique with DNA extracted from archival formalin-fixed paraffin-embedded (FFPE) tissue specimens usually has quantitative and qualitative limitations.
AIM: The aCGH application for testing DNA samples extracted from FFPE NB tissues by standard protocols not intended especially for FFPE samples.
MATERIALS AND METHODS: DNA was obtained from 2 FFPE NB tissue samples of 2 patients, using representative 6-µm sections of the tumor samples. Specimens were treated with xylene, and then washed with ethanol. DNA was extracted automatically using the QuickGene DNA tissue kit (Kurabo, Osaka, Japan) according to the manufacturer's protocol. The aCGH profiles were defined by SurePrint G3 CGH ISCA v2 Microarray Kit 8x60K (Agilent Technologies, Santa Clara, CA) according to the manufacturer's protocol. Chromosomal aberrations in selected patients have been previously verified by FISH and MLPA analysis on fresh tissues.
RESULTS: According to aCGH results for first patient, it has been revealed 100% compatibility with FISH and MLPA. It has been detected MYCN amplification, 1p36 deletion and 17q gain for this patient. Although for second patient, a discrepancy between raw aCGH and confirmatory tests has been indicated. However, a holistic view for data from all used techniques was consistent. It has been shown 2p gain, 1p36 deletion, 11q23 deletion, 17q gain and few additionally imbalances.
CONCLUSIONS: Our pilot study has shown that a material recovered from FFPE tissue specimens may be effectively examined by aCGH. It should be emphasized that an extremely important analysis of genomic imbalances in NB patients could be carried out also by aCGH method.

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Clinical characteristics and risk factor of transplantation-associated microangiopathy (TAM) in high-tisk neuroblastoma undergoing autologous peripheral blood stem cell transplantation (auto-PBSCT)

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Background:
Transplantation-associated microangiopathy (TAM) is a severe complication which occur after stem cell transplantation. TAM in high-risk neuroblastoma (NB) patients with autologous peripheral blood stem cell transplantation (PBSCT) is not yet well described in literature.
Patients and Method:
We retrospectively reviewed the medical record of 32 NB patients who received single auto-PBSCT from March 2002 to September 2014 at our institution. In this study, we defined TAM as all following criteria met;(1)twice elevation of creatinine(Cr) from baseline before PBSCT, (2)elevated lactate dehydrogenase(LDH) above the upper limit of normal, (3)hematuria, (4)increased transfusion requirements without other cause. Clinical characteristics and risk factors of TAM were analyzed and compared with other solid tumor patients received single auto-PBSCT.
Result:
Seven out of 32 NB patients experienced TAM (21.9%) which developed 7-90 days (mean = 39days) after PBSCT, comparing the incidence of 1 out of 27 patients of solid tumors other than NB (P=0.23). Three out of 4 patients with early onset (TAM occurred by day30) needed to receive hemodialysis more than that with late onset (TAM occurred after day30;0/3) (P=0.047).After hemodialysis, none required chronic hemodialysis, and six out of seven TAM patients were alive. To elucidate the risk factors for the occurrence of TAM, we compared clinical characteristics between TAM and non-TAM group. There were no significant differences between TAM and non-TAM group in patients characteristics, include age, sex, total dose of cisplatin and cyclophosphamide, radiotherapy and enucleation of kidney, and high dose chemotherapy (HDC) regimens. Mean systolic blood pressure (P=0.39), level of Cr (0.88) and 24-hr Cr clearance(P=0.31) before PBSCT weren't different, respectively.
Conclusion:
We could not identify risk factors of TAM after PBSCT in high-risk NB treatment. Although it is difficult to predict TMA before PBSCT, prognosis of TAM In NB patients is better than TAM after allogeneic transplantation previously reported.

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Survival and prognostic factors for children 12 to 18 months of age with stage 4 non-*MYCN* amplified neuroblastoma treated in the SIOPEN high-risk trial

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Background. Better outcome has been described in children 12-18 months old with stage 4 non-*MYCN* amplified (nonMNA) neuroblastoma. Our aim was to analyze the clinical and biological data at diagnosis in this cohort, and their impact on outcome.
Subjects and methods. Children 366-547 days old with stage 4 nonMNA neuroblastoma treated in the high-risk neuroblastoma (HR-NBL)/SIOPEN trial were included. Treatment consisted of induction chemotherapy (COJEC) with high-dose chemotherapy (HDC) and maintenance treatment. The amended HR-NBL1/SIOPEN(V1.5) trial excluded patients with only numerical chromosomal alterations (NCAs) from HDC. Central review of MIBG scans was performed by two independent reviewers. Kaplan-Meier estimator and estimates of cumulative incidences were compared with log-rank test and Gray test.
Results. Eighty-four patients met the inclusion criteria. Median age was 448 days (range: 366-540d). Bone marrow and bone involvement were detected in 60/84 and 54/84 patients, respectively; liver and lung disease in 12/84 and 10/84 patients. The median follow-up was 5.9 years (1.06-12.5yrs). Five-year event-free survival (EFS) and overall survival (OS) were 60%±6% and 70%±6%, respectively. Five-year recurrence rate was 27%±5%. Time to recurrence was 32.1 months (3.3-54.8mo). Five treatment-related deaths occurred: 3 with HDC (2 infections after Busulfan-Melphalan, 1 multi-organ-failure after Carboplatin-Etoposide-Melphalan) and 2 with surgery. Five-year EFS was better in children with LDH (p=0.01) and ferritin levels < 2xULN (p=0.01), and no lung disease (p<0.0001). In these patients, 5-year EFS was still less than 70%. Segmental chromosomal alterations (SCAs) were detected in 33/36 patients, and 1p loss (p=0.06) and 1q gain (p=0.008) were associated to worse prognosis.

The remaining 3/36 patients had no SCAs: 2/3 received HDC, and all of them were alive at the last follow-up.
Conclusion. Prognosis of children 12-18 months old with stage 4 nonMNA neuroblastoma needs to be further improved. NRM remains a major concern in this population.

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Isolation of circulating tumor and associated cells by microfiltration in patients with neuroblastoma

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Background: Circulating tumor cells (CTCs) and cancer-associated macrophage-like (CAMLs) cells have been identified in the peripheral blood of patients with a variety of malignancies and are not present in patients without cancer. Identification of CTCs and cancer-associated cells in blood may provide a non-invasive technique for evaluating cancer progression, relapse, or treatment response. As proof of principle, isolation of CTCs and CAMLs in neuroblastoma patients was attempted.
Methods: In a pilot study, ten peripheral blood samples were collected from patients with high-risk neuroblastoma at different stages of disease. Size-exclusion, low-pressure filtration was used to isolate CTCs and CAMLs from the blood. Cells were stained with fluorescent antibodies (anti-human disialoganglioside GD2/anti-vimentin/anti-CD45/DAPI) to identify CTCs (GD2+, vimentin+, CD45-) and CAMLs (atypical nucleus and any combination of the 3 markers). Immunofluorescent microscopy was utilized for cell characterization and imaging.
Results: Ten patients with neuroblastoma were included in this study. Two samples (20%) were found to have clusters of GD2-positive cells, thus detecting neuroblastoma CTCs. The presence of CTCs was not reliably associated with any clinical or biological features in this small cohort. CAMLs were found from 25 to greater than 100 µm in size. CAMLs were identified in 9 of the samples (90%). On average, more CAML cells were identified in samples from patients with Stage 4 disease (ranging 1 to 26, mean 10 CAMLs) compared to Stage 3 (ranging 1 to 8, mean 3 CAMLs), although this observation was not statistically significant (P=0.27). *MYCN* amplification was not associated with the number of CTCs/CAMLs identified. CAMLs were not identified in 30 healthy controls. In this limited cohort, CAML detection in patients with HR neuroblastoma had sensitivity of 0.9 (95%CI 0.54-0.99) and specificity of 1 (95%CI 0.85-1).
Discussion: Although further work is necessary, identification of CTCs and CAMLs in the peripheral blood of neuroblastoma patients is technically feasible and may provide a non-invasive and economically viable method for cancer surveillance.

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Impact of age and *MNA* amplification (MNA) on long-term survival rates: accurate estimation and refined modeling using innovative statistical approaches. A SIOPEN study from the high tisk neuroblastoma trial HR-NBL1/SIOPEN.

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BACKGROUND
The prognostic impact of MYCN amplification (MNA) in stage 4 neuroblastoma remains unclear and the findings of large studies differ.
METHODS
1005 stage 4 patients (pts) above one year of age in entered in HR-NBL1/SIOPEN treated with COJEC induction before November 2009 were evaluated. The primary endpoint was 5-year event free survival (5-yr EFS) and the pseudo-value regression model was used for statistical evaluation allowing separate investigation of short- and long-term survival probabilities without relying on proportional hazards.
RESULTS
The median age was 3yrs with 105pts 12-below18 months, 664pts 18mo-5yrs and 236pts above 5yrs. 591/1005pts (58%) were MNA (MNA+) and MNA was absent in 414pts (MNA-).
There is a significant interaction between age and MNA (p=0.032). The 5-yr EFS was 35% in pts 12-18 months for MNA+(n=70) and 62% for MNA- (n=35, p=0.011); in pts 18mo-5yrs. 33% in 294 MNA+ pts and 30% in 370 MNA- pts (p=0.503) and in pts> 5yrs 13% in 50 MNA+ pts and 14 % in 186 MNA- pts (p=0.933).
Metastatic CR after COJEC occurred more frequently in patients with MNA+ (46% vs 33%, p<0.001).
The 5-yr survival after relapse was 7% in MNA+ and

10% in MNA- pts. However, deaths occurred earlier in MNA+ pts: the in 1-yr post relapse survival was 25% in MNA+ and 59% in MNA- pts.

CONCLUSION

Pts below18 months without MNA have a superior long-term EFS. However, in patients with MNA, long-term EFS is similar in the younger age-groups (below18 months, 1.5-5yrs) but is significantly inferior in over 5yrs.

MNA is associated with a higher rate CR, but pts who relapse do so earlier. These differences should be considered in the design of clinical trials.

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Circulating tumor DNA for disease monitoring in neuroblastoma

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Introduction. Circulating tumor DNA (ctDNA) has been used for disease monitoring in several types of cancer. Therefore, the aim of our study was to investigate whether ctDNA can be used for treatment response monitoring in neuroblastoma.

Method 121 plasma samples from 50 patients were analyzed for the detection of hypermethylated RASSF1A DNA. DNA was isolated and total cell free DNA (cfDNA) was determined by qPCR for albumin and the amount of ctDNA was determined by qPCR for methylated RASSF1A after bisulfite conversion. Detection of ctDNA was compared with clinic-biological patient characteristics, such as BM and PB MRD (qPCR), MIBG scans and urinary catecholamines.

Results In 13/20 diagnostic samples ctDNA was detected. In all 7 diagnostic samples from patients with localized disease no ctDNA was detected. During induction chemotherapy (stage 4 patients only) in 13/42 samples ctDNA was detected (30%). At relapse in 8 out of 14 samples ctDNA was detected (57%). The amount of cfDNA was significantly higher in neuroblastoma patient at time of diagnosis and relapse than in healthy controls and stage 4 patients had the highest amount of cfDNA. There was a significant correlation between ctDNA and PB or BM MRD, when tumor levels were high or no tumor was detected. Discrepancies were observed in 20 samples and were studied in detail. The discrepancies were mainly observed in samples during treatment when tumor burden was lower.

Conclusion Hypermethylated RASSF1A can be used as marker for monitoring of ctDNA in neuroblastoma patients and highly correlates with the disease status at diagnosis. Our data indicate that there is no correlation when tumor load is lower because during treatment in several samples discrepancies were observed. It is likely that ctDNA can originate from both primary tumor as metastases and may be of special interest for disease monitoring in patients relapsing in other organs than the BM.

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A flow cytometry backbone panel as a first step in detection of circulating tumor cells in neuroblastoma

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Introduction: Circulating tumor cells (CTCs) and disseminating tumor cells from neuroblastoma tumors are detected by immunocytology and RQ-PCR. However, these techniques are unable to characterize heterogeneity at the cellular level. Since neuroblastoma shows high heterogeneity between and within tumors we believe that subpopulations of CTC might exist. It is not clear if these subpopulations are detected with current techniques and they could have a difference in drug response or metastatic potential, therefore affecting prognosis.

Aim: To develop a multicolour flow cytometry panel of antibodies consisting of a backbone panel identifying all neuroblastoma cells in blood and bone marrow, and additional heterogeneity markers to detect subpopulations.

Methods: Possible backbone markers were identified by literature study and bioinformatical analysis on tumors, primary tumor cell lines (PTCs) and cell lines. Candidate markers were confirmed, in vitro on a panel of cell lines, with flow cytometry.

A peripheral blood dump channel was designed, consisting of antibodies directed against markers present on blood cells but absent on neuroblastoma cells. In combination with a neuroblastoma backbone panel, this dump channel can be used to distinguish malignant cells from the healthy blood background.

Results: We identified six new potential backbone markers for the detection of all neuroblastoma cells; EBAG9, ITGAV, RAP1A, CKAP4, CDH2 and IL6ST.

Conclusion: The combination of markers commonly used in literature ($CD45^-$, $CD56^+$, $CD81^+$ and $CD9^+$) might not detect all neuroblastoma cells, since these markers were differentially expressed or even absent on cell lines and PTCs. A backbone panel with the new identified markers, in combination with a peripheral blood dump channel, is the first step in detection of distinct subpopulations in blood and bone marrow.

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Aurora Kinase A inhibition sensitizes neuroblastoma to ^{131}I -MIBG

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Background: Despite the use of intensive multimodal therapy, a substantial proportion of children with high-risk neuroblastoma have relapsed or refractory disease.. Novel agents and approaches are needed to improve outcomes. Targeted radiotherapy with ^{131}I -MIBG is one of the most effective salvage therapies for relapsed or refractory neuroblastoma, but the majority of patients do not respond to ^{131}I-MIBG. Aurora Kinase A (AURKA) inhibition has the potential to sensitize tumors to radiation by recruiting cells into G2/M phase. The AURKA inhibitor alisertib has shown efficacy in preclinical models for neuroblastoma and is currently in early phase testing in patients with neuroblastoma.
Methods: We tested alistertib alone and in combination with radiation therapy on cultured neuroblastoma cell lines and with ^{131}I –MIBG *in vivo* in a subcutaneous xenograft for efficacy and effects on cell cycle as well as the p53 apoptotic pathway.
Results: Alisertib caused G2/M phase arrest in neuroblastoma cells and significantly improved efficacy when combined with both external beam radiation in cultured cells. Alisertib combined with ^{131}I –MIBG yielded superior growth inhibition compared to either agent alone when tested in a mouse model of norepinephrine transporter (hNET) positive, radiation-resistant neuroblastoma.
Conclusions: The combination of alisertib and ^{131}I -MIBG shows at least additive activity in preclinical testing and provides preclinical rationale for combination testing in clinical trials.

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Ex vivo drug screening as a strategy for personalised therapy in high-risk neuroblastoma

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Background: Personalised medicine approaches may improve the poor survival rate of high-risk neuroblastoma, however the relatively low mutation rate and paucity of recurrent actionable mutations render therapy selection based on sequencing techniques challenging. We are currently using patient-derived xenograft (PDX) models to investigate whether unbiased, *ex vivo* drug screening is feasible as an alternative strategy.
Methods: PDX models of high-risk neuroblastoma were obtained from Children's Oncology Group and passaged in NSG mice. Tumours were harvested from five models, immediately processed using a semi-automated tissue dissociator and plated in 384-well tissue culture plates. *Ex vivo* screening was conducted using 136 established adult and paediatric anti-cancer drugs with cells treated at five doses (1–10,000 nM) to generate dose-response using an end-point viability assay.
Results: PDX models proved amenable to *ex vivo* manipulation, short-term culture and drug screening using a viability assay. For each of the five models, we were able to identify at least one drug for which that model was greater than 10-fold more responsive *ex vivo* compared to the average response of the other models based on IC_{50} values. In some cases this window was greater than 100-fold. Importantly, these included drugs approved for paediatric use, such as cytarabine, tretinoin (ATRA), gemcitabine and imatinib.
Conclusions: Drugs identified as effective in *ex vivo* screening will now need to be confirmed using these PDX models *in vivo*. Our results to date suggest that *ex vivo* drug screening can identify unexpected drug sensitivities that may aid in identifying therapy options for patients with high-risk neuroblastoma.

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A novel histone deacetylase inhibitor OBP-801 induces apoptosis in neuroblastoma tumor cells

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Background:
Neuroblastoma (NB) is the most common extracranial solid tumor in children. Despite advances in therapy, the prognosis of high-risk NB remains poor. Thus, alternate options of therapy are required to improve the survival rate and reduce the side-effects of existing therapies in neuroblastoma patients. Histone deacetylase (HDAC) proteins modulate transcription by deacetylation of the lysine residues in the histone tails of the chromatin and of several non-histone proteins. HDAC inhibitors comprise a diverse class of compounds targeting these HDAC proteins and are being rigorously studied for their role in several types of cancers. The aim of the present study was to investigate the antitumor effects of a novel HDAC inhibitor, OBP-801, as a potential therapeutic agent for treating NB.
Methods:
Human NB cell lines IMR32, GOTO, SK-N-AS, and SH-SY5Y were used in this study. Cell survival rate was evaluated by the WST-8 assay; cell cycle was analyzed by flow cytometry. Apoptosis was detected by Annexin V staining and analyzed by flow cytometry.
Results:

Induction of apoptosis and cell cycle arrest at the G2/M phase was observed in all the NB cell lines studied after 24-h exposure to pharmacological levels of OBP-801. Concentrations of OBP-801 required for 50% inhibition of proliferation for IMR32, GOTO, SK-N-AS, and SH-SY5Y were 1.5 ± 0.9 nM, 2.5 ± 1.1 nM, 3.6 ± 1.4 nM, and 2.6 ± 1.1 nM, respectively.
Conclusion:
Our results suggest that the novel HDAC inhibitor OBP-801 is a broad-range and effective inhibitor of NB cell lines and can be a potent therapeutic option for NB.

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Serum C-circles as biomarker of Alternative Lengthening of Telomeres (ALT) in neuroblastoma

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Unlimited proliferation of cancer cells requires activation of one of two telomere maintenance mechanisms: telomerase or homologous recombination-based ALT. C-circles (CC) (extrachromosomal partially single-stranded circular telomeric DNA) are a marker of ALT activity. We ask whether CC can be detected as cell-free circulating tumor DNA in the serum of patients with CC+ neuroblastoma tumor and can therefore be used as a serum ALT biomarker.
We first examined 149 high-risk neuroblastoma tumors and found 24% (n=36) of tumor DNA to be CC+, i.e. ALT+. The outcome of ALT+ neuroblastoma (all MYCN non-amplified) was as poor as that of MYCN-amplified neuroblastoma (n=55) (5-yr OS: 32% vs. 28%). Serum was available for analysis in a subset of 35 tumors where 40% (n=14) were CC+. CC were measured in the circulating DNA extracted from the serum (referred to as serum CC level). The amount of circulating DNA ranged from 8.6 to 373 ng/100uL of serum (median 46.2) and there was no significant difference in circulating DNA level between patients with CC+ and CC- tumor (median 41.5 vs. 47.4 ng/100uL; P=0.7).
Serum CC levels ranged from 1.2 to 1556 AU (median 50 AU) and there was no correlation between serum CC and circulating DNA levels (P=0.5). However, there was significant correlation between serum and tumor CC levels (P=0.001) and serum CC level was significantly higher in the CC+ than the CC- tumor group (median 340 vs. 25 AU; P<0.001). Serum CC for the normal control (individual with no known cancer) was 0.8 AU. In the ALT+ tumor group, 12 of 14 had serum CC >100 AU. In the ALT- tumor group, 3 of 21 has serum CC >100 AU. Using a cut-off of 100 AU, the sensitivity of serum CC for detecting ALT+ tumor was 86% (12/14), with specificity of 86% (18/21) and concordance 86% (30/35). The results of this study therefore support CC as a potential serum biomarker for ALT tumor activity.

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Proteomic analysis of high-risk neuroblastoma identifies nuclear distribution protein C as a marker of differentiation and prognosis

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Background: Despite widely heterogenous histological appearance and genetic markers, high-risk neuroblastoma share a commonly aggressive disease course and poor outcome. While the genome and transcriptome of these tumors have been studied in detail, little is known about its proteome. Using a proteomic analysis, this study aimed to identify potential biomarkers associated with this clinico-pathological risk group that may account for their consistently aggressive behavior.
Methods: Primary NB tumors obtained from untreated high-risk patients were analyzed using two-dimensional gel electrophoresis (2DE) and mass spectrometry. Proteins extracted were pooled to construct a reference proteome map. A total of 602 protein spots were excised from 2DE gels for protein identification. Identified proteins were classified by gene ontology analysis and searched in the literature for biological significance. Immunohistochemical staining of selected proteins was scored on a neuroblastoma tumor microarray (TMA), and correlated with survival outcomes derived from the Singapore Childhood Cancer Registry using Log-rank analysis.
Results: Biopsy specimens were obtained from 6 high-risk treatment naïve patients of different demographics, disease site, stage, and biology to represent the diverse heterogeneity seen in this clinico-pathological group. We established the first known proteome map of high-risk neuroblastoma and identified 382 unique proteins with a minimum of 2 unique peptides matched and an additional 42 proteins were identified based on one peptide match.
They included putative tumor suppressor proteins and oncoproteins not described in neuroblastoma before. Among them was nuclear distribution protein C (NudC), a nuclear movement protein that regulates microtubule organization. NudC staining correlated with degree of tumor differentiation, and was associated with INPC classification (p=0.023). In 86 TMA samples, 3+ NudC staining was associated with

poorer overall survival (p=0.03) in a subgroup of low- and intermediate-risk patients.
Conclusion: We identified novel biomarkers through proteomic profiling of high-risk neuroblastoma that may be associated with tumor biology and behavior. NudC expression correlated with established pathological and clinical prognostic indicators, and identifies a subgroup of non-high risk patients with poorer outcomes.

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Targeted drug delivery using nanoparticles (NPs) in neuroblastoma (NB) xenografts

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Background: Patients with high-risk NB require intensive, multimodality therapy, but have only a 40-50% survival rate. Survivors have significant short- and long-term toxicities, so more effective, less toxic therapy is needed. Agents are being developed that target specific genes, proteins and pathways, but targeted delivery of existing agents is also promising. Here, the use of NPs as a drug delivery system is explored to increase efficacy and decrease toxicity of these agents.
Methods: A subclone of the SY5Y NB line was used for the studies. Several active agents for NB were encapsulated in pegylated-polylactide NPs, including SN38, lestaurtinib, fenretinide (4HPR), and phenylbutyrate (PBA). The efficacy of free drug versus NP-drug was evaluated on both in vitro cell proliferation and in vivo xenografts growing in nude mice.
Results: We have tested NPs with SN38 conjugated to tocopherol succinate (SN38-TS) compared to free irinotecan; NP-lestaurtinib compared to free lestaurtinib (50-200 nM), and NPs with 4HPR-PBA compared to fenretinide (1-20 μM). In the in vitro experiments, inhibition of growth with NP-drug was as or more potent than free drug at the same concentration for all the agents tested. Furthermore, at 4 hr we achieved ~100x as much SN38 in NB xenografts using NP-SN38-TS as a comparable dose of irinotecan delivered as free drug. Mice were "cured" of their NB xenografts (>180 day relapse-free survival) at a fraction of the dose of free irinotecan that produced only transient remissions. NP-lestaurtinib was also more effective than free against NB xenografts, although the lestaurtinib was not retained in NPs as long as SN38-TS. Studies are currently underway with NPs containing 4HPR-PBA, with promising early results.
Conclusions: NP encapsulation allows the targeted delivery of conventional and biological agents, which dramatically improves efficacy and decreases toxicity compared to free drug administration.

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Bromodomain-inhibition as therapeutic option for *MYCN*-amplified neuroblastoma

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Background: The BRD4 inhibitor JQ1 inhibits growth and induces apoptosis in a number of MYC-overexpressing cancer types. This study aims to assess the efficacy of JQ1 in neuroblastoma cell lines and to identify the genetic background making neuroblastoma tumors sensitive to JQ1-treatment.
Methods: A panel of 24 neuroblastoma cell lines was screened for JQ1 sensitivity by using Alamar blue viability assays, soft agar assays and FACS live/dead and cell cycle staining. MYCN mRNA and protein levels were determined by quantitative PCR and western blotting. Reverse Phase Protein Analysis (RPPA) was performed with 15 cell lines. sh/siRNA-mediated knock down of MYCN or BRD4 was used to investigate the relationship between MYCN and BRD4. Global transcript expression was profiled by RNA-seq. ChIP-seq for 6 histone modifications was used to define epigenetic changes upon JQ1 treatment.
Results: Viability screens showed that MYCN-amplified neuroblastoma cell lines are either JQ1 sensitive or resistant, whereas most non-amplified cells show intermediate response to JQ1. Reduced viability was mirrored by the absence of anchorage-independent growth and an increase of G1 and sub G1 fraction after JQ1 treatment. MYCN protein levels were reduced in 6/10 MYCN-amplified cell lines, however, this did not correlate with sensitivity. BRD4 knockdown in IMR5/75 cells did not affect MYCN levels but reduced Cyclin D1 expression. Global expression analysis of IMR5/75 cells emphasizes the importance of TP53-related signaling pathways and cell cycle-regulating genes in the response to JQ1 treatment. This was confirmed by RPPA analysis. Additionally, DNA damage repair genes were up-regulated after 6 and 12 hours JQ1 treatment. RNA expression profiles of JQ1-treated cells and xenograft tumors showed a high correlation.
Conclusions: JQ1 treatment promotes a G1 arrest and cell death in neuroblastoma cell lines. MYCN-downregulation is not the central mechanism of action. Cell cycle-related genes are affected by JQ1 treatment.

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The Connectivity Map bioinformatics platform identifies agents that reverse the chemotherapy resistance phenotype in neuroblastoma

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Neuroblastoma (NB) is the most common extracranial malignant solid tumor in children which contributes to more than 15% of all pediatric cancer-related deaths. Treatment failure in high risk neuroblastoma is largely due to development of chemoresistance. Effective treatments for resistant and recurrent disease remain to be identified. We collected mRNA from six high risk neuroblastoma tumors both at diagnosis and after induction chemotherapy, using laser capture microdissection to obtain a pure population of neuroblasts. Gene expression changes associated with chemotherapy resistance were determined with the aid of the Connectivity Map bioinformatics platform. The analysis identified several therapeutic agents that were predicted to reverse the transcriptome changes associated with chemotherapy resistance. One of these agents, the EWS-FLI1 and RNA helicase A interaction inhibitor YK-4-279, was studied in further detail. Using a panel of neuroblastoma cell lines, including SK-N-AS, SH-SY5Y, IMR-32, CHLA-255, NGP, and NB-19 cells, we found that YK-4-279 had cytotoxic effects on neuroblastoma cells. YK-4-279 also had an inhibitory effect on anchorage-independent growth of neuroblastoma tumor cells and induced cell apoptosis of these cells in vitro. YK-4-279 could enhance Dox-induced cytotoxic effect and cell apoptosis. Moreover, YK-4-279 was able to overcome the established chemoresistance in LA-N-6 neuroblastoma cells. Using an orthotopic neuroblastoma mouse model, we found that YK-4-279 induced apoptosis in tumor cells through PARP and Caspase3 cleavage. Taken together, our results indicate that YK-4-279 might be a promising agent for treatment of refractory neuroblastoma. Furthermore, these results support the general validity of therapeutic strategies that target the chemotherapy-resistance phenotype in malignant tumors.

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A screening for natural products identifies a flavonol as a synergistic compound with 13-cis retinoic acid in neuroblastoma

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High-risk neuroblastoma has poor survival rates, and novel therapies are needed. Well-chosen drug combination strategy can provide new opportunities. 13-cis-retinoic acid (13-RA) is a differentiating agent used in most current treatment regimens while naturally occurring polyphenols have been shown to exert anti-proliferative effects in a wide variety of cancer cell lines. The purpose of this study was to identify natural products potentiating the impact of 13-CRA against neuroblastoma cells and investigate the molecular mechanisms behind.
A library of about 160 natural compounds was screened for the viability of CHP134 cells challenged with a sublethal dose of for 48h. The *in vitro* screening identified a flavonoid, isorhamnetin, as a potential enhancer of activity in cells. Median effect analysis of drug combination revealed that isorhamnetin exerted synergistic inhibition of the growth of neuroblastoma cells both at 24h and 48h treatment, leading to 80% reduction in cell viability.
Flow cytometry and microscope investigations also revealed cell cycle arrest in G0/G1 phase and enhanced apoptosis. Microarray gene expression analysis at polysomal RNA level pointed out that combination treatment of plus isorhamnetin led to coherent perturbations of gene expression including a negative regulation of cell proliferation and specific neural cell differentiation gene pathway and up-regulation of specific G protein-coupled receptor downstream signaling, cell cycle arrest, apoptosis gene sets. Our findings also allowed us to advance an hypothesis on the molecular mechanism of this pharmacological synergism, centered on the elicited 30-fold increased of the alpha-1 adrenergic receptor expression.
Taken together, these data demonstrate that the combination of with isorhamnetin at therapeutically relevant concentrations is more efficient than the sum of each single compound alone in inhibiting neuroblastoma cell growth *in vitro*, suggesting potential therapeutic capabilities.

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CYP26-mediated metabolism of retinoids is a putative mechanism of treatment resistance in neuroblastoma

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Introduction: 13-*cis* retinoic acid (13-*cis*RA) is used clinically in the treatment of high-risk neuroblastoma, but retinoid disease resistance remains a major challenge. Increased retinoid metabolism has been proposed to contribute to retinoid resistance. The CYP26 enzymes (CYP26A1, CYP26B1, and CYP26C1) are the intracellular metabolizers of all-*trans* retinoic acid, and drugs that inhibit their functions have been developed. However, the efficacy of these drugs alone or in combination with retinoid has not been evaluated in neuroblastoma.

Methods: Retinoid sensitive cell lines SHSY5Y and NB1648 and retinoid-resistant cell lines SK-N-RA and SK-N-AS were grown in the presence of vehicle, CYP26 inhibitor talarozole, nonspecific CYP inhibitor ketoconazole, 13-*cis*RA, or a combination of CYP inhibitor and 13-*cis*RA. Cell viability was measured by Alamar blue (ThermoSci), and cell morphology was analyzed with the Incucyte Zoom (Essen). Gene expression of differentiation biomarkers was evaluated by RT-qPCR, and protein expression was evaluated by Western blot. Synergy testing was performed with Compusyn software. Dataset evaluations were done with R2 and Oncogenomics online platforms.

Results: All four cell lines had decreased proliferation and cell viability when treated with talarazole, while none were affected when treated with ketoconazole. Combination treatment of talarazole with retinoic acid resulted in synergistic inhibition of proliferation, although no significant change in cell morphology was noted. Gene and protein expression evaluation demonstrated MYCN expressed decreased while NTRK1 and PBX1 expression increased, consistent with retinoid-induced differentiation. Low expression of CYP26B1 specifically was prognostic of survival in a two datasets of patient tumors (n=88, n=102).

Conclusions: CYP26 inhibition has a synergistic effect with retinoid therapy *in vitro*, with increased markers of retinoid activity and differentiation. Further evaluations including in *in vivo* models are warranted to evaluate the potential benefits of combination retinoid and CYP26 inhibitor therapy to improve therapy in neuroblastoma.

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Anti-tropomyosin agents enhance the antitumor effectiveness of microtubule inhibitors in preclinical models of neuroblastoma

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The actin cytoskeleton is an ideal chemotherapeutic target due to its role in numerous biological processes essential for tumor cell growth and survival. We developed a novel class of small molecule drugs that target actin filaments containing the tropomyosin Tpm3.1, upregulated in many cancers. Anti-tropomyosins (ATMs) are effective against neuroblastoma cell lines independent of NMYC status. Combination studies with ATMs show a high degree of synergy with anti-microtubule agents, both vinca alkaloids and taxanes, in neuroblastoma and other solid pediatric cell lines but not in untransformed cells. The combination of ATMs plus vincristine (VCR) resulted in a G2/M cell cycle arrest and apoptosis in neuroblastoma cell lines. More importantly, we found the synergy was maintained *in vivo* in a human neuroblastoma xenograft model. Animals treated with the combination showed a significant and profound regression of tumor growth compared to control animals including several with complete responses. The mechanism of synergy involves the ability of ATMs to sensitise neuroblastoma to the action of anti-microtubule drugs and results in multiple structural defects in the mitotic spindle. The outcomes of this study have significant therapeutic implications as microtubule inhibitors such as VCR are current standard of care for a wide range of solid tumors, including neuroblastoma. ATMs are currently being developed as adjunct therapy for the treatment of both adult and pediatric cancers.

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Inhibition of STAT3 with the generation 2.5 antisense oligonucleotide, AZD9150, decreases tumor-initiating potential of neuroblastoma cells and increases their chemosensitivity

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In neuroblastoma (NB), cytokines activating STAT3 have been associated with poor patient outcome, chemoresistance and have been proposed to mediate survival of a rare population of NB tumor-initiating cells. Thus targeting STAT3 may be an important therapeutic strategy for high-risk NB. To determine the biologic consequences of STAT3 ablation, we evaluated pharmacogenomic inhibition of STAT3 using AZD9150, a 16-nucleotide antisense oligonucleotide targeting STAT3 that is now in adult Phase I/II clinical trials. Studies were performed in MYCN-wt (AS) and MYCN-amplified (NGP, IMR32) NB cell lines. AZD9150 treatment caused a 70% reduction in STAT3 mRNA and protein levels causing decreases in STAT3 target genes, such as CyclinD1, D3, and MYC/MYCN. *In vivo*, AZD9150 treatment of established tumors had little effect on xenograft growth despite significant decreases in STAT3, P-STAT3 and target gene expression compared to ASO, a control anti-sense treatment. To assess whether inhibition of STAT3 altered the tumor-initiating potential of NB cells, varying numbers of tumor cells from the ASO or AZD9150 treated mice were re-implanted and secondary tumor growth assessed. At $2x10^5$ and $2x10^4$ ASO-treated NB cell inoculums, 100% of mice had tumors while at similar cell doses only 40 and 20%, respectively of AZD9150-treated mice had tumors. Limiting dilution analyses indicated that the precursor frequency for tumor-initiating cells in ASO-treated tumors was 1/5178 cells, but in the AZD9150-treated tumors only 1/187030 cells was competent to initiate tumor growth (P=1.93e-08). Since tumor-initiating or stem-like tumor cells are frequently more resistant to cytotoxic agents, we evaluated inhibition of STAT3 combined with cisplatin. In established NB tumor xenografts, STAT3 inhibition combined with cisplatin caused a 30% decrease in tumor size (P=0.0092) and increased the survival of AZD9150-treated tumor-bearing mice compared to ASO-treated mice (P=0.026). Our study supports the development of strategies targeting STAT3 in combination with conventional chemotherapy for patients with high-risk NB.

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Targeting the *MYCN* oncogene in *MYCN*-amplified neuroblastoma with a novel PI polyamide DNA-alkylating drug conjugate

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Background: *MYCN* gene amplification is found in ~25% of neuroblastoma patients and correlates with poor prognosis. Although development of MYCN inhibitor has been considered to be attractive, no drugs have yet progressed to clinical application due to lack of obvious surfaces of MYCN protein for small molecule binding. We have developed a novel *MYCN*-targeting DNA-alkylating drug using Pyrrole-Imidazole (PI) polyamide (MYCN-A3), designed to bind directly to minor groove of genomic DNA within the coding region of *MYCN* in a sequence-specific manner.
Methods: MYCN-A3 was designed to specifically alkylate on the template strand in 3'UTR of the *MYCN* gene and synthesized by an automated peptide synthesizer PSSM-8. To evaluate anticancer activity of MYCN-A3, we performed *in vitro* and *in vivo* assays using neuroblastoma cells and other type of cancer cells.
Results: Treatment of *MYCN*-amplified neuroblastoma cells with MYCN-A3 significantly suppressed MYCN expression at the mRNA and protein levels. Accordingly, MYCN-A3 induced apoptotic cell death and IC_{50} values of *MYCN*-amplified cells were lower than *MYCN*-non-amplified cells. Intriguingly, FISH analyses demonstrated that MYCN-A3 impaired the signal intensity of a probe specific for the *MYCN* gene loci (2p24.3), suggesting that MYCN-A3 directly binds to and alkylates the target sequence of the *MYCN* gene, interfering with the probe hybridization. Moreover, MYCN-A3 significantly inhibited tumor progression in human neuroblastoma xenograft mouse models.
Conclusion: MYCN-A3 is a promising and an innovative therapeutic drug candidate for aggressive neuroblastomas with *MYCN* amplification.

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Combined antitumor therapy with metronomic administration of topotecan and hypoxia-activated prodrug, evofosfamide, in neuroblastoma preclinical models

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Background: Tumor cells residing in tumor hypoxic zones are a major cause of drug resistance and tumor relapse. In this study we investigated the efficacy of evofosfamide,(formerly TH302)) a hypoxia-activated prodrug, and its combination with topotecan in neuroblastoma (NBL) preclinical models.
Methods: A panel of five NBL cells lines (SKNEB2 , CHLA15, CHLA20 CHLA90, LAN5) were tested in vitro to assess the effect of evofosfamide on cell proliferation, both as a single agent and in combination with low dose metronomic administration of topotecan daily x 2 weeks , both under normoxic and various hypoxic conditions. In vivo antitumor activity was evaluated in different xenograft models. Animal survival was studied with the NBL metastatic model.
Results: Under normoxic condition, all the tested lines responded to evofosfamide in a dose-dependent manner, with IC50 values ranging from 4.6 to 66.1µM. When tumor cells were exposed to hypoxia overnight, there was a 22 to 65-fold decrease of evofosfamide IC50 with the IC50 values ranging from 0.07 to 2.4µM. By adding 20nM topotecan, evofosfamide induced cytotoxicity was significantly enhanced under overnight hypoxia as indicated by decreased IC50s in most tested tumor cell lines (p<00.1). In SK-NBE(2) and CHLA-20 xenograft models, after 2 weeks of treatment, tumor growth delay was observed with evofosfamide or topotecan as monotherapies. Complete tumor regression was observed in the combined topotecan / evofosfamide treatment group. From SK-N-BE(2) metastatic model, evofosfamide or topotecan treatment showed a substantial increase in animal survival compared to the control mice with a median survival of 22.5 days for control group, 25 days for evofosfamide group and 36 days for topotecan group. However, treatment with a combination of evofosfamide and topotecan had the greatest impact on animal survival ($p < 0.01$) with a median survival of 46 days.
Conclusions: Evofosfamide shows antitumor effects in NBL xenografts. Compared to single-agent, evofosfamide / topotecan, combined therapy using a metronomic topotecan regimen improves tumor response and enhances animal survival in preclinical tumor models.

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EZH2 is highly expressed in neuroblastoma and plays an important role in neuroblastoma cell survival independent of its histone methyltransferase activity.

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Background
The Enhancer of zeste homologue 2 (EZH2) gene, a catalytic subunit of the Polycomb Repressive Complex 2 (PRC2) is a histone methyltransferase that targets lys-27 of histone H3 (H3K27me3). Previous studies have shown that EZH2 can via its histone methyltranseferase activity induce the silencing of known tumour suppressor genes. A functional role of EZH2 independent of its histone methyltransferase activity has been reported as well. In this study, we explored the functional role of EZH2 and therapeutic potential of inhibiting the histone methyltransferase activity of EZH2 in neuroblastoma with two known inhibitors GSK126 and EPZ6438 as well as the targeted downregulation of the EZH2 protein.
Results
We identified in one neuroblastoma tumour a regional gain in the 7q36 region that encompasses the PRC2 group protein EZH2 and its concomitant overexpression. High EZH2 expression correlated with poor prognosis and overall survival independent of MYCN amplification patient status. Inhibition of the histone methyltransferase activity of EZH2 by small molecule inhibitors GSK126 and EPZ6438 in EZH2 high-expressing cell lines IMR32, CHP134 and NMB led to a slight G1 arrest of the cell cycle despite a strong decrease H3K27me3. Additionally, we showed a reduction of colony forming capacity of all three cell lines but only at high nanomolar concentrations of both compounds even though strong target-specific inhibition of EZH2 histone methyltransferase activity was observed already at low nanomolar concentrations of both compounds. Knockdown of EZH2 by three shRNA resulted in a strong apoptotic response and a decrease in the cycle D1 gene while simultaneously overexpressing EZH2ΔSET, a truncated form of the EZH2 wild-type gene lacking the SET transactivation domain and consequently histone methyltransferase activity rescued the apoptotic phenotypes.
Conclusion
Taken together, our findings suggest that EZH2 might play a pivotal role in the survival of neuroblastoma cell lines independent of its histone methyltransferase activity, paving the way for the development of therapeutics which specifically targets the EZH2 protein as a whole.

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The MCM complex is a critical node in the miR-183 signaling network of *MYCN*-amplified neuroblastoma cells

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MYCN and HDAC2 jointly repress the transcription of tumor suppressive miR-183 in neuroblastoma. Enforced miR-183 expression induces neuroblastoma cell death and inhibits xenograft growth in mice. Here we aimed to focus more closely on the miR-183 signaling network using a label-free mass spectrometric approach. Analysis of neuroblastoma cells transfected with either control or miR-183 expression vectors identified 85 differentially expressed proteins. All six members of the minichromosome maintenance (MCM) complex, which is indispensable for initiation and elongation during DNA replication and transcriptionally activated by MYCN in neuroblastoma, emerged to be down-regulated by miR-183. Subsequent annotation category enrichment analysis revealed a ~14-fold enrichment in the "MCM" protein module category, highlighting this complex as a critical node in the miR-183 signaling network. Down-regulation was confirmed by western blotting. MCMs 2-5 were predicted by *in silico* methods as direct miR-183 targets. Dual-luciferase reporter gene assays with 3'-UTR constructs of the randomly selected MCMs 3 and 5 experimentally confirmed them as direct targets of miR-183. Our results reveal the MCM complex to be a critical and directly regulated node within the miR-183 signaling network in *MYCN*-amplified neuroblastoma cells.

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A genome-wide MYCN synthetic lethal screen identifies inhibition of PRC2 as drug target in *MYCN*-amplified neuroblastoma cells

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Background: Patients harboring *MYCN*-amplified neuroblastomas (NBs) have advanced disease and poor outcome. Current therapeutic options fail to cure this patient group and novel therapeutic concepts are urgently needed. Functional screens to identify synthetic lethal combinations with tumor-specific mutations are an exciting new approach to identify novel drug targets and pathways that upon inhibition disclose cancer-specific vulnerabilities.
Material and methods: We performed a genome-wide screen in a *MYCN*-amplified neuroblastoma cell line, harboring a tet-inducible shRNA against MYCN, using a siRNA library consisting of 3 different siRNAs targeting 11.000 genes. Positive hits were validated in different in vitro systems allowing MYCN modulation. Chromatin immunoprecipitation (ChIP-seq) coupled to sequencing for 6 histone modifications and RNA-sequencing was used to characterize MYCN-related transcriptional and epigenetic changes in cell lines and primary tumors.
Results: From a focused analysis of 334 epigenetic modifiers, we identified inhibition of several methyltransferases, including EZH2, as a synthetic lethal interaction with high MYCN. shRNA mediated knock down of MYCN in *MYCN*-amplified cells revealed massive transcriptional and epigenetic changes with 865 up-regulated genes and 887 down-regulated genes associated with reduction of H3K27me3 and H3K4me3 histone marks at the transcriptional start site (TSS) of regulated genes, respectively. Tumor ChIP-seq revealed higher abundance of H3K4me3 and H3K27me3 in MYCN amplified as compared to MYCN single-copy NBs. In line with this, several components of the PRC2 complex, such as SUZ12, EED, EZH2 and RBBP7 were up-regulated in MYCN-amplified tumors. Inhibition of EZH2 using the selective EZH2 inhibitor, EPZ-6438, reduces H3K27me3 globally and leads to re-expression of silenced genes and viability reduction.
Conclusion: Selective inhibition is a synthetic lethal interaction with high MYCN in neuroblastoma cells. Target inhibition of EZH2 using small molecules could be new approach to treat high-risk NBs with amplified MYCN.

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Epigenetic silencing of *CHD5* expression by histone modification in human neuroblastoma

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Background: *CHD5* is a tumor suppressor gene located on 1p36 able to form a NuRD-type chromatin-remodeling complex. It is preferentially expressed in the nervous system and testis, and expression is very low or absent in high-risk NBs, especially those with 1p deletion and/or *MYCN* amplification. EZH2, a Polycomb group protein and subunit of the Polycomb repressive complex 2 (PRC2), binds to gene promoters and causes histone-3 lysine-27 trimethylation (H3K27me3), leading to transcriptional suppression. Recent evidence suggests that *MYCN*contributes to the regulation of PRC2. We analyzed the H3K27me3 status, as well as the binding of EZH2 and MYCN at the proximal CHD5 promoter.
Methods: ChIP assays were performed using nuclear proteins prepared from NB cell lines NLF (1p deletion), NGP (1p translocation, no deletion), NBLS and SY5Y (no 1p deletion) by crosslinking and sonication. Immunoprecipitation was performed with antibodies EZH2, H3K27me3, MYCN and control IgG. Bound DNA samples were analyzed by PCR and qPCR with primers designed around the *CHD5* transcription start site with appropriate negative control primers.
Results: H3K27 trimethylation was found in *CHD5* promoter -250 bp and inside intron 1 in NLF and NGP cell lines. Both cell lines showed very low *CHD5* expression. EZH2 binding was also found, consistent with the H3K27me3 in both NLF and NGP. We also observed MYCN binding to the E-boxes around -250bp and -800 bp of the *CHD5* promoter in NLF and NGP. These 3 factors (H3K27me3, EZH2, and MYCN) were not found around the *CHD5* promoter of the NBLS cell line, which shows high *CHD5* expression.
Conclusion: Our data strongly suggest that H3K27 trimethylation by EZH2 contributes to the epigenetic suppression of CHD5 expression, and that MYCN binding may also contribute to the regulation of *CHD5* expression.

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R2: A public user-friendly website for integrated analysis of genomic data and associated clinical parameters in neuroblastoma

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With the avalanche of genomic information in neuroblastoma, software solutions that can integrate and make this information comprehensive are in great demand.
Methods:
We designed a web-based program to facilitate integrative analysis of genomic data for neuroblastoma. Next to a series of richly annotated neuroblastoma samples generated in our lab and profiled in various ways, the database also contains a large volume of other public neuroblastoma datasets and thousands of profiles from other tumor types and normal tissues.
Results:
The R2 program, which has its roots 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.
Aside from mRNA expression data, R2 is also being employed for various other technologies such as aCGH, SNP, methylation, ChIPseq, Exome and even whole genome sequencing data. Various different technologies can be combined in integrative analyses and visualizations.
Examples of R2 usage include: KaplanScan for prognostic analyses; GeneSignature metagene creation and successive use to classify patient cohorts from within the platform. MegaSampler allows for the comparison of neuroblastoma profiles against up to 70.000+ publicly available samples of various tissues/cancers that also reside in our ever expanding data repository. Integrative correlation analyses can be performed to find associations between gene expression, methylation and/or variation status. R2 also harbours an embedded genome browser with many features including ChIPseq (chromatin / TF), (somatic) mutation and variation viewing combined with gene expression or other high throughput data.
Conclusions:
R2 provides a valuable resource for high throughput data of Neuroblastoma. The R2 program and database has been used in more than 320 publications and is publicly accessible via http://r2.amc.nl. R2 will help researchers in identifying important genes and biological processes in neuroblastoma.

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Non-random pattern of whole chromosome gains and losses in neuroblastoma with numerical chromosomal aberrations.

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Introduction. In most types of adult cancer, aneuploidy leads to an unfavorable prognosis. In absence of segmental chromosomal alterations (SCA) infant neuroblastoma and low-risk tumors (INSS stage 1, 2, 4S) are frequently associated with hyperdiploidy or near-triploidy and have a clinical good prognosis. In pediatric acute lymphoblastic leukemia, hyperdiploidy is also associated with a good prognosis. The mechanisms leading to these types of genetic defects and associated good prognosis are poorly understood.
Methods. Array-CGH data from 96 neuroblastomas with only numerical chromosome alterations (NCA) were analyzed, using 68 cases from the R2 database (http://hgserver1.amc.nl/cgi-bin/r2/main.cgi) and 28 additional GHU cases.
Results. Analysis of the array-CGH confirmed a non-random pattern of whole chromosome gains and losses in NB with NCA only. The number of gains and losses was highly variable between the different tumors. In 14/96 tumors only chromosome gains were observed, 80/96 had a combination of gains and losses, in 2/96 tumors only loss of one chromosome was present. In tumors with gains (94/96), combination of chromosome 17 (n=87) and/or chromosome 6 and/or 7 gain was present in all cases (94/94). In 82/84 tumors with losses, these losses involved a combination of chromosome 14 (n=63), 11, 3, and 4. In tumors with gains and losses different combination patterns were seen. Chromosome 17 gain was accompanied by loss of 14 in 61/72 tumors, and with loss of chromosome 11/4/3 in 8/9 of the remaining cases. The 7 tumors without chromosome 17 gain always presented with a chromosome 6 or 7 gain and loss of chromosome 14.
Conclusion. In NB with NCA we found a typical pattern of combinations of gains of chromosomes 6/7/17 and losses of 3/4/11/14. As previously described, the typical segmental abnormalities present in tumors with SCAs involve the same chromosomes: 17q-gain, 3p/4p/11q/14q-deletions. Understanding the underlying pathophysiology requires further studies.

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Role of MiRNAs in the epigenetic silencing of CHD5, a tumor suppressor in neuroblastoma (NB)

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Background: We first identified *CHD5* as a tumor suppressor gene that is frequently deleted in NBs. Mutation of the remaining *CHD5* allele is rare in these tumors, yet expression is very low or absent, so expression is likely regulated by epigenetic mechanisms. MicroRNAs (miRNAs) are small RNAs that bind to the 3'UTR to mediate downregulation of target gene expression by translational repression or cleavage of the target genes' mRNA. In order to understand the role of miRNA regulation of CHD5 in NB cell lines we tested miRNAs predicted to target CHD5.

Method/approach: We used bioinformatic programs and identified 18 miRNAs that were predicted to bind to the *CHD5*-3'UTR. We used a renilla-luciferase reporter plasmid that contains 103 bp of the 3'UTR of CHD5 targeted by miRNAs. We performed transient transfections in NLF and SY5Y NB cell lines with the reporter plasmid and miRNA mimic. We also used control plasmids and Allstar siRNA as another negative control. Western blot analysis was performed using whole cell extracts of NBLS to further validate the functional regulation of CHD5 expression by miRNAs.

Results/Conclusion: We found seven miRNAs that significantly downregulated *CHD5* expression in NBs: miR-211, 17, -93, -20b, -106b, -204, and -3666. Interestingly, *MYCN* upregulates four of the candidates we identified: miR-17, -93, -106b & -20b. This suggests that miRNAs driven by *MYCN* and other genes represent a potential epigenetic mechanism to regulate *CHD5* expression. Our western results indicate there was almost complete reduction of CHD5 protein levels in NBLS cells transfected with miR-211, miR-17, miR-93 and miR-20b, but no changes were observed in CHD4, actin or MYCN protein levels. These results strongly suggest that miR-211, -17, -93 and -20b can dramatically downregulate CHD5 protein expression in NBs, and MYCN amplification and over expression can contribute to this.

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Clinical relevance of genomic and epigenomic classification of *MYCN*-non-amplified neuroblastoma

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Neuroblastoma is known to exhibit a wide range of clinical phenotypes, from spontaneous regression to highly resistant to chemotherapy. One of the challenges in the clinic is to develop adequate therapeutic strategies for the intermediate risk-type of patients (stage 3 or 4 without *MYCN* amplification) whose prognosis still tends to be poor in terms of long-term follow-up (10 years survival rate was 51%, n=161). To identify key therapeutic targets involved in the patient group with unfavorable phenotypes, we have analyzed 63 primary tumors (dead:32, alive:31) without *MYCN* amplification nor *ALK* alteration by integrated genomic analyses such as array CGH, whole exome sequencing (SureSelect XT Human All Exon V4, average read depth:157), RNA sequencing, as well as Methylome profiling (Infinium HumanMethylation450 BeadChip). We found so far 816 non-synonymous somatic single nucleotide variations (SNVs) in coding exons in total (approximately 20 per tumor in average) and number of alterations in each tumor strongly correlated with age at diagnosis (p<0.0001) and patient prognosis (p=0.0077). Recurrent somatic SNVs (n=2 to n=5) were found in 66 genes, 19 of those were involved only in the tumors from the patients with fatal outcome. Gene Ontology and pathway analysis using the 66 genes showed that mutations occurred in the features including cytoskeleton, ion channel and extracellular matrix were significantly correlated with patient survival of the 63 tumors with logrank p-values of 0.0039, 0.0482 and 0.0011, respectively. *ATRX* mutations were observed in four tumors (6.3%), all of them were occurred in patients with unfavorable phenotypes (3 dead, 1 alive but relapsed twice). Methylome signature subdivided the 63 tumors into at least three clusters, which also showed strong correlation with patient prognosis (cluster-1 vs. cluster-2 and -3, p=0.0368). Unfavorable cluster-2 and -3 exhibited hyper-methylation in CpG islands and the former was quite similar to that of *MYCN*-amplified tumors. These genomic features could be useful markers for risk classification and help understanding molecular mechanism for aggressive phenotypes of *MYCN*–non-amplified neuroblastomas.

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Genomic characterization of high-risk neuroblastoma in Japan: A retrospective study of 537 cases by using updated follow-up data based on INRG variables [Japan Neuroblastoma Study Group (JNBSG)]

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[Purpose]

Neuroblastoma (NB) is known to exhibit a wide range of clinical behavior and genome-based molecular signature of tumor specimens has been considered as one of the useful risk markers to define high-risk NB. In this study, we collected follow-up data for more than 2,200 NB cases diagnosed in 122 hospitals from 1995 to 2014 according to the common INRG queries as a cooperative effort of the Japan Neuroblastoma Study Group (JNBSG). Array CGH (Agilent microarray system) was conducted with 537 cases (INSS stage 1:73; stage 2:46; stage 4s:41; stage 3:96; stage 4:281) and clinical relevance of each genome signature was assessed with the updated follow-up data. Detailed genome analysis of the 50 patients with high-risk NB enrolled in the JNBSG phase II study (JN-H-07) was also included.

[Results]

Tumors were sub-grouped into three genomic groups (GGs): silent (S), partial (P) and whole (W), which were further segregated by *MYCN* amplification (a;*MYCN*-amp), 1p-loss, 11q-loss and 17q-gain. Concordant with our previous results, Ws (s:*MYCN* single copy) and Ss showed favorable prognosis with 8 year survival rates (8y-SR) of 90% and 87%, respectively, whereas Pa and Wa exhibited poor prognosis (8y-SR:38% and 25%, respectively). As compared with the previous follow-up data in 2008 with same GGs, survival rates have been progressed especially in those with *MYCN*-amplification (cf. Wa from 0% to 45%). On the other hand, genome analysis of unfavorable cases (death within 2 years) in JN-H-07 suggested that additional chromosomal aberrations such as 12q amplification and 2p gain as well as mutations including *ATRX*, *ARID1 and MYCN* seems to affect the prognosis of these patients.

[Conclusion]

Our results suggested that array CGH-based GG subgroups can be useful for the tumor risk

classification. Correlation with other clinical markers and additional genomic markers such as gene mutations and expression signature will be also discussed.

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MicroRNA-204 suppresses neuroblastoma tumour growth through down-regulation of MYCN oncogene

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MYCN acts as a major oncogenic driver through pleiotropic effects controlled by multiple protein encoding genes as well as microRNAs (miRNAs) in neuroblastoma. MYCN activity is tightly regulated at the level of transcription and protein stability through various mechanisms. MYCN is further controlled by miRNAs, but the full complement of all miRNAs implicated in this process has not been determined through an unbiased approach. To elucidate the role of miRNAs in regulation of MYCN in neuroblastoma, we investigated the global miRNAs and mRNAs expression pattern in the *TH–MYCN* neuroblastoma mouse model and age-matched wild-type mice during the pre-tumour stage. Five miRNAs were selected to be examined further for their correlation to human patient prognosis and *MYCN* amplification. One of these miRNAs, miRNA-204 showed reduced expression in ganglia tissues of *TH–MYCN* versus wild-type mice. High miR-204 expression is significantly associated with better overall and event-free survivals, and also associated with low clinical stage. Moreover, high miRNA-204 expression correlated with non-*MYCN*-amplified human neuroblastoma tumour tissues. miRNA-204 expression is increased with MYCN siRNAs knockdown in *MYCN*-amplified BE(2)-C and Kelly neuroblastoma cells. Furthermore, chromatin immunoprecipitation assays revealed MYCN directly bound to the DNA region which encodes the miRNA-204 primary transcript in BE(2)-C cells. Importantly, transient transfection of miRNA-204 mimic or stably overexpression of miRNA-204 using doxycycline-inducible lentiviral construct significantly reduced MYCN mRNA and protein expressions, reduced cell proliferation and colony forming capacity both BE(2)-C and Kelly cell lines. Preliminary data from doxycycline-induced miRNA-204 overexpression in subcutaneous xenografts of stable BE(2)-C and Kelly cell lines showed reduced tumour growth in the animal models. For the first time, our data suggest that MYCN transcriptionally suppressed miRNA-204 by direct binding to its promoter, and miR-204 suppressed neuroblastoma cell growth through down-regulation of MYCN.

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Exploring m6A mRNA methylation for novel therapeutic chances in neuroblastoma

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Neuroblastoma is a tumor that poses important challenges to establishing more effective and less toxic therapies, and industry is hardly engaged in developing new specific drugs. Reasons are the marked heterogeneity of the tumor, a substantial lack of deep understanding of its molecular basis, the paucity of targetable driving mutations.
The ascertained role of segmental chromosomal aberrations and, therefore, gene expression imbalances in guiding aggressiveness and the embryonic derivation of neuroblastoma initiating cells could suggest an involvement of post-transcriptional control of gene expression in the phenotypic derangement of this tumor.
The importance of mRNA methylation has remained a mystery for decades until rapid advances in the field of epitranscriptomics and RNA sequencing have raised the exciting hypothesis that reversible *N*6-methyl-adenosine (m6A) modification of mRNA may constitute an essential mechanism of post-transcriptional regulation. Currently, the function of mRNA methylation and the signaling pathways controlling mRNA methylation are still in the route of being uncovered, but m6A-mediated gene expression control has already proved to be an indispensable process guiding self-renewal and differentiation of embryonic stem cells.
We found that the expression of the methyltransferase METTL14 is specifically higher in neuroblastoma compared to other solid-tumor cell types. We also found that increased levels of the METTL14 methyltransferase, along with that of the m6A-binding protein YTHDF1, are associated with worse clinical features and poor prognoses. When overexpressed, METTL14 increases the growth rate of neuroblastoma cells, while its depletion reduces proliferation and tumor-spheroid formation. Further data will be required to substantiate the hypothesis that modulation of mRNA methylation might have a direct impact on gene expression during neuroblastoma progression although the possibility of reversing m6A modifications through enzymatic inhibitors may open the way to conceive new prospects for therapeutic intervention.

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The mitochondrial genetic landscape in neuroblastoma from tumor initiation to relapse

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The mitochondrial (mt) genome differs from the nuclear genome by its high copy number, small size, intronless genomic structure, different mode of replication, exposure to oxygen radicals and increased DNA damage. While it is well-established that tumor-specific mt variants and mutations are present in many cancers, little is known about changes within the mt genome during tumor progression in general and during initiation and progression of neuroblastoma (NB) in particular. Analysis of mt sequences extracted from whole exome sequencing data of corresponding normal tissue, primary (diagnostic) tumors and relapsed tumors of 16 NB patients that we previously had investigated for nuclear mutations, revealed that most NB harbor tumor-specific mitochondrial variants. In relapsed tumors, the status of mt variants changed, as shown by increased number and spatio-temporal differences of tumor-specific variants, and by a concomitant decrease of germline variants. The spectrum of mutations at initial disease was consistent with replication-induced but not with reactive oxygen species-induced DNA damage and, in contrast to the nuclear genome, did not change at relapse, suggesting different mutating mechanisms. As mt variants are present in most NB patients, change during relapse and have a higher copy number compared to nuclear variants, they represent a promising new source of biomarkers for monitoring and phylogenetic analysis of NB.

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CPEB1 down-regulated the expression of MYCN *via* tumor-suppressor miRNA let-7 in human neuroblastoma cells

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Background/Purpose:
The four cytoplasmic polyadenylation element binding proteins (CPEB 1 – 4) function as translational regulator for mRNAs thus linking with tumorigenesis and tumor suppression. In our previous study, CPEB1 was found as a tumor-suppressing protein in neuroblastoma cells with significant changes in the expression of certain mRNAs as well as miRNAs. In this study, we focused on the molecular mechanism of how CPEB1 down-regulated oncogene expression and whether miRNA species play a role in such mechanism.
Methods:
Two human neuroblastoma cells, BE(2)-M17 and SH-SY5Y, were used in this study. Control and CPEB1-overexpressing neuroblastoma cells were harvested for RNA extraction. The levels of miRNAs and mRNAs were quantified by real-time RT-PCR. The relative expression was calculated by setting the individual miRNA or mRNA level in control cells as 100% or 1-fold.
Results:
In CPEB1-overexpressing cells, certain oncogenes were found with lower expression then control: MYCN (76.3% in BE(2)-M17 and 39.2% in SH-SY5Y cells), MYC (47.3% in BE(2)-M17and 35.1% in SH-SY5Y cells), and RAS (63.7% in BE(2)-M17 and 70% in SH-SY5Y cells). When examining the corresponding miRNAs targeting these oncogenes, let-7 miRNA family displayed a significant elevation in response to CPEB1 expression. Among let-7 members, let-7a showed a 2.27-fold increase in BE(2)-M17 and 1.77-fold increase in SH-SY5Y cells.
Conclusions:
Up-regulation of miRNA let-7 and concomitant down-regulation of MYCN may contribute to the tumor-suppressing function of CPEB1 in human neuroblastoma cells.

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Genetic characteristics of 494 neuroblastomas using genome-wide analysis combined with immunohistochemistry

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Background
Neuroblastomas (NB) exhibits substantial heterogeneity; while some cases show spontaneous regression, the prognosis of advanced NBs is still poor in spite of recent developments in treatments. The best-characterized genetic alterations include amplification of *MYCN*, amplification/mutation of *ALK*, and losses of 1p and 11q. However, genotype/phenotype correlations in NB are still to be elucidated. Thus, to explore genetic basis of NB, we performed integrated genetic analysis combined with immunohistochemistry in a large series of NBs.

Methods
We analyzed 494 NB samples, including stage 1, 2 (n = 106), stage 3 (n = 86), stage 4 (n = 282) and stage 4S (n = 20), using targeted deep sequencing for 10 NB related-genes (including *ALK*, *ATRX*, *ARID1A/1B*, *PHOX2B*, *PTPN11*). Copy number alterations were analyzed with SNP array. ALK Expression was also evaluated by immunohistochemistry in 241 NB samples obtained from Japan NB Study Group (JNBSG).

Results
In this series, deep sequencing allows the detection of *ALK* (7.7% of cases), *ARID1A /1B* (7.3%), *ATRX* (4.9%). Based on genomic alterations, six major NB subgroups with different genetic signatures were identified; group A (*ALK*), group B (*MYCN* and 1p LOH), group C (other mutations), group D (11q LOH), group E (chromosome 17 gain), and group F (silent). Group D showed higher age of onset (median 44 months), whereas group E contained younger patients (median 7 months), mainly infants ($p<0.0001$). There was a significant association of high ALK expression and 2p gain, suggests that *ALK* copy number gain is one of the potential mechanisms of high ALK expression. Of note, among cases with high ALK expression, *ALK* mutation is a significant prognostic factor rather than *MYCN* amplification.
Conclusion
In this study, we disclosed genetic and pathological landscapes of a large NB series, which provide novel insights in to the pathogenesis of NB.

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End of life care for children with neuroblastoma: a retrospective study from the Royal Children Hospital Brisbane

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Introduction. Despite advances in the treatment of Neuroblastoma and increased survival rates, the needs of those patients who cannot be cured must still be addressed. One in four patients will die of disease progression. These patients present a unique set of challenges at end of life beyond those inherent to the palliation of paediatric patients. These challenges are often related to the location of the primary tumour, the location and degree of spread of distal metastases, the sequelae of treatment, and the psychosocial burden of the disease and its symptoms.
Methods. We conducted a retrospective chart review of Neuroblastoma Patients treated at the Royal Children Hospital Brisbane between 2000 and 2014. One-hundred-and-one neuroblastoma patients were diagnosed and treated during this period, 29 died of disease progression, and 8 were excluded due to incomplete records.
Results. Of the 21 included patients, 8 received palliative chemotherapy and radiotherapy, 6 received palliative chemotherapy alone, and three palliative radiotherapy alone. Eight patients were managed exclusively by the Oncology team and 13 were referred to the Palliative Care team for input on symptom management and/or end of life care. Six of these 13 patients were referred in their last two weeks of life. The most common symptoms requiring management in these patients were: pain (12), nausea and vomiting (7), constipation (5)lethargy (3), and agitation (3).
Conclusions. This study highlights some of the challenges in the end-of-life care of Neuroblastoma patients. A palliative approach to management is often initiated early by the oncology service even when treatment is still focused on cure. A formal transition to palliative care may occur later in the illness trajectory. Markers of such care include the provision of an afterhours telephone support number for parents and a specialised paediatric palliative care service.

A better understanding of needs of these patients, and how to integrate palliative care early, is an area that could be further researched to enhance the quality of life of those patients whose disease cannot be cured.

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Physeal arrest leading to angular deformity after therapy with isotretinoin for high risk neuroblastoma (HR-NBL)

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Background: Isotretinoin is part of standard therapy of HR-NBL given for 6 courses at 160mg/m2/day. Partial or complete growth arrest, physeal narrowing, short stature, and osteopenia have been reported with isotretinoin use. However this has primarily been reported in the dermatology literature where dosing is lower but given on a protracted schedule.

Methods: Clinical data on all patients between 5/2006 – 1/2016 with HR-NBL treated at Children's Hospital Los Angeles who had orthopedic consults not related to fractures were retrospectively reviewed. Data abstracted were: disease/therapy history, orthopedic presentation history, type of bony defect and surgical intervention.

Results: 111 patients with HR-NBL (69 male) were identified. The median age at diagnosis was 3 years (5 m – 15 y). 3 (2 male)/111 were noted to have distal femoral physeal growth arrest, all with isotretinoin exposure at 7 years of age (two stage 3, one stage 4). All presented with a valgus deformity of the knee on exam 1-4 years from initiation of isotretinoin therapy. These patients developed a physeal bridge of the distal femur, involving the central and lateral physis, confirmed by radiographs and MRI. All had surgical correction with one who developed a significant leg-length discrepancy, requiring further surgical intervention.

Conclusions:

Growth arrest leading to angular deformity was noted in 3% of our patient population who were treated with isotretinoin. The same age of exposure to istotretinoin, the pattern of presentation (valgus deformity) as well as physeal arrest of the central and lateral aspect of distal femur suggest a common mechanism of action. Careful consideration to bony abnormalities, particularly for this age group may be warranted.

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A multidisciplinary team care improved outcomes for children with high-risk neuroblastoma

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Background and aims: Neuroblastoma (NB) is the most common extracranial solid tumor in children. The current treatment for NB is risk-adapted multimodal therapy. Poor coordination and miscommunication potentially exist between different health-care professionals. Here we reported the treatment results of NB patients in our hospital and the effects of the multidisciplinary care for the outcomes of NB patients.
Methods: Newly diagnosed NB patients in National Taiwan University Hospital began receiving TPOG N2002 protocol for NB treatment since 2002. We started multidisciplinary team approach, including nurse-led case management, for our patients treated by this protocol in the end of 2009 to enhance communication among relevant disciplines and improve timeliness of care. We compared the treatment results before and after the multidisciplinary approach of these patients.
Results: Fifty-eight patients treated by above protocol from 2002 to 2014 were included for analysis. There were 29 patients received treatment during 2002 to 2009 (group 1) and another 29 patients were during 2010 to 2014 (group 2). The 5-year overall survival (OS) and event-free survival (EFS) rate for all 58 patients were 59% and 54.7%, respectively. When comparing treatment results before and after the multidisciplinary approach, group 2 patients showed significant superior outcome to group 1 in 3-year EFS (P=0.046), but not OS (P=0.162). In further analysis, patients with HR, stage 4 disease, non-amplified MYCN belonged to group 2 had significant better 3-year EFS (P<0.05) than those belonged to group 1. In multivariate analysis of prognostic factors among 58 patients, the multidisciplinary approach is the only significant independent prognostic factor.
Conclusions: Multidiscipline team approach can benefit patients with high-risk NB. The long term effects of this multidiscipline care for our patients needs further followed-up.

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The requirement for accurate standardization and methodology of dosimetry in international trials incorporating molecular radiotherapy (MRT) in the treatment of high-risk neuroblastoma

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Molecular radiotherapy (MRT) using radiopharmaceuticals ^{131}I-mIBG and ^{177}Lu-DOTATATE is increasingly utilized in the treatment regime of primary refractory or relapsed high-risk neuroblastoma. Whilst individualized dosimetric treatment planning is mandatory for external beam radiotherapy and brachytherapy, there is a need for treatment planning or post-therapy dosimetric verification to be implemented in MRT.
Indeed, the avoidance of unacceptable bone marrow and renal toxicities is essential for safety, and knowledge of tumour dose is desirable for correlation for response. Owing to the complexity of the individual disease status and metabolism, there is an unknown relationship between administered activity and absorbed dose. There is therefore a need for precise and accurately delivered tumour and organ sensitive absorbed doses, which will correlate with improved outcomes.
Currently, almost all trials in Europe incorporating MRT are multi-centre as there are too few patients for national statistics or nationally based randomized clinical trials to give reliable and meaningful data in a short time frame. There is therefore an absolute requirement for standardization of methodology for the assessment of dosimetry, which is essential for major European trials incorporating MRT.
UCLH is one of the leading centres in Europe providing MRT for Neuroblastoma. As such, we are key implementers and advisors in the role of dosimetry in MRT for other European sites.
We are using, or intending to use MRT dosimetry in the following open or proposed trials:
A phase IIa trial of ^{177} Lutetium Dotatate in children with primary refractory or relapsed high risk neuroblastoma (LUDO trial)
An international multicenter phase II randomized trial comparing two intensification treatment strategies for metastatic neuroblastoma patients with a poor response to induction chemotherapy (VERITAS trial).
An international phase I trial of ^{131}I-mIBG therapy with Nivolumab and ch.14.18 anti-GD2 immunotherapy.
We present here our methodology and initial dosimetry results that we aim to see incorporated into all future international trials incorporating MRT, and our role in standardization across centres.

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Antibodies specific for O-acetyl-GD2 mediates the same efficient anti-neuroblastoma effects as therapeutic ch14.18 antibody to GD2 without antibody induced allodynia.

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Introduction
Monoclonal antibodies against GD2, such as dinutiximab—a chimeric mAb ch14.18— have become benchmarks for neuroblastoma therapies. Pain, however, can limit immunotherapy with anti-GD2 therapeutic antibodies like ch14.18. This adverse effect is attributed to acute inflammation via complement activation on GD2-expressing nerves. Thus, new strategies are needed for the development of treatment intensification strategies to improve the outcome of these patients.
Methods
We established the mouse-human chimeric antibody c.8B6 specific to OAcGD2 in order to reduce potential immunogenicity in patients and to fill the need for a selective agent that can kill neuroblastoma cells without inducing adverse neurological side effects caused by anti-GD2 antibody immunotherapy. We further analyzed some of its functional properties compared with anti-GD2 ch14.18 therapeutic antibody.
Results
With the exception of allodynic activity, we found that antibody c.8B6 shares the same anti-neuroblastoma attributes as therapeutic ch14.18 anti-GD2 mAb when tested in cell-based assay and in vivo in an animal model.
Discussion
The absence of OAcGD2 expression on nerve fibers and the lack of allodynic properties of c.8B6-which are believed to play a major role in mediating anti-GD2 mAb dose-limiting side effects-provide an important rationale for the clinical application of c.8B6 in patients with high-risk neuroblastoma

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Importance of quality assurance in radiotherapy for optimal local control. A report from the SIOPEN radiotherapy committee of the High Risk Neuroblastoma Trial (HR-NBL1/SIOPEN)

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Background: Local control plays an important role in high risk metastatic and MYCN-amplified (MNA) localized neuroblastoma, as part of the multimodality treatment approach. In a subset of 100 patients with complete imaging and radiotherapy (RT) data sets treated in the current HR-NBL1/SIOPEN trial, we previously reported on the radiotherapy quality assurance (RTQA), identifying unjustified deviations in 17% of patients. The majority of these were an inappropriate target volume and/or an inappropriate dose. Methods: The aim of this analysis was 1) to investigate local control and event-free survival (EFS) in the full cohort of 1467 patients (stage 4: 1180, MNA: 170, unknown: 117) having received irradiation according to the HR-NBL1/SIOPEN trial recommendation after high dose chemotherapy and autologous stem cell reinfusion between 2002-2015. 2) to correlate local control with RTQA in the subset of 100 patients with previously performed RTQA. Results: In the group of 1467 patients no difference was detected neither in the cumulative incidence of local relapse nor in event-free survival between patients receiving < 21Gy, 21Gy as prescribed or > 21Gy. However, only in the subgroup of 170 patients with localized MNA disease a trend towards improved 5-year EFS was observed in patients receiving 21Gy as prescribed in contrast to those patients who did not (5yr-EFS:70% versus 62%). In the subset of 100 patients with previous RTQA a significantly increased local recurrence rate of 44% (10/23pts) was found when RT had not been delivered according to protocol in contrast to 22% (17/77pts) in patients treated with correct volume and dose (p=0,042). Conclusion: These early results show the importance of RTQA for local control in the treatment of neuroblastoma. Further confirmation in a larger patient population is currently being performed. Prospective central validation of RT planning prior to RT delivery could improve overall disease control.

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Stromal collagen type XI alpha 1 COL11A1 expression in neuroblastoma

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Purpose: Alpha1 (XI) collagen COL11A1 is one of the tumor microenvironment that support collagen remodeling and it has been associated with carcinogenesis in malignant tumors. However, COL11A1 levels in Neuroblastoma (NB) have not been evaluated. The aim of this study is to clarify the COL11A1 expression and its relation with Neuroblastoma outcome in patients.
Methods: A total of 80 NB patients in Children Hospital II were enrolled in this study. The expression levels of COL11A1 genes were measured using quantitative reverse transcription polymerase chain reaction (qRT-PCR). Immunohistochemistry was used to localize COL11A1 expression in tumor or stroma region. Association with clinoco-pathological NB and COL11A1 were assessed in specimens collected from primary, metastases and recurrent NB.
Results: COL11A1 expression was detected in 23% of 83 NB and correlated with stage 3/4 (P=0.23), recurrence (n=8, P=0.01), MYCN amplification (P=0.04). Immunostaining showed that the COL11A1 co-localized with Vimentin in intratumoral stroma, but not the peripheral stroma. Remarkably, such COL11A1 mRNA expression is significantly associated with MYCN amplification p<0.05 in the public microarray database. Finally, our data the sorting of tumor vs stroma, was highly expressed intra-stroma tumors isolated from amplified MYCN specimens and INSS stage 3,4,4S.
Conclusion: Our findings examine the COL11A1 expression in advantaged cancer and suggest that microenvironment of stromal COL11A1 is a significant risk for cancer prognosis and disease progression.

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Dinutuximab combined with chemotherapy in patients with multiply relapsed/refractory high risk neuroblastoma (HR-NBL)

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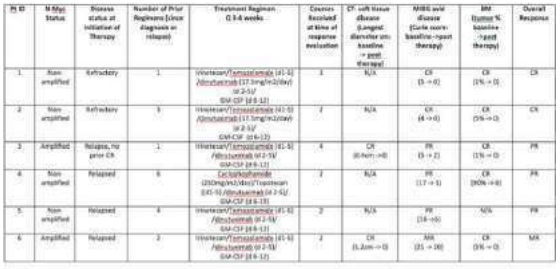
Background: Dinutuximab is part of standard upfront therapy for patients with HR-NBL.

Conventional chemotherapy with immunotherapy has successfully been administered to patients with first relapse/refractory neuroblastoma but limited data exists on efficacy in patients resistant to multiple prior therapies.

Methods: Patients with HR-NBL with refractory/relapsed disease enrolled on NANT biology study and treated with dinutuximab concurrently with chemotherapy at Children's Hospital Los Angeles were reviewed. Disease and treatment history, adverse events, and response per NANT criteria v1.2 were analyzed.

Results: Six HR-NBL patients (refractory n=2, relapse n=4) were identified (table 1). Monthly cycles of dinutuximab (17.5mg/m2/dose), with either combination of irinotecan (50mg/m2/day) and temozolamide (100mg/m2/day) or combination of cyclophosphamide (250mg/m2/day) and topotecan (0.75mg/m2/dose), followed by 7-14 days of GMCSF were administered. No serious adverse events, including lack of fever or neutropenia, were noted despite limited marrow reserve. Complete remission (CR) was observed in 2/6, partial response (PR) in 3/6, and minor response (MR) in 1/6. Two patients with target lesions achieved CR. MIBG response showed 2/6 CR, 3/6 PR, and 1/6 stable disease. All patients with evaluable bone marrow (BM) achieved CR (n=5); including patients achieving first ever tumor-negative BM (n=3) from diagnosis or since relapse (n=2). Elevated catecholamines normalized within a week of therapy when elevated.

Conclusions: Combination chemotherapy and dinutuximab is well tolerated. The exceptional response rate (5 out of 6 with PR/CR) in patients resistant to multiple prior therapies is encouraging and this approach warrants further use and study in this cohort of patients.



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Evaluation of genetic modified anaerobic *Salmonella* typhimurium as therapy for neuroblastoma: Comparison of response of orthotopic mouse models with different immunological backgrounds

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Background: Neuroblastoma is an aggressive paediatric tumor and highly resistant to the current therapeutic approaches. Here we proposed a new treatment strategy by using an engineered anaerobe *Salmonella* (Sal-YB1).

Method: Nude and NOD-SCID mice were used in the intra-adrenal orthotopic neuroblastoma mouse models. They were divided into no intervention and Sal-YB1 treated arms. Sal-YB1 was administered through the tail vein. The therapeutic effectiveness, biosafety and related mechanism were studied.

Results: No mice from treatment and control arms died of treatment. Tumor regression rate was 70% in nude mice and 30% in NOD-SCID mice; and the treatment effect was significantly different ($P < 0.05$). *Salmonella* could not be detected in urine; 75% mice had Sal-YB1 found in stool initially but they all turned negative within two weeks. Tumor tissues had a relatively higher Sal-YB1 positive rate and necrotic change in Sal-YB1 treated mice. It was significant difference for IRAK ($P < 0.05$) and IkBα ($P < 0.001$) in the tumor mass between YB1-treated & -untreated group. Sal-YB1 could significantly up-regulate TLR4 expression in YB1-treated tumor samples ($P < 0.001$). There were higher release of TNFα found in macrophage and mouse tumor tissue with Sal-YB1 treatment ($P < 0.05$). Neuroblastoma cells supplemented with the supernatant of Sal-YB1 pre-treated macrophage revealed significantly higher cell death ($P < 0.05$). TNFα and pan-Caspase inhibitors could reverse the anti-neuroblastoma effect by macrophage ($P < 0.001$).

Conclusion: Our approach provides a new paradigm in treaating neuroblastoma. The macrophages may play a critical role in Sal-YB1 directed therapy.

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Preclinical studies of anti-PDL-1/PD-1-based combination immunotherapy for Neuroblastoma

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Background: IL-21 is an immune enhancing cytokine produced by T helper cells that showed promising results in pre-clinical and clinical cancer immunotherapy. We previously observed that the administration of anti-CD4 cell-depleting antibody strongly enhanced the anti-tumor effects of recombinant (r)IL-21immunotherapy in a syngeneic model of disseminated Neuroblastoma (NB). The removal of pre-existing and NB-induced CD4+CD25high Treg cells by the anti-CD4 mAb synergized with IL-21 in the induction of anti-NB CD8+ T cell responses. Murine NB cell lines express Programmed Death-Ligand (PD-L)1 and its interaction with PD-1 on immune cells may induce immune tolerance favoring tumor growth and spread. Here we studied the therapeutic effects of a combination of rIL-21 and PD-1/PD-L1 immune checkpoint blockers in disseminated syngeneic NB.
Methods: A/J mice were injected intravenously with either Neuro2a-Luc (GD2-) or NXS2-Luc (GD2+) NB cell lines to induce disseminated disease. rIL-21 therapy (1 µg/dose) was administered for 5 times. Immune checkpoint blocker, anti-PD-L1 mAb (200 µg/dose), was administered intra peritoneally for 5 times from intravenous injection of tumor cells. The tumor-free survival of treated mice was evaluated.
Results: Both anti-PD-L1 mAb and rIL-21 alone had a limited effect on NB development. The combination of anti-PD-L1 mAb and rIL-21 could not cure mice from disseminated NB. We then found that murine NB cell lines express PD-L2, which is an alternative ligand for PD-1 and thus NB could escape anti PD-L1 blockade.
Conclusion: Our data indicate that the use of anti PD-L1 mAb cannot cure disseminated NB even if associated with rIL-21. We are currently investigating the effects of anti-PD-1 mAb either alone or in combination with IL-21 or antibodies targeting Treg cells to further evaluate the role of this immune checkpoint in pre-clinical NB models.

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Which miRNAs should be developed into novel therapeutics for neuroblastoma?

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MicroRNAs (miRNAs) are a class of non-coding RNAs which repress the translation of mRNA target genes. A handful of miRNAs have already entered early-stage clinical trials in other cancer types.
A genome-wide functional screen of 1280 miRNAs using both miRNA mimics (overexpression) and antisense inhibitors was carried out in two neuroblastoma (NB) cell lines (including MYCN-amplified Kelly). This screen was also performed in the presence of low dose chemotherapy, which allowed us to discover 22 synthetic lethal miRNAs which synergize with chemotherapy, in addition to 114 outright lethal miRNAs. Having validated the majority of these in a secondary screen that included an additional MYCN-amplified cell line (SK-N-DZ), we are now proceeding to investigate mechanisms of action and direct target genes. Through integrating several established bioinformatics tools we can investigate patterns of over-represented target genes/pathways among several miRNAs with similar phenotypes. Clinical relevance of miRNAs and their targets will be examined using datasets from NB tumours with known outcomes. In order to assess the miRNAs in vivo as potential therapeutics we aim to use nanoparticle delivery vectors in patient-derived xenograft models of high-risk NB.

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Generation and characterization of a new chimeric human/mouse anti-idiotype antibody ganglidiximab for active immunotherapy against neuroblastoma

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Introduction: Vaccination with proteins mimicking ganglioside GD_2 is a promising strategy to induce an active anti-tumor immune response against neuroblastoma (NB). We previously showed *in vivo* efficacy of a murine anti-idiotype antibody (anti-Id Ab) ganglidiomab acting as a GD_2 surrogate. To reduce murine origin for human studies, we generated a human/mouse chimeric anti-Id Ab ganglidiximab by replacing murine with corresponding human constant regions and report here its GD_2-mimicking properties.
Methods and Results: DNA sequences encoding for variable regions of ganglidiomab heavy and light chain were synthesized by RT-PCR using RNA of ganglidiomab-producing hybridoma cells and inserted into mammalian expression vectors encoding for human IgG1 heavy and light chain constant region, respectively. Next, CHO cells were stably co-transfected with both vectors to establish a cell line permanently producing ganglidiximab. After purification from cell culture supernatants, binding of ganglidiximab to anti-GD_2 Abs of the 14.18 family (14G2a, ch14.18, ch14.18-dCH2, ch14.18-IL-2, hu14.18 and hu14.18-IL-2) was shown by ELISA and binding affinities were determined using Biacore technique. Importantly, GD_2-specific NB cell lysis mediated by ch14.18 as well as binding of anti-GD_2 Abs to the nominal antigen GD_2 was competitively inhibited by ganglidiximab confirming its anti-idiotypic function. Finally, vaccination of mice with ganglidiximab resulted in induction of GD_2-specific Abs.
Conclusion: We generated and characterized a new chimeric human/mouse anti-Id Ab for active immunotherapy against NB and demonstrated induction of a GD_2-specific humoral immunity in mice providing a baseline for protein vaccine development.

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Role of Surgery in Patients older than 18 months with localized Neuroblastoma (Stage 1-3)

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Purpose
Although several studies have been conducted on the role of surgery in localized neuroblastoma the impact of surgical timing and extent of primary tumor resection on outcome remains controversial.
Patients and Methods
Patients from the German clinical trial NB97 with localized neuroblastoma INSS stage 1-3 age >18 months were included for retrospective analysis. Imaging reports were reviewed by two independent physicians for Image Defined Risk Factors (IDRF) and operation notes and corresponding imaging reports were analyzed for surgical radicality. The extent of tumor resection was classified as complete resection (95-100%), gross total resection (90-95%), incomplete resection (50-90%), and biopsy (<50%) and correlated with local control rate and outcome.
Results
A total of 181 patients were included in this study At diagnosis, 45.1% of all patients had complete resection, 12.5% gross total resection, 4.3% incomplete resection, and 36.4% biopsy only. Complication rate was 16.9%. Image defined risk factors (IDRF) predicted extent of resection.
76 patients underwent more than one primary tumor resection. At best surgery, 70.2% of patients achieved complete resection of the primary tumor, 15.5% gross total resection, 13.8% incomplete surgery, and 0.05% biopsy only. Cumulative complication rate was 20.1% and surgery associated mortality rate was 0.8%. Patients with complete resection had a better local-progression-free survival (LPFS), event-free survival (EFS) and OS than the other groups. Subgroup analyses showed better EFS for patients with complete resection in INSS stage 3 disease and in localized neuroblastoma with MYCN amplification. Multivariable analyses revealed resection (complete vs. other), and MYCN (non-amplified vs. amplified) as independent prognostic factors for EFS and OS .
Conclusion
In patients with localized neuroblastoma age 18 months or older, especially in patients with MYCN amplification, extended surgery of the primary tumor site improves local control rate and survival with an acceptable risk of complications.

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More is less: radiation exposure to family caregivers and health care providers of paediatric neuroblastoma patients receiving ^{131}I-MIBG therapy in Canada

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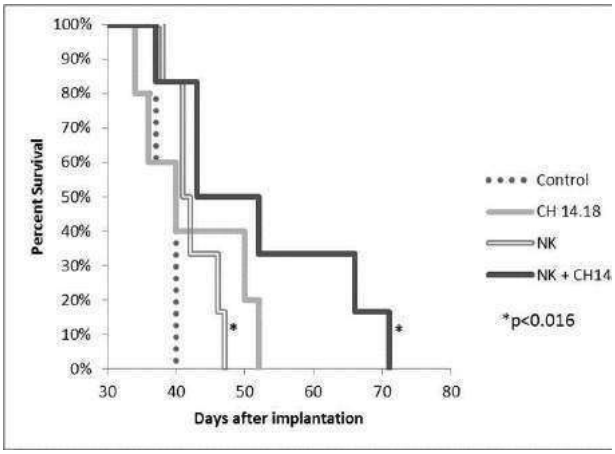
Purpose: This retrospective study determined the measured radiation exposure to family Caregiver (FCG) and Health Care Providers (HCP) who cared for patients receiving ^{131}I-MIBG therapy during hospital admissions during year one of operation.
Methods: Design of our new ^{131}I-MIBG therapy suite was guided by Canadian Nuclear Safety Commission (CNSC) regulations for occupational exposure to the general public (non- NEW). Special attention was paid to the urine drainage system. HCP completed mandatory class instruction and online education modules on caring for ^{131}I-MIBG patients. FCG were provided with radiation safety education from a Radiation Safety Officer (RSO) and Neuroblastoma team. ALARA principles were a key component to the education programs. ^{131}I-MIBG therapy was administered to 12 children (13 treatments) (average age 8, range 3-14 years) for relapsed and refractory neuroblastoma.Direct read dosimeters were used to measure radiation exposure to the FCG and HCP from radioisotope administration until discharge.
Results: The administered activity ranged from 8.51 to 32.23 GBq (average 18.13 GBq). The average FCG exposure for 13 treatments was 0.309 mSv (range 0.028 to 0.947 mSv per FCG). The average HCP exposure was 0.038 mSv with a range from 0 to 0.165 per treatment. Length of stay was between 4 and 10 days (mean 8 days). Average length of stay is longer than comparable ^{131}I-MIBG programs outside Canada.
Discussion: Length of stay was dependent on CNSC requirements, pre-determined discharge levels, distance and travel requirements and family housing conditions. Exposure to HCP and FCG was well below CNSC regulatory limits and considerably less than that reported from other comparable institutions. The design of our facility enhanced shielding and unique urine drainage system likely contributed to the very low exposure for caregivers.

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Anti-GD2 antibody combined with activated natural killer cells leads to improved survival and decreased metastasis in a minimal residual disease mouse model of neuroblastoma

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Introduction: Relapsed metastatic disease is the primary cause of death for high-risk neuroblastoma patients following tumor resection. The FDA-approved anti-GD2 antibody (ch14.18) has been shown to improve survival in high-risk patients, and synergistic therapies are being sought. As previous studies demonstrated that natural killer (NK) cells augment the cytotoxicity of ch14.18 activity, we hypothesized that activated NK cells combined with ch14.18 would decrease metastasis and increase survival in a minimal residual disease mouse model of neuroblastoma.
Methods: $1x10^6$ CHLA-255 neuroblastoma cells were intrarenally implanted into NSG mice, which were then randomized into 4 groups: control (n=5), ch14.18 (n=5), NK cell (n=6), NK cell+ch14.18 (n=6). Primary tumors were surgically resected on day 7, and mice were then treated 2x a week for 4 weeks. Incidence of liver and bone marrow metastasis was assessed by bioluminescent imaging. Survival was analyzed by Kaplan-Meier technique using log-rank test to determine significance. The incidence of metastasis was analyzed using Fisher's exact test.
Results: At week 5, the NK cell+ch14.18 group had significantly less incidence of metastasis than the control and ch14.18 groups (1/6 vs 5/5 vs 5/5 liver, p=0.015; 0/6 vs 4/5 vs 4/5 femur, p=0.015). Survival in the NK cell+ch14.18 group was significantly increased compared to control (mean survival 52 vs 38 days, p=0.0157).
Conclusions: NK cells combined with ch14.18 following primary tumor resection significantly decreases incidence of metastasis and increases survival in NSG mice. This combination may serve as a valuable therapeutic option for the treatment of metastatic disease in children with high-risk neuroblastoma.



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TLR3-Mediated innate immune response in the treatment of neuroblastoma

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Purpose

Toll-like receptor 3 (TLR3), an initiator of the innate immune response to pathogens, predicts a favorable prognosis when highly expressed in neuroblastoma (NB). *In vitro*, TLR3 showed a high expression in *MYC*-nonamplified NB cells. Since the expression of c-MYC/MYCN proteins is high in aggressive NB, it is uncertain to what degree that TLR3 agonists may exert their effects on NB cell death.

Materials and Methods

92 NB patients, 47 nonobese/severe combined immunodeficiency mice, six human NB cell lines were involved in this study. Treatment with TLR3 agonist-polyinosinic-polycytidylic acid [poly(I:C)] was used to examine cell viability, apoptosis and tumor growth in NB. TLR3- induced signaling patterns in NB cell lines were detected by Western blot and in human or mice NB tissue samples were evaluated by immunohistochemistry or quantitative RT-PCR.

Results

Poly(I:C) significantly suppressed the tumor growth of SK-N-AS mice, but not of SK-N-DZ mice. Significant downregulation of c-MYC and upregulation of p-IRF3, active NF-kB, MnSOD, 8-OHdG, activate caspase-3 and cleaved poly (ADP-ribose) polymerase were induced by poly(I:C) in SK-N-AS mice. Knockdown of TLR3 attenuated the inhibition of c-MYC protein expression regulated by poly(I:C) in SK-N-AS cells. In children with NB, positive melanoma differentiation-associated gene 5 (MDA5) staining strongly correlated with *MYCN* non-amplified NB tissues. In AS cells, simultaneously targeting MDA5 and TLR3 showed the best effect to rescue poly(I:C) induced up-regulation of mitochondrial antiviral signaling protein, caspase-9, active caspase-3, and apoptosis. Patients with double positive staining of MDA5 and TLR3 had the most favorable clinical outcome.

Conclusion

c-MYC overexpression may increase sensitivity to poly(I:C) treatment in NB, and be suppressed by TLR3 to induce ROS-mediated apoptosis. Activation of MDA5 may serve as a complementary role in the TLR3 activated suppression of NB. The role of TLR3, c-MYC and MDA5 expression in NB may not only shed light on the pathogenesis of NB but also point out a potential therapy of NB by targeting innate immune system.

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Interleukin-2 adds toxicity to long term infusion treatment regimen of ch14.18/CHO antibody without measurable additional activity in relapsed/refractory neuroblastoma patients.

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Background: Long-term infusion (LTI) emerges as a promising new delivery method of ch14.18/CHO for relapsed/refractory neuroblastoma patients. The role of concomitant treatment with scIL2 is unclear in this setting.

Methods: Pts (74) were treated with 5 cycles of LTI with 100 mg/m^2 ch14.18/CHO (d8-17) combined with 6x10^6 IU/m^2 sc IL-2 (d1-5; 8-12) (53 Pts) and without IL2 (21Pts) in a single center program. Surrogate parameters for activity and pain toxicity were determined using complement dependent cell mediated cytotoxicity (CDC), the whole blood cytotoxicity test (WBT) (d1, 8, 15) and analysis of the intravenous (iv) morphine usage (d8-17).

Results: In CDC and WBT assays, GD2 specific killing of neuroblastoma cells was found in all patients and cycles. The cytotoxic activity in base line samples preceding ch14.18/CHO applications increased over cycles, indicating persistent lytic activity over the entire treatment period of 6 months. This effect GD2 specific and was inhibited by anti-idiotypic antibody ganglidiomab. Interestingly, there was no difference cytotoxic activity at any time point between patients treated with or without IL2.

Analysis of iv morphine usage in cycle 1 of patients treated in combination with scIL2 revealed a cumulative dose of 2.5 mg/kg/cycle in contrast to patients treated with ch14.18/CHO only (1 mg/kg/cycle). This corresponds to a >60% increase of the pain toxicity by additional scIL2 treatment. Reduction of iv morphine usage in subsequent treatment cycles was observed in both cohorts. However, in patients treated without additional scIL2, 0% of patients required iv morphine in cycles 3 or greater in contrast to 29% in the scIL2 combination group.

Conclusion: Neither the surrogate WBT activity parameter nor the higher pain toxicity profile support the combined treatment of antibody with IL2 in above pilot studies. Ongoing SIOPEN trials (EudraCT 2009-018077-31; EudraCT2006-001489-17) randomizing LTI ch14.18/CHO ± scIL2 in front line and relapsed/refractory patients will clarify the optimal approach.

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Galactose-α-1,3-galactose (a-Gal) glycosylation determinant on ch14.18 antibodies produced by CHO- or SP2/0 cell lines – potential clinical impact

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Background:
While α-gal is present as major carbohydrate in non-primate mammals, anti-α-gal is the most abundant natural immunoglobulin in humans. α-gal glycosylation of therapeutic antibodies may thus have an impact on their clinical properties, especially regarding their potential for allergic reactions. Murine production cell lines such as SP2/0 are known to lead to α-gal glycosylation, whereas CHO cell lines usually lack this property. In this regard, we tested anti-GD2 antibodies ch14.18 derived from murine SP2/0 and CHO cell lines.
Methods:
A detailled comparative head-to-head glycan profiling of ch14.18/CHO and ch14.18/SP2/0 (including dinutuximab) was performed. The glycans were cleaved from the antibody backbone enzymatically using PNGase F and were labelled with a 2AB fluorescent tag for determination. Labelled glycans were seperated using Hydrophilic Interaction Chromatography (HILIC), and the identity of the eluted glycans was determined based on the calculated Glucose Unit (GU) values as well as by ESI-QTOF mass spectrometry.
Results:
The presence of α-gal structures in ch14.18 manufactured from murine SP2/0 cell lines was clearly demonstrated. None of these structures were identified in any of the CHO cell derived ch14.18 batches. Whereas levels of up to 8.17% of α-gal structures were determined for ch14.18/SP2/0 (a product comparable to that used within COG study ANBL0032 (Yu, 2010)), a level of 1.12% was determined for dinutuximab.
Discussion:
The glycosylation analysis of ch14.18 antibodies derived from SP2/0 or CHO cells confirmed literature data regarding α-gal glycosylation. SP2/0 derived ch14.18 batches contain α-gal although percentages may differ considerably. No α-gal whatsoever was found in ch14.18/CHO. Clinical consequences of α-gal glycosylation of ch14.18 may relate to their allergic potential. As published in the EPAR for Unituxin (dinutuximab) out of 798 neuroblastoma patients 81.1% reported an allergic reaction-related event, 29% were reported severe (Grade 3-4) and 18% had anaphylactic reactions. Out of 514 neuroblastoma patients treated with ch14.18/CHO, 20% reported an allergic reaction-related event (including 0.8% with anaphylactic reactions), 6% were reported to have severe (Grade 3-4) allergic reaction-related events.

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Entrectinib is a potent inhibitor of Trk-driven neuroblastomas in a xenograft mouse model

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Background: Neuroblastoma (NB) is characterized by clinical heterogeneity, and the Trk neurotrophin receptors play important roles in this behavior. High-risk NBs frequently overexpress TrkB and its ligand, leading to invasion, metastasis, angiogenesis and drug resistance. We wanted to determine if entrectinib (RXDX-101, Ignyta, Inc.), an orally bioavailable pan-Trk, Alk and Ros1 inhibitor, was effective in our TrkB-expressing NB model.
Methods: We tested the *in vitro* effects on growth of entrectinib either alone or in combination with the chemotherapeutic agents Irinotecan and Temozolomide (Irino-TMZ) on an SH-SY5Y NB cell line stably transfected with TrkB. These inhibition results were confirmed by both RT-CES and SRB assays. We also studied the *in vivo* growth inhibition of entrectinib in NB xenografts as a single agent or in combination with Irino-TMZ.
Results: Entrectinib significantly inhibited the growth of TrkB-expressing NB cells *in vitro*, and it significantly enhanced the growth inhibition of Irino-TMZ when used in combination. Single agent therapy resulted in significant tumor growth inhibition in animals treated with entrectinib alone compared to untreated control animals [$p<0.0001$ for event-free survival (EFS)]. The addition of entrectinib to Irino-TMZ also significantly improved the EFS of animals compared to vehicle or Irino-TMZ treated animals ($p<0.0001$ for combination vs. control, $p=0.0012$ for combination vs. Irino-TMZ). There was no apparent toxicity of entrectinib treatment alone, and no additional toxicity when entrectinib was added to Irino-TMZ.
Conclusions: Our data show that entrectinib significantly inhibits growth of TrkB expressing NB cells both *in vitro* and *in vivo*. It also significantly enhances the efficacy of conventional chemotherapy *in* vitro and in our NB xenograft model. Thus, Entrectinib is a very promising targeted therapy for NBs and other Trk-expressing tumors. Entrectinib is currently entering an industry-sponsored, multi-institutional phase I clinical trial for children and adolescents with NBs and other recurrent or refractory solid tumors.

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Impact of radiotherapy and curie score on bone relapse in high-risk neuroblastoma

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Background: Our center has treated bone metastasis of high-risk neuroblastoma with severe bone destruction at diagnosis with 20-Gy radiotherapy even after disappearance of MIBG positivity at the completion of induction therapy. Our purpose of this report is to analyze the impact of Curie score (CS) and radiotherapy to the outcome of high-risk neuroblastoma.
Methods: Thirty-three consecutive patients with stage 4 neuroblastoma were treated between 2002 and 2014. Patients received induction chemotherapy with five cycles of standard agents in Japan, resection of the primary tumor, followed by high-dose chemotherapy (HDC; TT+LPAM (n=17), BU+LPAM (n=9), MEC (n=4)) with autologous PBSCT and local irradiation. We analyzed CS at completion of induction chemotherapy.
Results: The 5-year OS and PFS for 33 patients of stage 4 neuroblastoma were 60.5+/-9.5% and 51.4+/-9.2% respectively. Three patients died of therapy-related toxicity during the protocol, so we exclude them from the analysis of bone metastasis. There was a significant EFS difference according to CS at the completion of induction chemotherapy, CS=0 (n=20) vs CS>0 (n=10), with 5-year EFS 60.2±12.3% vs 18.0±15.1% (p=0.035) respectively. Sixteen patients with CS=0 were irradiated on metastatic bone sites, and 5 of them relapsed at non-irradiated sites (n=4) or irradiated bones (n=1). In contrast, 2 patients out of 4 patients without irradiation and CS=0 relapsed at bone metastatic sites previously existed. There was no difference of EFS between irradiated and non-irradiated patients with CS=0 (p=0.738). In CS>0 patients, 9 out of 10 patients were irradiated on metastatic bone sites, but 6 patients relapsed at previously irradiated sites. Of note, 5-year EFS of patients with *MYCN* amplified and CS=0 (n=11) was 80.8±12.2% compared with 55.6±21.3% of patients with *MYCN* non-amplified and CS=0 (n=9) (p=0.130).
Conclusion: CS before HDC is useful to predict the outcome of advanced neuroblastoma as previously reported. Although we could not identify the efficacy of bone irradiation to the patients with CS=0, EFS of patients with *MYCN* amplified and CS=0 has been improved.

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Phase II study of the combination of bevacizumab plus irinotecan and temozolomide for relapsed or refractory neuroblastoma

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Background: The rationale for studying the combination of bevacizumab plus irinotecan and temozolomide (BIT) in neuroblastoma is based on: (a) Vascular endothelial growth factor (VEGF) expression is associated with aggressive phenotype. (b) Anti-VEGF antibody bevacizumab enhances irinotecan-mediated suppression of xenografts. (c) Irinotecan+temozolomide (IT) is a standard salvage chemotherapy. (d) Bevacizumab safety was established in pediatric phase I studies.
Methods: We completed a phase II study to evaluate tumor responses to BIT in patients with measurable/evaluable refractory or relapsed high-risk neuroblastoma (www.clinicaltrials.gov NCT01114555). Each cycle consisted of bevacizumab (15mg/kg intravenously) (days 1 and 15) plus irinotecan (50mg/m^2/day intravenously) and temozolomide (150mg/m^2/day orally) (days 4-8). Patients could have previously received but not relapsed on IT. Patients were monitored for toxicity and response assessed after every two cycles; cycles could be repeated every four weeks after reversal of toxicities, and if progressive disease (PD) was not observed. An early stopping rule for efficacy mandated continuing study therapy only if >5/27 evaluable patients achieved partial (PR) or complete response (CR) based on INRC after 4 cycles.
Results: 33 heavily-pretreated (25 previously received IT) patients (9 primary refractory; 24 relapsed) received 1 (n=7 patients), 2 (n=7), 3 (n=1), 4 (n=10), 5 (n=1), 6 (n=5), 7 (n=1) or 8 (n=1) cycles respectively. All toxicities were expected and transient. Grade 4 toxicities were neutropenia (n=11, 30%), thrombocytopenia (n=8; 24%) and proteinuria (n=2; 6%). Grade 3 toxicities included hepatic transaminitis (n=5;15%) and diarrhea (n=1;3%). Overall responses were: 3 CR (all in prior IT-treated patients with relapsed disease), 12 PD and 18 no response. Only 1/23 patients assessable for early stopping rule regarding efficacy achieved CR/PR. Median progression-free and overall survival post-BIT was 7.7±1.7 and 31.5±5.6 months respectively; all patients received further anti-neuroblastoma therapy post-BIT.
Conclusion: Although BIT was well tolerated, the addition of bevacizumab did not improve response rates in relapsed or refractory neuroblastoma compared to historical data for IT. The study was terminated due to lack of efficacy of the combination.

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Event-free survival (EFS) and overall survival (OS) of *MYCN*-amplified stage 2/3 neuroblastoma with or without autologous stem-cell transplantation (ASCT)

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Background: The literature on *MYCN*-amplified stage 2/3 neuroblastoma is limited because this high-risk subset is uncommon. Its prognosis improved with ASCT. Reports on anti-G_{D2} immunotherapy do not provide details on this entity. We now present our experience since 2000 when anti-G_{D2} antibody 3F8/GM-CSF+isotretinoin became routine. ASCT was used 2000-2003 at our center but not thereafter, though patients transplanted elsewhere remained eligible for 3F8/GM-CSF.
Methods: This report on *MYCN*-amplified stage 2/3 has two components. Part 1 covers an unselected series of 24 consecutive newly-diagnosed patients treated with dose-intensive induction chemotherapy. Part 2 concerns 3F8/GM-CSF+isotretinoin as consolidation of first complete remission (CR), including 20 (15 non-ASCT and 5 ASCT patients) of the aforementioned 24 newly-diagnosed patients, plus 6 patients referred post-ASCT. Consolidation included local radiotherapy.
Results: Part 1: 21/24 (88%) newly-diagnosed patients achieved CR with induction; 20 received consolidation but one CR patient did not (social reasons) and eventually died of progressive disease (PD). Three patients had PD with induction and died early. At 5 years post-diagnosis, these 24 patients had EFS/OS 67%/77%. Part 2: Regarding consolidation with 3F8/GM-CSF+isotretinoin, the 15 non-ASCT patients include 12 relapse-free 12-151 (median 40) months from diagnosis and two who relapsed but remain in 2nd CR at 24 and 84 months post-relapse (their salvage included 3F8/GM-CSF+isotretinoin again). These 15 non-ASCT patients had EFS/OS 72%/92% at five years from start of 3F8. Of 11 ASCT patients, 10 remain relapse-free 75-204 (median 164) months from diagnosis, with EFS/OS 91%/91% at 5 years from start of 3F8. There were no significant differences in EFS/OS between non-ASCT and ASCT cohorts (p=.34/.92). Minimal residual disease (bone marrow) was detected in only 3 patients, cleared with 3F8/GM-CSF, and did not predict relapse.
Conclusions: This subset has a good prognosis with contemporary multi-modality therapy, even without ASCT. Repeating anti-G_{D2} immunotherapy post-relapse may be warranted.

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The iTHER (individualized THERapy) program; personalized cancer treatment for relapsed pediatric cancer

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Background
A vast amount of clinical effective targeted drugs against genes involved in oncogenic processes are becoming available. In parallel the technical developments in genetic tumor cell characterization have made it possible to analyse the complete DNA sequence, the total mRNA gene expression profile of an individual's cancer relative quickly. Thereby, a rational selection of targeted drugs matching an individual patient's tumor characteristics is realistic in a clinically-relevant time span. This personalized cancer treatment approach is being implemented in new trial protocols from pharma as well as academia. Thereby a strong need is generated to systematically perform biological characterization of relapsed or refractory tumors for patients that might benefit from these new trials.

Methods and results
Therefore we have initiated in 2016, the iTHER (individualized THERapy) project which aims to realize personalized treatment for children with relapsed or refractory, incurable cancer in the Netherlands including all neuroblastoma relapse patients. Within this program we are currently testing the feasibility of 4 components:
Selection and inclusion of patients with relapsed or refractory pediatric cancers for whom no curable options are available.
Molecular genomic tumor characterization for events in actionable genes/pathways within 21 days
Selection and prioritization of 'actionable events' in a Molecular Tumor Board setting.
Register the follow-up and potential treatment with targeted drugs.
The feasibility phase will take 2 years which will be followed by a clinical trial phase of 3 years. This should result in full implementation of personalized cancer treatment approach in the clinical setting of centralized pediatric cancer care in the Netherlands. This program is closely linked to similar initiatives in other European countries and will be part of the E-smart ITCC 'basket' trial.

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The BEACON-Neuroblastoma ITCC/SIOPEN phase 2 trial for children with relapsed and refractory neuroblastoma: a progress report

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Background: Current therapy for relapsed/refractory neuroblastoma is not evidence based. Long-term disease control is poor and there is a need for randomised controlled trials (RCT). BEACON-Neuroblastoma is the first randomized European study for relapsed/refractory neuroblastoma and is a critical element of the SIOPEN/ITCC strategy for the development of new agents
Patients and Methods: Factorial phase 2, European, open-label, RCT (EudraCT 2012-000072-42) sponsored by the University of Birmingham, UK, that will evaluate the role of bevacizumab (B) and three backbone chemotherapy regimens (temozolomide (T), irinotecan(I)-temozolomide and topotecan(To)-temozolomide) relapsed/refractory neuroblastoma. The trial incorporates molecular characterisation, functional imaging to elucidate the role of anti-angiogenic therapy, measurement of neuroblastoma mRNAs and is due to be completed in 2017.
Results: As of January 2016, 65 patients have been randomized to T (16), BT (17), IT (15), BIT (16) and BTTo (1) from 26 sites in 8 European countries. Patients had refractory (35), early (22) or late (8) relapsed neuroblastoma. Median age was 5.7 (range 1.6-21.2) years and *MYCN* amplification was present in 22%. Thirty nine of 50 cases with available data had measurable disease per RECIST, 49 had MIBG positive disease and 24 had bone marrow involvement. Most common sites of disease were bone, bone marrow and primary tumor. Progression-free survival (PFS) at 6 and 12 months is 57% (95% CI: 40%-70%) and 36% (CI: 18%-55%); overall survival is 77% (CI: 61% to 87%) and 52% (CI: 29%-71%) respectively. 6-monthPFS is: 50% (CI: 25%-71%) and 63% (CI: 41%-79%) for relapsed versus refractory neuroblastoma; 51% (CI: 19%-76%) and 57% (CI: 36%-72%) for MYCN amplified versus non-amplified patients.
Conclusions: The BEACON-Neuroblastoma trial has established a network and infrastructure to evaluate new drugs efficiently in Europe and will provide reliable evidence on treatments for relapsed/refractory NBL. The results will inform the design of future relapse and frontline studies.

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Viability Of cryopreserved peripheral blood stem cells (PBSC) does not cuarantee functional activity: important implications for quality assurance of stem cell transplant programmes

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Background: Cryopreserved peripheral blood stem cells (PBSC) are used following myeloablative therapy (MAT) in the autologous, and occasionally allogeneic, setting. In paediatric practice, MAT for high-risk neuroblastoma constitutes the majority of such procedures. Standard quality assurance (QA) uses post-thaw CD34+ cell counts and assessment of viability using vital dyes. In 2013, concerns were raised at Great Ormond Street Hospital (GOSH) about a series of patients who experienced delayed engraftment after MAT with cryopreserved cells, despite adequate cell viability and counts in all cases.
Methods: Root cause analysis was undertaken including all aspects of the pathway: patient factors, PBSC mobilisation, apheresis, cryopreservation, reinfusion and count recovery. Investigations suggested the freeze process itself might be a contributing factor to suboptimal engraftment. A series of experiments were undertaken in which a single PBSC product was divided into three and cryopreserved in parallel using a control-rate freezer (CRF) or passive freezing method (-80°C freezer) at GOSH, or the same passive freezing at another centre. Colony Forming Unit-Granulocyte-Monocyte (CFU-GM) assays were used to assess potency.
Results: Comparison of parallel cryopreservation methods revealed equivalent and adequate CD34+ viability in all experiments. However, although CFU-GM assays demonstrated colonies from the products cryopreserved using passive freezing (both at GOSH

and at the other laboratory), products cryopreserved using the CRF did not form any colonies.
The CRF device was shown to be operating within manufacturer's specifications with freeze profile within acceptable limits. The cryopreservation process at GOSH was amended in 2014, since when there has been a statistically significant improvement in time to neutrophil and platelet engraftment and reduction in duration of admission.
Conclusions: This experience has important implications for quality assurance for all transplant programmes, particularly those using cryopreserved products. The failure of post-thaw CD34+/7AAD- counts, the most widely used routine QA test available, to ensure PBSC function is of great concern and should prompt a reassessment of protocols and QA procedures in all transplant centres.

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CAMKV is a candidate immunotherapeutic target in *MYCN*-amplified neuroblastoma.

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Background: GD2-targeting dinutuximab therapy set a precedent for the use of immunotherapy to treat of high-risk neuroblastoma. However, this therapy is associated with toxicity and relapses on or after therapy do occur, highlighting the need for new tumor-specific immunotherapy targets.
Methods: We developed a computation pipeline designed to used RNA sequencing (n=136 high-risk tumors) and gene expression profiling data from 250 neuroblastoma tumors to identify cell surface proteins predicted to be highly differentially expressed in *MYCN* amplified neuroblastomas compared to a normal human tissues. We then performed ChIP-seq in the *MYCN* amplified cell lines KELLY and NGP to identify gene promoters that are occupied by MYCN protein to define the intersection with the differentially expressed gene list.
Results: From this pipeline, we identified 116 putative immunotherapy targets with predicted transmembrane domains. The most significant differentially expressed cell surface molecule in high-risk neuroblastomas harboring *MYCN* amplification was the calmodulin kinase-like vesicle-associated gene (*CAMKV*, $p=2x10^{-5}$), which encodes a protein that binds calmodulin in the presence of calcium, but lacks the kinase activity of other calmodulin kinase family members. We have confirmed that the CAMKV protein is selectively expressed in 7 (of 7 tested)*MYCN* amplified neuroblastoma cell lines and the transcription of CAMKV is directly controlled by MYCN. From membrane fractionation and immunohistochemistry, we have verified that CAMKV is membranous in *MYCN*-amplified neuroblastoma cell lines and xenografted tumors. Immunohistochemistry also showed that CAMKV is not expressed in human pediatric normal tissues with the expected exception of neural tissues.
Conclusions: CAMKV is a differentially expressed cell surface protein that is transcriptionally regulated by MYCN, making it a candidate for immunotherapeutic targeting. Due to the normal CNS expression of CAMKV, we are pursuing an antibody drug conjugate strategy (does not cross blood-brain barrier).

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Significantly reduced relapse rate after KIR ligand incompatible allogeneic cord blood transplantation with nonmyeloablative conditioning for primary stage IV neuroblastoma

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Background: We initiated prospective clinical trial of allogeneic cord blood transplantation (CBT) from Killer immunoglobulin-like receptor (KIR) ligand incompatible donor for stage IV neuroblastoma patients in 2008. **Methods:** Eligibility criteria of this study was newly diagnosed stage IV neuroblastoma patients with one of the following three criteria 1)Chemo-resistant disease 2)10 years or older at diagnosis 3)MYCN amplification. We scheduled CBT with reduced intensity conditioning regimen about three months after high-dose chemotherapy followed by autologous PBSCT. Single inhibitory KIR expressed NK cells with no corresponding recipient's HLA were monitored by flowcytometry before and after CBT to assess the expansion of alloreactive NK cells in vivo. We also retrospectively analyzed the outcome of 82 patients with high risk neuroblastoma treated without KIR ligand incompatible CBT in Nagoya University Hospital between 1982 and 2014 as a control cohort. **Results:** Twenty consecutive patients who matched eligibility criteria underwent KIR ligand incompatible CBT (11 chemo-resistant, 7 MYCN amplification and 2 older age). The median age was 4 (range: 1-10) years old at diagnosis, consisting of 8 boys and 12 girls. No patients developed grade III or more acute GVHD and chronic GVHD. Two patients died in this group because of BU related lung toxicity and only one patient relapsed 7 months after CBT. Significantly reduced cumulative incidence of relapse (7.1%) with the median follow-up period of 52 (5-84) months was observed compared with 51.0% in control cohort. 3-year progression free survival in KIR ligand incompatible CBT group was significantly better than others (83.4% vs 40.7%, p=0.042). Single inhibitory KIR expressed NK cells significantly expanded after CBT (p=0.0007). Finally, multivariate analysis revealed only KIR ligand incompatibility was significantly better

covariate factor for relapse.
Conclusions: CBT from KIR ligand incompatible donor significantly reduced the relapse rate with regard to better progression free survival in stage IV neuroblastoma.

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Validation of a test-dose strategy prior intravenous melphalan in children with renal failure undergoing high-dose chemotherapy with autologous stem cell transplantation

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Introduction
Previous pharmacokinetic (PK) studies of intravenous high dose melphalan based in surface area have shown wide interindividual variations in the PK parameters. For children with renal failure, high melphalan exposure has been previously related with increased toxicity. We studied the possibility of using a test-dose of melphalan to determine the individual clearance and subsequently, the optimal therapeutic dose for each child with renal failure undergoing high-dose chemotherapy (HDCT) with autologous stem cell transplantation (ASCT).
Patients and methods
A test-dose of melphalan, consisting of 10% of the full scheduled dose of melphalan was performed in 13 patients with renal failure several days before melphalan-based HDCT and ASCT since 2011 at Gustave Roussy. The target AUC of melphalan was set at 423 mg/L.min. The patient received the lower dose between the calculated and the theoretical dose. After the full dose, PK was performed to evaluate real melphalan exposure. Toxicity and efficacy were evaluated after the completion of treatment.
Results
PK estimations led to reduce doses in 9 patients since the median predicted AUC was 572 mg/L.min (232-1522). A correlation test between EDTA and melphalan clearances during the test-dose showed that renal function and melphalan clearance were linked (p=0.0279). Melphalan full dose was administered according to the theoretical full dose in 4 patients and to the reduced dose calculated according to PK estimations in 9 patients. The median obtained AUC after full dose was 396 mg/L.min (224-956) with a target AUC after full dose (+/-50%) reached for 9 patients. There was no increased toxicity of HDCT in these patients.
Conclusion
This PK study allowed us to administer melphalan in patients with renal failure avoiding increased toxicity. These results confirm the useful of a test-dose to determine the optimal full dose to achieve the targeted exposure based on AUC calculation.

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Dextran-Catechin conjugate targets copper metabolism in neuroblastoma

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Background: Despite the use of aggressive therapy survival rates for neuroblastoma patients are poor, and survivors often experience long-term side effects from the treatment. Catechin is an natural antioxidant under evaluation in multiple clinical trials, however, poor serum stability of catechin has limited its clinical application. We overcame this issue by conjugating catechin with dextran (Dextran-Catechin) and evaluated its potential as a treatment for neuroblastoma.
Methods: Viability assays were performed using Alamar-Blue. Apoptosis using PARP-cleavage and western-blotting. CTR1 gene and protein levels using qPCR and western-blotting, respectively. Copper levels measured using spectrophotometric analysis. Induction of oxidative stress using ROS-fluorescent probes and NADH/NAD$^+$ ratio measured using FLIM. Levels of glutathione examined by colorimetric assay. Copper uptake in tumours quantitated using Cu^{64}-PET-imaging. Dextran-Catechin anti-cancer activity assessed in xenograft and syngeneic models of neuroblastoma.
Results: Dextran-Catechin significantly decreased neuroblastoma cell viability in SH-SY5Y, IMR-32, BE(2)C and doxorubicin-resistant BE(2)C-ADR cell lines (IC_{50} 9.7-18.2 µg/ml). Importantly, in non-malignant MRC-5 cells, no IC_{50} was reached at doses as high as 60µg/ml. In IMR-32-Cisplatin-resistant cells, Dextran-Catechin resistance was observed compared to parental cells prompting us to investigate copper-transporter-1 (Ctr1), the major cisplatin transporter. Mechanistically, Dextran-Catechin generated oxidative stress in neuroblastoma cells containing high intracellular copper, but not in MRC-5 with low copper levels. Dextran-Catechin was found to react with copper, generating ROS and inducing cell death. Decreased NADH/NAD+ ratio and GSH levels further confirmed induction of oxidative stress. To investigate copper metabolism *in vivo*, Cu^{64}-PET-imaging of bio-distribution in a xenograft neuroblastoma model was performed and confirmed high accumulation of copper in the tumour mass. Finally, we showed that Dextran-Catechin significantly reduced tumour growth in human xenograft and syngeneic models of neuroblastoma.

Conclusion: Dextran-Catechin mediates its effects via copper metabolism and has the potential to be used as a treatment for neuroblastoma and other cancers.

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Development of a novel transgenic neuroblastoma tumor model using genome editing

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Genome editing via CAS9-mediated DNA strand breaks permits the rapid and accurate germline genetic modification of murine tumor models. Previous results from our laboratory demonstrate that G-CSF mediated activation of pSTAT3 is critical for the survival and function of a small tumor subpopulation expressing the G-CSF receptor (CD114). These cells are highly tumorigenic, self-renew and generate differentiated progeny in vivo, fulfilling the accepted definition for cancer stem cells. Furthermore, G-CSF regulates important interactions among immune subsets, stroma and tumor within the microenvironment. G-CSF Knockout mice ($CSF3^{-/-}$) are viable, reproductive, and have intact immune systems with mild neutropenia.
To further evaluate the functional significance of G-CSF signaling in murine MYCN-driven neuroblastoma, which is highly strain dependent, we sought to directly knockout $CSF3^{-/-}$ in the pTH-MYCN (SVJ/129) transgenic model of NB via CRISPR/CAS9 targeted deletion. As the *CSF3* genomic region spans only 3,317 bps, we deigned two guide sequences targeting 5' of exon 1 and 3' of exon 5 within the 3'UTR to excise the entire coding sequence (exons 1-5) for embryonic stem cell injection. Blastocysts harvested from super-ovulated female pTH-$MYCN^{+/-}$ mice mated to wild-type stud males were injected with both guide RNAs as well as CAS9 mRNA, and transferred into pseudo-pregnant surrogate females. Genomic PCR based genotyping demonstrated three founder mice with the following phenotypes: ($CSF3^{-/+}$, $MYCN^{+/+\ Female}$), ($CSF3^{-/+}$, $MYCN^{+/-\ Male}$), ($CSF3^{-/+}$, $MYCN^{+/-\ Male}$). DNA sequencing confirmed knockout of the coding sequence on one allele in each founder. These mice are currently breeding to generate large cohorts with the following phenotypes: ($CSF3^{-/-}$, $MYCN^{+/+}$), ($CSF3^{-/+}$, $MYCN^{+/+}$), $CSF3^{+/+}$, $MYCN^{+/+}$).
These genome edited immunocompetent transgenic mice will permit us to directly evaluate the impact of G-CSF deficiency on tumor incidence, prevalence, and rate of progression in murine model of aggressive neuroblastoma. Administration of exogenous G-CSF will permit in vivo phenotypic rescue in $G\text{-}CSF^{-/-}$ mice.

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IGF2BP1 harbours prognostic significance by gene gain, diverse expression and interplay with MYCN

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Previous reports have shown chromosomal 17q21-ter gain in neuroblastoma is both a common and highly prognostic event. The oncofetal, Insulin-like growth factor-2 mRNA-binding protein 1 (IGF2BP1), is located near the proximal edge of this gained region. IGF2BP1 is also essential for neurocrest migration during development. This study analyzes the DNA copy number and expression of the IGF2BP1 gene in neuroblastoma, and importantly dissects the molecular interplay of the IGF2BP1 and MYCN oncogenes. In two microarray datasets, 77-100 % of tumours had substantial IGF2BP1 mRNA expression. High IGF2BP1 transcript abundance was significantly associated with stage 4 tumours ($p = 0.019$, 1.1e-06), and decreased patient survival (both $p < 0.0001$)(1). Additionally, IGF2BP1 was also associated with MYCN gene-amplification and MYCN mRNA abundance. In the 69 neuroblastoma samples IGF2BP1 DNA copy number (increased in 84 % of tumours), mRNA and protein abundance were significantly higher in stage 4 as compared to stage 1 tumours. Importantly, IGF2BP1 protein levels were associated with lower overall patient survival ($p = 0.012$) and positively correlated with MYCN mRNA, even when excluding MYCN amplified tumours. Although widely expressed in neuroblastoma tumours, the other protein family members IGF2BP2 and IGF2BP3, failed to clearly demonstrate any prognostic trend. *In vitro*, IGF2BP1 clearly affected MYCN expression and neuroblastoma cell survival. Moreover, we have evidence showing MYCN promotes IGF2BP1 expression, including that the MYCN protein binds the IGF2BP1 promoter, and also that IGF2BP1 is significantly upregulated in precancer cells in a MYCN-driven neuroblastoma mouse model. IGF2BP1 is also expressed highest in cell lines with MYCN amplification. In conclusion, IGF2BP1 was expressed in the majority of neuroblastoma specimens analyzed and was associated with lower overall patient survival and MYCN-abundance. These data demonstrate, for the first-time, IGF2BP1 as a potential oncogene and biomarker in neuroblastoma, which has an important role within MYCN-driven carcinogenesis.

IGF2BP1 harbors prognostic significance by gene gain and diverse expression in neuroblastoma. Bell JL, Turlapati R, Liu T, Schulte JH, Hüttelmaier S. J Clin Oncol. 2015 Apr 10;33(11):1285-93. doi: 10.1200/JCO.2014.55.9880. Epub 2015 Mar 9. PMID: 25753434

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Aldehyde dehydrogenases activity plays a key role in NB aggressive behavior

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Background: The successful targeting of neuroblastoma (NB) associated tumor-initiating cells (TICs) is a real challenge in developing new therapeutic strategies. By combining serial neurosphere (NS) passages with gene expression profiling, we have previously identified ALDH1A2 and ALDH1A3 as potential NB-TICs markers in patient-derived xenograft (PDX) tumors. ALDH1A2, ALDH1A3, with ALDH1A1, belonging to the subfamily of aldehyde dehydrogenases 1 (ALDH1) isoenzyme involved in the synthesis of retinoic acid, have been identified as functional stem cell markers in various cancers. In this study, we explored the involvement of ALDH1 isoforms and the related ALDH activity in NB aggressive properties.
Methods: ALDH activity and ALDH1A1/A2/A3 expression levels were measured using the ALDEFLUOR™ kit, and by real-time PCR, respectively. ALDH activity was inhibited using the specific ALDH inhibitor diethylaminobenzaldehyde (DEAB), and ALDH1A3 gene knock out was generated through the CRISPR/Cas9 technology.
Results: Here, we confirmed the enrichment of ALDH1A2 and ALDH1A3 mRNA expression in NB cell lines and PDX tumor during NS-passages. We found that ALDH1 expression was associated to less aggressive NB cell lines and correlated with better prognosis, while ALDH1A3 is more widely expressed in NB cell lines and is associated with poor survival and high-risk prognostic factors. We also observed an important ALDH activity in various NB cell lines and PDX tumors. Specific inhibition of ALDH activity with DEAB resulted in a strong reduction of NB cell clonogenicity, and self-renewal potential, and partially restored NB cells sensitivity to 4-hydroxycyclophosphamide. Finally, the specific knock out of the *ALDH1A3* gene mediated a cell type-dependant inhibition of NB clonogenic and TICs self-renewal properties.
Conclusion: Altogether our data revealed that ALDH activity plays a key role in NB aggressive phenotype, and that the involvement of ALDH1A3 isoenzyme in this process is cell type-dependent, possibly due to the various expression profiles of other ALDH isoforms.

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Rab6B mediates the progression of neuroblastoma through the interaction with MTMR5

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Background: Neuroblastoma is one of the most aggressive pediatric tumor that is characterized by its biological and clinical heterogeneity. Despite the current intensive therapy, more than half of high-risk patients have experienced a tumor relapse. Neuroblastoma recurrence is primarily driven by chemoresistant cancer stem cells that can be grown as spheres. Although Rab family small G proteins (Rabs) are implicated in the progression of growing number of cancers, the impact of their pathophysiological role in the progression of neuroblastoma remains to be determined.
Methods: Spheres of neuroblastoma BE(2)-C cells were grown in sphere medium with a non-adherent dish. Total RNA was extracted from parental cells and spheres of BE(2)-C cells and 64 Rabs expression was analyzed by real time RT-PCR. Rab6B, MTMR5 and Rab28 shRNAs and cDNAs were expressed in BE(2)-C cells and analyzed for intracellular localization, cell proliferation, sphere formation, colony formation, and xenograft tumor formation activities.
Results: When mRNA expression of all 63 Rabs in neuroblastoma BE(2)-C cells was examined, 48 Rabs were detected. Rab6B expression was most strongly induced in spheres compared to parental cells. During sphere formation, the primary intracellular localization of Rab6B was changed from the Golgi to the late endosome. By using Mass spectrometry, we identified a novel Rab6B-binding protein, myotubularin-related 5 (MTMR5) that was originally isolated as a SET-domain binding protein and known to activate Rab28 resided at the late endosome. Whereas MTMR5 and Rab28 decreased the sphere, colony and xenograft formation activities of BE(2)-C cells, Rab6B increased them by competing with Rab28 for the binding to MTMR5.
Conclusion: These results suggest that Rab6B mediates the progression of neuroblastoma through the interaction with MTMR5.

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Characterization of patient-derived xenograft neuroblastoma cells

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We recently established a neuroblastoma patient-derived xenograft (PDX) model from which we have isolated cells and established PDX sublines. The PDX cells express common neuroblastoma markers and retain patient-specific genomic aberrations over several passages. We believe that PDX cells are relevant models for identifying new treatment targets in neuroblastoma since they retain *in vivo* tumorigenic and metastatic capacity, including to bone marrow. The PDX cells are grown in serum-free, stem-cell promoting medium as spheres in order to maintain an immature phenotype. Serum induces spontaneous differentiation of neural stem cells, which results in loss of stem-like properties. By culturing PDX cells in the presence of serum, we observed a trend towards a more differentiated phenotype as cells started to adhere, proliferate slower and increased their expression of known differentiation markers. Thus, culturing PDX cells in serum-free conditions is important for maintaining a more undifferentiated phenotype.

Furthermore, the PDX cells express Hypoxia Inducible Factor 2α (HIF-2α) at normoxia. We have previously shown that presence of HIF-2α positive neuroblastoma cells in a perivascular niche associates with poor prognosis. Moreover, these cells seem to have a less differentiated phenotype. It has also been shown that HIF-2α is expressed in sympathetic neuroblasts during early sympathetic nervous system development/embryogenesis (w6.5) in humans. This might suggest that the PDX cells are arrested in an early developmental stage.

Finally, for future drug screening, we grew PDX cells as a monolayer culture on laminin since sphere culturing is more cumbersome. PDX cells grown on laminin showed a differentiated morphology and increased expression of various differentiation markers. An increased survival was also observed in laminin-cultured cells. Although laminin seems to promote differentiation, PDX cells grown on laminin retained tumorigenic and metastatic capacity *in vivo* and formed spheres *in vitro* after short-term laminin culture.

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Differentiation of Neuroblastoma is controlled by cdk-mediated regulation of the master regulator transcription factor Ascl1.

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Achaete-scute homolog 1 (Ascl1) is a proneural transcription factor that plays a central role in regulating differentiation of noraderinergic neuron precursors during embryonic development, the cell type from which neuroblastoma is thought to arise. In a developmental model, we have shown that Ascl1 transcriptional activity is inhibited by multi-site phosphorylation on serine/proline sites, potentially by cdk kinases that drive cell cycle progression. Ascl1 is highly expressed in multiple neuroblastoma cell lines but the endogenous protein is usually phosphorylated. We are characterizing how phospho-regulation of Ascl1 by cyclin-dependent kinases regulates its ability to drive differentiation by genome-wide characterization of its targets. We are also exploring Ascl1 phospho-regulation of chromatin and co-factor binding.

Using chemical inhibitors and western blotting to characterise the phospho-regulation of Ascl1 in SHSY5Y neuroblastoma cells, we see that phosphorylation of Ascl1 is cell cycle regulated, and that Ascl1 is a target of the mitotic kinase Cdk1. Expression of a phospho-mutant Ascl1 or addition of Cdk1 inhibitor both cause cell cycle exit and morphological clustering, reminiscent of ganglion formation during development. We are also using inducible expression of wild-type and phosphomutant Ascl1 in these cells to characterize phosphorylation status-related morphological changes, DNA binding alterations (by ChIP-seq) and genome-wide transcriptome changes (by RNA-seq). We have identified over 6000 transcripts that are upregulated when Ascl1 is overexpressed. 1780 of there are specifically upregulated by phospho-mutant Ascl1 and these are highly enriched for differentiation-specific genes. Many of these genes are bound directly by Ascl1.

In summary, we find that Ascl1 is phosphorylated by Cdk1, while its dephosphorylation potentiates neuroblastoma cell differentiation. These findings indicate that cdk inhibitors may have therapeutic benefit by both arresting cell cycle and potentiating differentiation. Moreover, we additionally identify potential new biomarkers of neuroblastoma differentiation.

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MYCN-regulated nuclear hormone receptors impact differentiation and survival in neuroblastoma patients

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Neuroblastoma is the most common extra-cranial solid tumor in children and arises from neural crest cells involved in the development of sympathetic nervous tissue. This childhood tumor is characterized by heterogeneity and amplification of the MYCN oncogene is highly associated with aggressive tumors and poor outcome. The aim of this study was to investigate the role of the MYCN-driven miR-17-92 cluster in neuroblastoma pathogenesis. In order to identify putative targets of miR-17-92 we performed in silico prediction analysis and found that miR-17-92 target sites are significantly enriched in the nuclear hormone receptor (NHR) super family, indicating a role for hormonal regulation in neuroblastoma tumorigenesis. Importantly, high expression of several of the NHRs correlated with increased event-free survival of neuroblastoma patients. By using non-MYCN amplified neuroblastoma cells with a tet-regulatable miR-17~92 we could verify a differentially expressed NHR profile in induced compared to uninduced cells. Interestingly, one of the most significantly dowregulated NHRs was the glucocorticoid receptor (GR). Moreover, luciferase reporter assays containing the wildtype or mutated 3'UTR from the gene encoding GR demonstrate that it is a direct target of miR-17~92. Importantly, both MYCN and miR17~92 are able to downregulate GR. We further show that activation of GR signaling by dexamethasone induces differentiation markers and contributes to neural differentiation. Inhibition of MYCN activity and/or activation of GR signalling induced a significant reduction in tumor growth in a xenograft model of neuroblastoma. Taken together, our findings indicate an important role for miR-17-92 cluster in regulation of NHRs in neuroblastoma biology, with important implications for future therapeutic approaches in patients with MYCN-amplification.

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CDX1 regulates cancer stemness pathway in neuroblastoma

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Background: Cancer stem cells (CSCs) are regarded as essential for tumor maintenance, recurrence and distant metastasis. However, CSC or tumor-initiating cells were not identified in neuroblastoma (NB). We previously reported that stem cell related gene CD133 was expressed in neuroblastoma (NB) spheres and controls tumor sphere formation (Takenobu et al., 2010).
Methods: Gene expression profiling of adherent (control) and sphere forming NB cell lines as well as primary NB cells were conducted by microarray and RNA seq using illumina next generation sequencer. CDX1 knockdown and over-expression were performed by lentiviral system.
Results: To study the regulatory mechanism of *CD133* transcription in NB tumor sphere, we analyzed activity of the promoter region of *CD133*, and found a promoter region which mainly regulates *CD133* transcription in sphere-forming NB cells. To identify the responsible transcription factors, we analyzed the promoter region by in Silico and wet-lab analysis and identified sphere specific gene *CDX1*. Intriguingly, CDX1 specifically bound to the sphere-specific *CD133* promoter region and promoter regions for reprogramming factors, including *OCT4* and*NANOG*; CDX1 also regulates sphere formation efficiency in NB cells as well as CD133. In NB sample analysis by expression microarray, CDX1 high expression related to the unfavorable prognosis. These results indicate that CDX1 plays a role in NB cell stemness and aggressiveness. We also performed that comprehensive analysis in CDX1-expressing NB cell and sphere forming NB cells to identify NB-CSC specific genes and signaling pathways. The pathway analysis and Gene Set Enrichment Analysis (GSEA) suggested that upregulated genes were enriched for ES-related transcription and developmental signaling pathways in both the CDX1-expressing and sphere-forming NB cells. Taken together, CDX1 regulates the stemness-related pathways and seems to be a target of cancer stem cell-targeted therapy development.

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BMCC1, a tumor suppressor protein that facilitates DNA-damage response and apoptosis, is associated with favorable prognosis of neuroblastoma

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Background: *B*CH *m*otif-containing molecule at the *c*arboxyl terminal region *1* (*BMCC1*)/*PRUNE2* is highly expressed in patients with favorable neuroblastoma. It encodes a 340-kDa protein with a conserved BNIP2 homology scaffold domain that may regulate signaling networks and multiple cellular functions, including apoptosis. Recent studies have suggested that BMCC1 acts as a tumor-suppressor because of its loss-of-function mutation or down-regulation in several types of tumors.
Methods: Quantitative RT-PCR was employed for the detection of *BMCC1* mRNA expression in 102 primary neuroblastoma samples. To assess the functional role of BMCC1 in neuroblastoma, we performed the overexpression and/or knockdown of BMCC1 and E2F1 in neuroblastoma cell lines expressing BMCC1, such as SK-N-AS and NBL-S cells. To introduce DNA damage into neuroblastoma cell lines, cisplatin, adriamycin, or VP-16 was used.
Results: From a clinical perspective, multivariate analysis showed that the *BMCC1* expression level was highly associated with survival after controlling for other well-known prognostic factors, such as *MYCN*, INSS stage, age, and *TrkA* expression level, indicating that the *BMCC1* expression level acts as a reliable independent prognostic factor from these existing factors in the neuroblastoma patients. Our functional analysis demonstrated that BMCC1 was induced by DNA damage, one of the triggers of apoptosis, and it was transactivated by an ATM-E2F1-dependent mechanism. Accordingly, we investigated whether BMCC1 expression impacts intracellular signals involved in the regulation of apoptosis and found that BMCC1 promotes apoptosis at multiple steps in the AKT-mediated survival signal pathway via its BNIP2 homology domain. These steps include physical interaction with BCL2 and attenuation of the AKT-dependent inhibition of FOXO3a functions, such as the transcriptional induction of BIM and phosphorylation of ATM after DNA damage.
Conclusion: Our findings suggest that the downregulation of BMCC1 expression facilitates tumor development and genomic instability of neuroblastoma by abrogating apoptosis and ATM-mediated DNA damage repair.

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DENN domain protein DENND2A regulates the progression of neuroblastoma

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Background: Neuroblastoma is one of the most frequent solid tumor in children, which accounts for 15% of childhood cancer death. More than half of patients experienced tumor relapse driven by cancer stem cells that can be isolated as spheres. DENN domain proteins serve as GDP/GTP-exchanging factor of Rab family small G protein (DENN/RabGEFs) and are implicated in the progression of several cancers possibly through the regulation of intracellular membrane traffic. However, their involvement in the progression of neuroblastoma has not been elucidated.
Methods: Spheres of neuroblastoma BE(2)-C cells were grown in sphere medium with a non-adherent dish. Total RNA was extracted from parental cells and spheres of BE(2)-C cells and mRNA expression of 18 DENN/RabGEFs isoforms was analyzed by real time RT-PCR. Overexpression and knockdown of DENND2A in BE(2)-C cells were achieved by stably transfecting DENND2A cDNA and shRNA, respectively, and subjected to the sphere formation, colony formation and xenograft formation assays. The correlation of DENND2A expression with overall survival probabilities of neuroblastoma patients was analyzed by the bioinformatics program R2 (http://r2.amc.nl).
Results: Among 14 DENN/RabGEFs expressed in BE(2)-C cells, DENND2A showed the most profound difference between spheres and parental cells. Knockdown of DENND2A significantly promoted the sphere, colony and xenograft tumor formation, whereas they were significantly suppressed by overexpression of DENND2A. Furthermore, high DENND2A expression was significantly associated with high overall survival probabilities of neuroblastoma patients.
Conclusion: These results suggest that DENND2A is involved in the progression of neuroblastoma

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Transmembrane adaptor protein PAG1 is a novel tumor suppressor in neuroblastoma

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The tyrosine kinase c-Src plays an important role in the differentiation and survival of neuroblastoma (NB) cells, however the mechanism(s) activating this pathway remains poorly defined. Here, we characterize PAG1 (Cbp, Csk binding protein), an inhibitor of c-Src and other Src family kinases, as a novel tumor suppressor for neuroblastoma. Gene expression data from multiple independent NB clinical cohorts demonstrate that low expression of *PAG1* is a significant prognostic factor for poor overall and event-free survival ($p<0.0001$), independent of MYCN status. Other disease factors, such as relapse or tumor progression, risk of death due to disease, high stage disease are also found to be inversely correlate with *PAG1* expression (pPAG1 and *MYCN* expression levels ($p<0.001$) in all patient cohorts. *PAG1* overexpression in NB cell lines significantly reduces proliferation and anchorage-independent colony formation in vitro with inhibition of pSRC, pAKT and pERK downstream of c-Src, while *PAG1* Knockdown rescues these effects. Furthermore, *PAG1* overexpression dramatically inhibits in vivo tumorigenicity of orthotopic xenografts (MYCN-amplified NGP ($p=0.007$) and non-amplified SH-SY5Y ($p=0.001$). The clinical data above and the lack of a canonical E-box site for MYCN binding at the *PAG1* promoter, suggests this gene is indirectly repressed by MYCN possibly via microRNAs. Of note, the *PAG1* 3' UTR contains target sites for mir-17-92, miR-34a, miR-26 and other MYCN regulated microRNAs implicated in MYCN mediated pathogenesis. These miRs also target additional targets down stream of Src as noted by Ingenuity analysis. Overall our data establish PAG1 as a potent tumor suppressor in neuroblastoma and suggest that reactivation of *PAG1* and inhibition of c-Src kinase activity represents a novel therapeutic approach for high-risk neuroblastoma.

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The identification of hypoxia regulated genes that confer a poor prognosis in neuroblastoma patients

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Background: Hypoxia is well known to confer a more aggressive phenotype in neuroblastoma. We sought to integrate gene expression data from diagnostic tumors and hypoxic neuroblastoma cell lines to identify genes that correlate with poor patient outcome and are involved in the hypoxia response which may represent novel therapeutic targets.
Methods: Linear models identified DEG from tumors of two cohorts of 567 total neuroblastoma patients who survived compared to those that did not. Mixed linear models identified differentially expressed genes (DEG) between 12 neuroblastoma cell lines grown in hypoxia or normoxia. qPCR validated expression differences in multiple neuroblastoma cell lines. shRNA was used to knock down expression of *HK2*, the most overexpressed of the identified genes in hypoxia.
Results: Eight genes, *SLCO4A1*, *ENO1*, *HK2*, *PGK1*, *MTFP1*, *HILPDA*, *VKORC1*, and *HIST1H1C*, were differentially expressed in all datasets and significantly associated with outcome on Kaplan-Meier analysis. Six of these eight had hypoxia inducible factor (HIF) binding sites on CHIP-seq analysis verifying that they are indeed hypoxia targets. Three of these genes are part of the glycolytic pathway and three more are directly involved in cellular metabolism. We functionally validated our computational findings and showed that six of the eight genes are significantly up regulated in five independent neuroblastoma derived cell lines. shRNA knockdown of the *HK2* gene leads to decreased cell growth by MTT and a G0/G1 arrest in flow cytometry analysis in the La1-55n cell line and may represent an important potential drug target for this disease.
Conclusions: Our findings highlight the utility of combining diverse data sets to identify key genetic drivers of disease that may be otherwise be overlooked as we have identified a compact and robustly validated group of eight genes which may represent new therapeutic and prognostic targets for children with neuroblastoma.

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The MAD Family members, MXI1 and MXI0, display distinct subcellular localization patterns in neuroblastoma

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The Myc family regulates cell growth and is implicated in the etiology of many cancers, including neuroblastoma. Mxi1, a MAD family member, inhibits N-Myc activity. Mxi0 is an alternatively spliced variant of Mxi1 with a different first exon (Exon 0) whose function has not been determined. These proteins have differential functions in neuroblastoma: Mxi1 inhibits neuroblastoma cell proliferation, while Mxi0 promotes it. While Mxi1 and Mxi0 are mostly homologous, including their repressive domains, they possess distinct N-terminal exons, suggesting a critical role of Exon 0 in the distinct function of Mxi0. To determine the role of subcellular localization as a mechanism for the differential function of Mxi0, we created GFP-tagged constructs of Mxi1, Mxi0, and Exon 0. After expression in 293T cells, localization of Mxi1, Mxi0, and Exon 0 was detected by immunofluorescence. Examination of subcellular location reveals that Mxi1 resides in the nucleus, while Mxi0 is found primarily in the cytoplasm. Exon 0 also displays cytoplasmic localization, indicating that it contributes to differential localization. We then blocked nuclear export with Leptomycin B (LMB) to assess nuclear/cytoplasmic translocation. LMB treatment resulted in nuclear accumulation of Mxi0, suggesting that Mxi0 cycles in and out of the nucleus in response to appropriate signals. This appears to be mediated by Exon 0, as it also accumulates in the nucleus after nuclear export inhibition. In summary, Mxi0 and Mxi1 exhibit distinct subcellular localization patterns, with Mxi1 residing in the nucleus and Mxi0 found in the cytoplasm. Exon 0 directs the cytoplasmic localization of Mxi0. Finally, nuclear export inhibition leads to nuclear accumulation of Mxi0, suggesting that Mxi0 translocates in response to appropriate signals. A better understanding of how Mxi0 impacts neuroblastoma physiology and how Exon 0 imparts the differential function of Mxi0 may aid in developing more effective targeted therapies for children with neuroblastoma.

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The expression of Mxi1 and Mxi0 lead to differential effects on neuroblastoma pathogenesis and chemosensitivity.

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Neuroblastoma is the most common extracranial malignancy of childhood and advanced cases are difficult to treat. The Myc family of proteins regulates cell growth and *MYCN* amplified neuroblastoma is associated with a poor prognosis. Mxi1 is a member of the MAD family that inhibits N-Myc activity. Mxi0 is an alternatively spliced variant of Mxi1 with a different first exon (Exon 0) whose function has not been determined. To test the hypothesis that Mxi1 and Mxi0 differentially impact N-Myc-dependent neuroblastoma cell proliferation, we expressed Mxi1 and Mxi0 in SHEP neuroblastoma cells, and SHEP cells stably transfected to express high levels of *MYCN* (SHEP/*MYCN*). We also utilized native neuroblastoma cell lines with inducible expression of Mxi1 and Mxi0. Cell proliferation and survival were quantified using BrdU and MTT assays, respectively. Impact of Mxi1 and Mxi0 on N-Myc expression was measured by RT-PCR and immunoblot analysis. Overexpression of Mxi1 inhibits neuroblastoma cell viability. Conversely, overexpression of Mxi0 in neuroblastoma cell lines leads to increased viability, suggesting that Mxi0 is a counter-regulatory role of Mxi1. Further examination reveals that these changes are partially due to alterations in cell proliferation, with Mxi1 decreasing proliferation and Mxi0 augmenting it. Additionally, we observed that N-Myc levels decrease in response to Mxi1 expression and increase when Mxi0 is expressed. Compared with Mxi1, expression of Mxi0 enhanced chemoresistance of neuroblastoma cells to doxorubicin or etoposide. In summary, overexpression of Mxi1 in neuroblastoma cell lines leads to inhibition of N-Myc activity, while Mxi0 appears to promote it. These effects may partially be due to alterations in N-Myc expression. A better understanding of the interactions among Mxi1, Mxi0 and N-Myc and how the relative expression levels of these proteins affect neuroblastoma physiology may aid in developing more effective targeted therapies to improve outcomes in pediatric neuroblastoma patients.

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Targeting MYCN-amplified neuroblastoma through RORα activation.

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Background: *MYCN* activation is a hallmark of advanced tumor stage in neuroblastoma (NB), characterizing high-risk patients prone to resistant disease. *MYCN* is also a potent regulator of metabolic reprogramming that favors NB adaptation to its microenvironment. The retinoic acid receptor-related orphan receptor α (RORα) is a key regulator of cell metabolism, immunity, as well as the circadian rhythm. Importantly, RORα activates the transcription (via recruitment of transcriptional co-activators) of BMAL1, a master circadian transcription factor frequently deregulated in human cancers.
Material and Methods: Multivariate logistic regression analysis identified low levels of RORα as independent predictors of EFS and OS survival in large NB patients' cohorts (n=890). *MYCN* inducible overexpression and knock down lines were generated and Q-PCR assays used to assess *MYCN*-mediated disruption of molecular clock genes' expression. Growth-suppressive and pro-apoptotic effects of the RORα agonist SR1078 were tested in a panel of *MYCN*-amplified and non-amplified lines, as well as in MYCN inducible MYCN-3 cells (Tet-ON), using MTT and caspase 3/7 activation. *MYCN*-amplified xenografts were used to test *in vivo* therapeutic response to SR1078.
Results and Conclusions: The circadian clock is profoundly disrupted in *MYCN*-amplified NB lines and tumors. Specifically, RORα and BMAL1 expression are uniformly repressed in MYCN-amplified NB lines and tumors ($p<0.0001$) and their reduced levels strongly correlate with poor survival (890 patients, $p<0.0001$). Moreover, RORα re-activation via SR1078 reduces cell viability and induces apoptotic cell death to a higher extent in *MYCN*-amplified compared to *MYCN*-non amplified lines. Importantly, RORα re-activation strongly suppresses *MYCN*transcription and induces BMAL1 expression in *MYCN*-amplified cells and tumors, inhibiting cell survival. Lastly, tumor weights of xenografts treated with SR1078 were significantly ($p=0.020$) smaller than control. Together, our data suggest that: 1) reactivating RORα may represent an effective way to inhibit *MYCN*-mediated metabolic functions, and 2) RORα agonists can be developed as effective therapy for *MYCN*-amplified NB.

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Identification of a novel protein that suppresses the ability of NDPK-A to promote the invasiveness of neuroblastoma cells

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Nucleotide diphosphate kinase A (NDPK-A) acts as a metastasis promoter in neuroblastoma. Overexpression or the S120G mutation of NDPK-A, detected in patients with advanced neuroblastoma, promotes neuroblastoma cell invasiveness and metastasis *in vitro* and *in vivo*. Relative to the wild type, NDPK-A^{S120G} appears to be a more potent promoter. Currently it is unclear how NDPK-A mechanistically promotes neuroblastoma metastasis. Using the yeast two-hybrid system, we have identified a novel protein (termed Protein Y) that interacted with NDPK-A, but not with NDPK-A^{S120G}. Immunocytochemistry shows that Protein Y was translocated in human neuroblastoma NB69, IMR32 and, to a lesser degree, SH-SY5Y cell lines, and which is correlated with mutations in the *Protein Y* mRNA. In NB69 derivatives, ectopic expression of NDPK-A or NDPK-A^{S120G} increased cell migration by two fold, whereas ectopic expression of Protein Y decreased cell migration by 47% in the transwell assay. In the clonogenic assay Protein Y reduced the colony number by 17%, whereas NDPK-A or NDPK-A^{S120G} increased the number by 20-25%. Co-expression of Protein Y almost completely abolished the invasiveness-promoting ability of NDPK-A, but not NDPK-A^{S120G}. Furthermore, deletion of the N-terminus of Protein Y abolished its ability to suppress the invasiveness-promoting ability of NDPK-A and, to a lesser degree, NDPK-A^{S120G}. Knockdown of Protein Y expression restored the ability of NDPK-A to promote cell invasiveness. In xenograft zebrafish, NDPK-A or NDPK-A^{S120G} increased NB69 extravasation by 3.9 and 1.9 folds, respectively. In contrast, Protein Y decreased NB69 extravasation by 14%. Co-expression of Protein Y reduced the extravasation-promoting ability of NDPK-A and NDPK-A^{S120G} by 19.5% and 2.5%, respectively. In conclusion, Protein Y behaved as a suppressor and counteracted the invasiveness-promoting ability of NDPK-A and, to a lesser degree, NDPK-A^{S120G} in NB69 cells. However, this invasiveness-suppressing ability of Protein Y is likely abrogated by mutations, as detected in the human neuroblastoma cell lines examined.

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Reduced endoplasmic reticulum (ER)-mitochondria tethering as a cause of multidrug resistance in neuroblastoma

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Background: High-risk neuroblastoma patients often respond to therapy before relapsing with lethal multidrug-resistant disease. We propose loss of ER-mitochondria physical tethering, selected for during therapy, contributes to drug resistance.
Methods: We studied isogenic neuroblastoma cell line pairs derived from the same patients both at diagnosis (DX) and progressive disease on/after chemotherapy (PD). Tumor-isolated mitochondria were studied for functional responses to death stimuli (BH3 profiling), biomass (citrate synthase activity), and mtDNA (content by qPCR; sequence by MitoChip v2.0). Electron microscopy (EM) was used to quantify ER-mitochondria morphology and tethering. The impact of chemical and genetic manipulation of tethering on mitochondrial response and chemosensitivity was assessed.
Results: DX/PD pairs showed no changes in mitochondrial biomass or mtDNA. However, 7/7 pairs showed markedly attenuated cytochrome c release at relapse in response to tBid and BimBH3 peptides (terminal death effectors downstream of therapeutic stress), corresponding to relative chemotherapy resistance (up to 800-fold across diverse agent classes). Marked reductions in ER-mitochondria tethering (up to 70%) were seen by EM in post-relapse tumors. Attenuated mitochondrial responses were phenocopied by limited protelolysis of ER from mitochondria in DX (sensitive) lines, as well as by enforced de-tethering using a cyclophilin-D inhibitor; extent of mitochondrial attenuation was proportional to chemoresistance. A similar phenotype was observed in ALK-mutant cells selected for ALK-inhibitor resistance, leading to mitochondrial attenuation and broad therapy resistance.
Conclusions: We identify a novel contributor to multidrug resistance attributable to attenuated mitochondrial stress-responses. Our data support this attenuation derives from reduced ER-mitochondrial tethering with consequent loss of ER-derived signals required for mitochondrial competency. This mechanism is not exclusive to any other contributor to therapeutic resistance and may also contribute to resistance to targeted agents. Altered ER-mitochondria tethering has been linked to neurodegeneration and diabetes suggesting regulation of this inter-organelle contact may have broad relevance to human disease.

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An embryonic stem cell activated FOXM1 transcriptional program marks ultra-high-risk primary neuroblastoma patients for FDI-6 small molecule inhibition

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Introduction: Chemotherapy resistance is responsible for high mortality rates in high-risk neuroblastoma patients. MYCN is a major oncogenic driver in these tumors controlling pluripotency genes including LIN28B. Therefore, we hypothesized that enhanced embryonic stem cell (ESC) gene regulatory programs could mark tumors with increased risk for therapy failure enabling the selection of patients for novel targeted therapies.
M&M: A microRNA expression ESC-signature was established based on publically available data. In addition, an mRNA ESC-signature of top 500 protein coding genes with highest positive correlation with the microRNA ESC-signature score was generated.
Results: High ESC-signature scores were significantly correlated with worse neuroblastoma patient survival, both in the global patient cohort as well as in the subset of stage 4 tumors without MYCN-amplification. In addition, both in neuroblastoma and other embryonal tumors exhibiting MYCN-activation, the scores were significantly higher. This was confirmed in MYCN cell model systems where the scores altered upon MYCN-overexpression/knock-down. Using GSEA, we identified that genes implicated in DNA damage response and cell cycle control were strongly enriched in the signature. One of the genes in the signature is the transcription factor FOXM1, which is a master regulator driving those pathways. The upstream activator of FOXM1, MELK, was also part of the signature. Inhibition of FOXM1 in neuroblastoma cells using the small molecule FDI-6 significantly reduced cell viability. In addition, MELK inhibitors are currently tested in vitro and both FOXM1 and MELK inhibitors are evaluated in MYCN transgenic zebrafish models.
Conclusion: A novel ESC-signature score marks neuroblastomas with poor prognosis enabling the identification of ultra-high-risk neuroblastoma patients that may benefit from targeted therapies using FOXM1 or MELK inhibitors.

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Molecular control of neuroblastoma stem cell metabolism

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Background: A growing and proliferating cell requires enhanced metabolic capacity for accumulation of biomass and replication of the genomic DNA. It is well documented that cancer cells alter their metabolism to meet the biosynthetic challenge of growth and proliferation. The metabolic pathways critical for driving neuroblastoma development are poorly understood.
Methods: We isolated and propagated a population of neuroblastoma stem-like cells from tumors developed in *TH-MYCN* mice, a well-characterized mouse model of human neuroblastoma. We performed microarray gene expression profiling of these tumor stem cells in comparison with their parental primary neuroblastoma cells and identified metabolic pathways that are activated in neuroblastoma stem cells. We further conducted loss- and gain-of-function studies to investigate the functional significance of these metabolic pathways in regulation of neuroblastoma stem cell activities and the molecular mechanisms for the activation these pathways in neuroblastoma stem cells.
Results: We found that neuroblastoma stem cells display significantly higher expression of genes coding for enzymes in the serine-glycine synthesis pathway, which generates biosynthetic precursors essential for the production of proteins, nucleic acids, fatty acids, and the membranes needed for cell proliferation. Transcriptional activation of the pathway genes are also observed in high-risk human neuroblastoma tumors and are significantly associated with poor prognosis in neuroblastoma patients. The serine-glycine synthesis pathway is required for the survival, proliferation, and tumorigenicity of mouse neuroblastoma stem cells and human neuroblastoma cell lines. Our investigation further revealed a key role of histone H3 lysine 9 methylation states in transcription control of the serine-glycine synthesis pathway.
Conclusions: Our findings identify an epigenetic program for transcriptional activation of cancer metabolism in neuroblastoma stem cells and suggest new therapeutic strategies for high-risk neuroblastoma.

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New, highly selective MRP1 inhibitors show promising preclinical activity in neuroblastoma

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We have previously shown that multidrug resistance protein 1 (MRP1) is highly prognostic of clinical outcome in neuroblastoma (1,2) and contributes to chemoresistance in a neuroblastoma mouse model (3). Although MRP1 inhibitors would have substantial clinical potential, allowing improved cancer control and reduction in the dose of chemotherapeutics used, there are no available agents that are both selective for MRP1 and suitable for *in vivo* use. Based on our previously identified MRP1 inhibitor Reversan (3), we have developed a series of highly promising new MRP1 inhibitors with a view to identifying a candidate molecule suitable for clinical use.
Methods: To determine *in vitro* activity and selectivity, cells overexpressing clinically relevant drug transporters (MRP1, P-glycoprotein or ABCG2) were treated with established drug substrates in combination with MRP1 inhibitors and viability assessed. Levels of the endogenous MRP1 substrate glutathione (GSH) were assessed by GSH recycling assay. *In vivo* activity was assessed in the Th-*MYCN* mouse neuroblastoma model in combination with the MRP1 substrate drug etoposide.
Results: Our inhibitors demonstrated unprecedented selectivity for MRP1 over P-glycoprotein and ABCG2, and sensitized neuroblastoma cell lines three-fold to the frontline cancer drugs etoposide and vincristine *in vitro* ($P<0.001$). The inhibitors showed very promising preclinical activity in the Th-*MYCN* mouse model, doubling median survival over etoposide alone (11 days to 21–23 days; $P<0.001$) without impacting on etoposide pharmacokinetics. Surprisingly, these inhibitors also strongly depleted intracellular GSH in an MRP1-dependent manner. This effect was synergistic with the GSH synthesis inhibitor buthionine sulfoximine.
Conclusion: The selectivity of our inhibitors for MRP1 over other clinically relevant drug transporters and their demonstrated preclinical activity is a major advance over previously developed compounds. MRP1-dependent depletion of GSH may provide an additional therapeutic window in MRP1 overexpressing tumours.

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The MRN complex controls replication stress and allows proliferation and survival in MYCN amplified neuroblastoma

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Finding an effective treatment for MYCN amplified (MNA) high-risk neuroblastoma patients represents an open challenge in pediatric oncology. We have recently shown that MYCN upregulates the three components of the MRE11/RAD50/NBS1 (MRN) complex during granule cell progenitors expansion in postnatal cerebellar development to restrain the deleterious effects of MYCN-dependent replication stress. Analysis of multiple datasets in R2, indicated that MRE11 and RAD50 are significantly more expressed in MNA compared to MYCN single copy primary neuroblastomas. Moreover, all MRN components are upregulated in MYCN-dependent medulloblastomas arising in mice with constitutively active Hedgehog pathway. Thus, we tested whether the MRN complex is necessary for proliferation/survival in MNA neuroblastoma. MRE11 or NBS1 RNAi-mediated knock down impaired proliferation and colony formation in a MYCN-dependent way. Pharmacological inhibition of the MRN complex via mirin, a MRE11 exonuclease drug inhibitor, selectively induced cell death in MNA compared to MNSC cells or non-neuroblastoma cancer models. A mirin analog selective for MRE11 exonuclease inhibition, but not an endonuclease specific inhibitor, induced cell death in MNA cells. While mirin caused accumulation of 53BP1 foci, a marker of DNA damage associated to replication stress, it also inhibited the ATR/CHK1-dependent checkpoint/s, preventing any arrest in the S and G2 phases of the cell cycle. In contrast, mirin induced early occurrence of DNA double strand breaks and a typical DNA damage response (DDR) characterized by ATM, H2AX and p53 phosphorylation culminating in the accumulation of pro-apoptotic p53 target genes. Gain and loss of function experiments confirmed that mirin-induced cell death in MNA cells is p53 dependent. Injection of mirin encapsulated in nanoparticles significantly inhibited tumor growth in a neuroblastoma xenograft model. Overall, these data support the idea that targeting the MRN complex, and perhaps other components of the replication stress response, might be exploited for therapeutic purposes in MNA neuroblastoma.

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TRPM7 promotes Neuroblastoma progression

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Neuroblastoma (NB) is the most common extra-cranial pediatric solid tumor in children. High-risk and refractory NB signifies poor prognosis, and is difficult to treat due to the lack of response to current therapies and aggressive disease progression. Novel drugs and alternative treatments are being investigated for patients with refractory or high-risk NB. However, finding an effective treatment strategy for these patients continues to be a major challenge due to a myriad of complex mechanisms that promote NB progression, including increased tumor growth and metastasis, and resistance to treatments. Previous studies have shown that MycN, a prognostic indicator for advanced and high-risk NB, regulates the expression of TRPM7, a calcium-permeable ion channel that plays a role in NB progression. The current research investigated the mechanism by which TRPM7 regulates NB progression. The results show that TRPM7 expression is significantly increased in high-risk and drug resistant NB, and down-regulating TRPM7 sensitizes NB cells to chemotherapeutic agents and induces cell death. The preliminary data also demonstrate that TRPM7 reside in the membrane of vesicles that are mobilized to the cleavage furrow during mitosis. Accordingly, inhibition of TRPM7 prevented the mobilization of TRPM7 vesicles, inhibited mitosis, and led to multi-nucleated cells and eventually cell death. The results from this study suggest that TRPM7 plays a critical role in regulating proper mitotic progression, and promotes drug resistance in NB. Accordingly, loss of TRPM7 activity sensitizes drug resistant NB to chemotherapeutic drugs by inducing mitotic catastrophe and cell death.

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Curcumin mediated apoptosis in human neuroblastoma cells via ROS and inhibition of sphingomyelin synthase and glycosylceramide synthase

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Background:
Curcumin induces apoptosis and inhibits proliferation, angiogenesis, and metastasis. The mechanism of cytotoxicity in neuroblastoma is unclear.
Objectives:
In this study we investigated the effects of curcumin on SMS-KCNR and CHLA-20 cells.
Methods:
SMS-KCNR and CHLA-20 cells were treated with increasing concentrations of curcumin. Cell viability was determined by Alamar Blue assays. Real-time PCR and western blotting of apoptotic pathways was performed. Measurement of endogenous sphingolipids was performed by LC/MS. Sphingolipid pathway enzyme activities were also determined.
Results: Curcumin was cytotoxic at 10 and 20 uM concentrations. PARP cleavage was noted at 24 hours, but cleavage of caspases 3, 8 and 9 was not observed. Treatment with the pancaspase inhibitor z-VAD did not reverse the cytotoxicity in curcumin treated cells, confirming that curcumin induced cell death was caspase-independent. Since perturbation in complex sphingolipids is associated with apoptosis, LC/MS measurement of endogenous sphingolipids was performed and showed increases in both dihydroceramides and ceramides. Dihydroceramide desaturase (DEGS-1) enzyme activity was inhibited. DEGS-1 activity was inhibited *in-situ* in a dose dependent manner. There was no change in the mRNA or protein levels of DEGS-1, supporting that curcumin inhibited this enzyme indirectly. Next, the mechanism of ceramide generation was investigated by measuring the activity of sphingomyelin synthase (SMS) glycosylceramide synthase (GCS), acid ceramidase, neutral ceramidase, acid sphingomyelinase and neutral sphingomyelinase (SMase). At 6hrs, curcumin downregulated SMS activity by 30% and 54% GCS activity by 40% and 42% at concentrations of 10 and 20uM respectively. Curcumin has been demonstrated to induce ROS generation. Pre-treatment with the antioxidants N-acetylcysteine or glutathione abrogated curcumin mediated apoptosis and sphingolipid generation. Furthermore, curcumin mediated SMS and GCS inhibition was blocked by these antioxidants.
Conclusions:
ROS plays a key role in sphingolopid and curcumin induced-cytotoxicity in neuroblastoma cells. Modulation of sphingolipid signaling pathways may provide a more effective and novel approach for the treatment of pediatric solid tumors. Curcumin is a potential novel therapy for neuroblastoma.

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PPP3CB is a novel prognostic indicator of high-risk neuroblastoma contributing to aggressive behaviors

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We have previously identified a stop-gain mutation in *PPP3CB*, which encodes a catalytic subunit of calcineurin, in a primary neuroblastoma (NB) with *MYCN* amplification by whole-exome sequencing of 57 paired neuroblastoma samples. The stop-gain mutation identified in *PPP3CB* results in a constitutive active form of the PPP3CB protein. In this study, we investigated the clinical and functional roles of PPP3CB in NB. Expression levels of *PPP3CB* in 72 NB clinical samples were measured by quantitative RT-PCR method. We found that high expression of*PPP3CB* was significantly associated with poor prognosis in our sample set. Multivariate Cox regression analysis showed that PPP3CB was an independent prognostic factor predicting poor outcome. On the other hand, overexpression of wild-type and mutated PPP3CB promoted cell growth, while PPP3CB knockdown decreased cell growth in NB cells. Enforced expression of mutated PPP3CB enhanced anchorage-independent growth in NB cells. Both wild-type and mutated PPP3CB activated the Nuclear Factor of Activated T cell (NFAT) signaling, accompanied with activation of Akt and inhibition of the GSK3β activity, which were inhibited by treatment with calcineurin inhibitors. Treatment with calcineurin inhibitors suppressed cell proliferation and induced apoptotic cell death in several NB cell lines. Thus, our data indicate that as a catalytic subunit of calcineurin, PPP3CB is an independent indicator of NB predicting poor prognosis, which may contribute to malignant biological behaviors of NB cells through activation of NFAT signaling. Inhibition of calcineurin activity might have therapeutic potential against high-risk NB.

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Clinical significance of a seven-gene hypoxia signature in neuroblastoma

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Background:
Survival of high-risk neuroblastoma (NB) patients remains poor. Advances in our understanding of neuroblastoma biology are critical for improving outcome. Hypoxia is a condition of low-oxygen tension occurring in poorly or abnormally vascularized tissues related to tumor aggressiveness. We report on the clinical significance of new 7 genes hypoxia signature.

Methods:
Gene expression of 498 samples from NB patients of all stages was measured by RNA-seq technology. Dataset was split into two groups of 49 and 449 patients to build a classifier and test its prognostic power. Classifier was built utilizing Logic Learning Machine algorithm (LLM).

Results:
We defined a new seven-gene signature (NB-hop) to assess hypoxia in primary NB tumors (PGK1, PDK1, EGLN1, ALDOC, FAM162a, AK4, and MTFP1 genes). A multi-gene classifier was built based on the expression of the aforementioned genes and patients' outcome. In the test cohort, the clinical significance of NB-hop predictions was assessed in relevant subgroups of patients defined by combinations of established clinical risk factors. NB-hop stratified patients into groups with significantly divergent OS (P =.006) and EFS (P =.03) in the population of high-risk patients with metastatic neuroblastoma (INSS 4) diagnosed after 18 months of age. Furthermore, NB-hop classifier distinguished patients with different probability of relapse in the population of localized (INSS 1, 2) or metastatic (INSS 4s) MYCN not amplified tumors, which are notoriously at low-risk of relapse (P=.004). The NB-hop classifier emerged as an independent risk factor in multivariate cox regression models on both OS and EFS (OS HR 2.4; 95% CI 1.4-4.2 and EFS HR 2.5; 95% CI 1.7-3.8, both P<.001).

Conclusions:
NB-hop is a novel independent prognostic factor capable of improving stratification of clinically relevant neuroblastoma populations while measuring tumor hypoxia. NB-hop may guide the use of hypoxia-activated prodrugs.

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The CXCR4/CXCR7/CXCL12 axis is involved in a secondary but complex control of neuroblastoma metastatic cell homing

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Background: The CXCR4/CXCR7/CXCL12 chemokine axis has been involved in the progression and organ-specific dissemination of various cancers. In NB, CXCR4 expression was shown to be associated to highly aggressive undifferentiated tumors, while CXCR7 expression was detected in more differentiated and mature neuroblastic tumors. As investigated *in vivo*, using an orthotopic model of tumor cell implantation of chemokine receptor-overexpressing NB cells (IGR-NB8), the CXCR4/CXCR7/CXCL12 axis was shown to regulate NB primary and secondary growth, although without any apparent influence on organ selective metastasis.

Methods: The selective role of CXCR4 and CXCR7 receptors in the homing phase of metastatic dissemination was addressed using an intravenous model of tumor cell implantation into NOD-scid-gamma mice.

Results: Tail vein injection of transduced IGR-NB8 cells overexpressing CXCR4, CXCR7 or both receptors revealed that all transduced cell variants preferentially invaded adrenal gland and typical NB metastatic target organs, such as liver and bone marrow (BM). However, CXCR4 expression favored NB cell dissemination to liver and lungs, while CXCR7 was able to strongly promote NB cell homing to adrenal gland and liver. Finally, coexpression of CXCR4 and CXCR7 receptors significantly and selectively increased NB dissemination toward BM.

Conclusion: CXCR4 and CXCR7 receptors may be involved in a complex and organ-dependent control of NB growth and selective homing, making these receptors and their inhibitors, potential new therapeutic targets.

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Stathmin expression regulates miR-382/PTPN14 expression in neuroblastoma cells

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Advanced stage neuroblastoma is highly aggressive and drug refractory metastasic disease is a major cause of treatment failure. We have recently shown that the phosphoprotein stathmin, which is overexpressed in neuroblastoma, mediates cell migration and invasion *in vitro* and neuroblastoma metastasis *in vivo*[1]. Several miRNAs have been identified to play an important role in neuroblastoma cell proliferation, migration, invasion and metastasis. To investigate whether stathmin regulates miRNA expression and metastasis, miRNA and gene arrays were performed on control versus stathmin depleted neuroblastoma cells. A number of miRNA were found to be significantly altered in stathmin depleted neuroblastoma cells compared to control cells, with analysis of their target genes identifying key signalling pathways involved in cell migration, metastasis and cell survival. Expression of three of these miRNAs was validated by qPCR and found to be consistently altered in stathmin depleted neuroblastoma cells. The protein tyrosine phosphatase, non-receptor type-14 (PTPN14) is a predicted target for three of the differentially expressed miRNAs, and miR-382, was prioritised for further studies. Consistent with gene array data, qPCR analysis showed that PTPN14 expression was downregulated in stathmin depleted neuroblastoma cells. In breast cancer, PTPN14 has been shown to prevent metastasis by restricting protein trafficking[2] and mutations in PTPN14 were identified in relapsed neuroblastoma samples[3]. Analysis of RNAseq data (Tumor SEQC data - 498 primary samples) showed that high expression of PTPN14 gave better overall survival in the neuroblastoma cohort compared to low expression. The same trend was also found when analysing the MYCN amplified subset separately. Collectively, we have identified miR-382 and its predicted target gene, PTPN14 to be modulated by stathmin and these are being investigated to understand their role in stathmin mediated metastasis and to explore their potential as therapeutic targets for treating neuroblastoma.

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Functional Genomics identifies novel therapeutic targets for retinoic acid combinations

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Background. Our laboratory employs a functional genomic siRNA array platform to identify novel therapeutic targets. Using this approach we have interrogated the human kinome for novel targets for neuroblastoma (NB) therapeutics.
Goals. To identify novel kinases as future drug targets to be used as a combined therapy with Retinoic Acid in NB.
Methods. We employed high-throughput siRNA screening in three molecularly defined NB cell lines with different sensitivities to RA. Using a kinome-focused siRNA library, we identified 49 kinase encoding genes out of 713 genes which were selected for their statistical significance and for their biological relevance.
Results. We tested 40 genes across three cell lines +/- RA. Fourteen out of the 40 kinases were validated as sensitizers through multiple growth inhibition assays with independent siRNAs. In SKNAS, a MYCN non-amplified cell line, we identified kinases in the phosphatidylcholine biosynthesis and involved in neurite differentiation as well as others with important roles in non-homologous-end-joining repair of DNA damage pathway. In SKNBE2, a MYCN-amplified cell line, we also identified kinases involved in cell motility such as a bifunctional enzyme implicated in N-acetylmuramic acid biosynthesis, in differentiation such as tyrosine-kinase receptor involved in phosphatidylserine modulation and other kinases in the homologous recombination repair pathway. We confirmed that, for a total of fourteen genes, knock-down strongly decreased cell viability in combination with RA. Interestingly, several genes in common between the two cell lines were involved in the DNA damage repair pathway.
Conclusion. Our experiments identified novel therapeutic targets to use in combination with RA in high-risk NB. The detection of a concordance between different cell lines indicates DNA repair and differentiation-linked genes as promising pathways to be targeted for RA combinations. Furthermore, linkage of these kinase sensitivity with specific genomic features of NB will allow to tailor these combinations to individual patients.

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A multidisciplinary approach to antigen discovery and immune profiling of Opsoclonus-Myoclonus Ataxia Syndrome associated with Neuroblastoma

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Pediatric Opsoclonus Myoclonus Ataxia Syndrome (OMAS) is a rare but devastating autoimmune disorder characterized by acute onset of opsoclonus, myoclonic jerking and ataxia, and disordered mood/behavior in a previously well child. At least 50% of children with OMAS have a detectable neuroblastoma (NB) at the time of diagnosis. However, only 2-3% of children with NB develop OMAS, raising the question of what triggers autoimmune disease in this subset of patients. It is also striking that tumor-related survival rates are significantly higher among children with OMS than among NB patients at large.
Though this condition was first described over 50 years ago, no specific molecular marker of this disease is known, and the identity of the antigen responsible for autoimmune attack remains unknown. The current standard of care for treating OMAS involves relatively non-specific immunosuppressive therapies, with variable success: although frank ataxia and opsoclonus typically resolve with treatment, most children with OMAS suffer from lasting neurocognitive and/or motor deficits. Identification of the specific antigen(s) underlying OMAS pathology, or other molecular markers of disease, would represent critical advances en route to its effective diagnosis and treatment. Further, if the immune component of OMAS is related to improved NB survival in OMAS patients, then understanding the basis of this tumor immunity could have clinical relevance for NB in a broader context. We are using RNAseq and immune sequencing of TILs in OMAS-associated NBs, and non-OMAS NBs of both low-risk and high-risk subtypes, to better understand how OMAS associated NB is different. Our results will be presented.

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Composite Neuroblastoma: Unique tumours with morphologically and genetically defined intratumoral heterogeneity

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Background. Peripheral neuroblastic tumours make one of the most common paediatric neoplasms, and are characterized by clinical and biological heterogeneity. Intratumoral heterogeneity is well documented in Ganglioneuroblastoma, nodular (composite, Schwannian stroma-dominant/stroma-rich and stroma-poor) category. However little is known about "Composite Neuroblastoma" with intratumoral heterogeneity in Neuroblastoma (Schwannian stroma-poor) category.
Methods. First, a series of 12 neuroblastomas showing morphological heterogeneity were identified by the International Neuroblastoma Pathology Committee. All of those tumours were composed of 2 clearly distinct histology areas: i.e., 2 different components based on the grade of neuroblastic differentiation and/or mitosis-karyorrhexis-index. Then genetic intratumoral heterogeneity was analyzed by the fluorescence in situ hybridization (FISH) method using the formalin-fixed and paraffin-embedded sections from each of 24 distinct components of those tumours. FISH performed in this study tested (1) *MYCN* genomic status using a *MYCN* probe in 2p24 and a reference DNA probe *Laf* (2q11), and (2) Gene rearrangements (gain) using a Dual Fusion Probe containing a mixture of two probes for *PML* gene (15q22) and *RARA* gene (17q21.1).
Results. The first FISH analysis with *MYCN* and *Laf* probes demonstrated genetic difference between the two morphologically distinct components for 10 of 12 cases: namely one component showed disomy and the other had polysomy/gain. None of 24 components in this series had amplified *MYCN* defined by the presence of *MYCN* signals more than 4 times reference signals. The second FISH analysis also demonstrated difference of 17q21.1 status (disomy vs. gain) between the two components for 11 of 12 cases. Overall, the two morphologically distinct areas

(components) of all the 12 tumours demonstrated different genetic characteristics by the FISH tests.
Conclusion. This study verified the intratumoral heterogeneity; i.e., presence of different clones, in "Composite Neuroblastoma". Those different clones in the individual tumours were first identified morphologically and then completely confirmed genetically.

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The histone H3 lysine 4 presenter WDR5 is a potential therapeutic target in N-Myc-induced neuroblastoma.

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Neuroblastoma is a cancer of the sympathetic nervous system occurring mostly in infants and young children. It accounts for more than 10% of all paediatric cancer deaths. Despite considerable progress in the treatment of neuroblastoma, 5-year survival rates have remained less than 50% for the majority of children with high-risk disease. One of the most powerful prognostic markers for this disease is amplification of the *MYCN* oncogene, which has been found in approximately one third of neuroblastomas and nearly half of high-risk cases. Although *MYCN* has a clearly central role in neuroblastoma tumorigenesis, the critical molecular mechanisms of high-risk neuroblastoma have not yet been well understood for clinical use. Histone H3 lysine 4 (H3K4) trimethylation at target gene promoters is a strict pre-requisite for Myc-induced transcriptional activation. We have identified WD-repeat protein 5 (WDR5), a core component of many H3K4 methyltransferase complexes, interacts with N-Myc, leading to WDR5-mediated H3K4 trimethylation and N-Myc-regulated transcriptional activation in neuroblastoma cells. Furthermore, we have demonstrated RNAi-mediated attenuation of WDR5 upregulated expression of wild type but not mutant p53, an effect associated with

growth inhibition and apoptosis in neuroblastoma. In addition, WDR5 was over-expressed in pre-cancer ganglia and neuroblastoma cells from *MYCN* transgenic mice, compared with normal ganglia cells. In neuroblastoma patients, high levels of WDR5 expression in tumor tissues independently predicted poor overall survival. Importantly, we have identified that OICR-9429 exerts a dose-responsive, inhibitory effect in *MYCN*-amplified neuroblastoma cells viability. Moreover, we have founded that OICR-9429 modulates the expression of N-Myc target genes CCNE1, MDM2 and p53. Of note, we have showed that OICR-9429 blocks WDR5-N-Myc protein interaction. Our findings therefore identify WDR5 as an important biomarker for N-Myc-regulated transcriptional activation and tumorigenesis, and as a novel therapeutic target for N-Myc over-expressing neuroblastoma.

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The signaling complex of tyrosine phosphatase SHP2 and docking protein ShcC regulates oncogenicity of neuroblastoma cells in a tyrosine-phosphorylation dependent manner

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Background: Recent studies revealed that gene amplification and series of oncogenic mutations of anaplastic lymphoma kinase (ALK) are potent oncogenic factors of neuroblastoma (NB). In addition, enhanced ALK expression even without genetic alteration of ALK.is a poor prognostic factor in NB patients. We investigated the specific downstream signaling molecules of ALK in NB to investigate the unique role of ALK in oncogenesis of NB.
Methods: To elucidate the role of ALK in oncogenesis of NB cells, phosphotyrosine-containing proteins associated with ALK were investigated by purification and mass-spectrometry analysis.
Results: SHP2 (SH-PTP2/PTPN11), a tyrosine phosphatase was identified among the ALK-binding tyrosine-phosphorylated proteins. Since oncogenic contribution of SHP2 phosphatase has been reported in several cancers, and point mutations of SHP2 was recently reported in neuroblastoma as well, we examined the role of SHP2 phosphatase in the neuroblastoma cells. The ALK-dependent N'-terminal tyrosine phosphorylation of SHP2, which is known to regulate phosphatase activity of SHP2, was revealed in NB-39-nu NB cells which harbor gene amplification of wild-type ALK. It was also shown that SHP2 is associated with ALK through SH2 domains of SHP2 via ShcC, a docking protein we originally found as a major binding partner of ALK (Miyake et al, 2002) which regulates NB oncogenicity in a tyrosine phosphorylation-dependent manner. SHP2 appeared to mediate ALK-dependent oncogenic property such as proliferation or migration of NB-39-nu cells, while SHP2 also induced dephosphorylation of ALK protein in NB-39-nu cells. These data suggest that SHP2-ShcC complex control ALK-dependent malignant characteristics of NB by enzymatic interaction between molecules. Further studies are currently on-going to clarify the precise mechanism.

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The role of $p75^{NTR}$ during neuronal differentiation of neuroblastoma cells

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Neuroblastoma is the most common solid tumor in infancy and the high-risk form has still a dismal outcome although multimodal treatment. MYCN amplification is a negative prognostic marker and contributes to maintain an undifferentiated phenotype of neuroblastoma. The overall aim of this study is to explore regulatory pathways of controlling differentiation of neuroblastoma cells and the potential of activating these pathways as a mode of action of therapy.
We found that $p75^{NTR}$, a cell surface receptor that gets internalized upon ligand binding, is regulated by estrogen signaling. Thus, the aim of this study was to further investigate the role of the receptor in differentiation of NB cells. Interestingly, $p75^{NTR}$ was mainly located perinuclear in NB cells overexpressing ERα. Activation of ERα resulted in upregulation of $p75^{NTR}$ and redistribution throughout the cytoplasm, whereas control cells remained unaffected by the estradiol treatment. A more detailed analysis showed that $p75^{NTR}$ was located at the microtubule-organizing center (MTOC) and the golgi at low concentrations, whereas it was detected in vesicles at high expression levels. $p75^{NTR}$ can regulate the depolymerization of actin through inhibition of Rho-GTPases. Since Rho-GTPases are important players during the organization of the cytoskeleton in neuronal cells, we analyzed if $p75^{NTR}$ is involved in the cytoskeletal rearrangements during differentiation. Temporary disruption of the microtubules using nocodazole resulted in a dispersion of $p75^{NTR}$ in the cytoplasm. After nocodazole removal, a rapid localization of $p75^{NTR}$ to the center from where the microtubules regenerated was observed and the original structure was restored.
Here we identified a putative role of $p75^{NTR}$ during the regulation of the cytoskeleton in neuronal differentiation of neuroblastoma cells. Further studies will be needed to elucidate the role of p75NTR in neuronal differentiation of neuroblastoma cells.

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Impact of the involvement of the separate body regions in the modified Curie and the SIOPEN mIBG-scoring systems in patients with stage 4 neuroblastoma

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Background: Semi-quantitative scoring of initial I-123-mIBG-scans using the modified Curie- or the SIOPEN-score allows prediction of outcome in patients with stage 4 neuroblastoma[1]. However, the impact of involvement of the separate body regions on outcome and effect of the degree of subdivision has not been investigated yet.
Methods: Initial mIBG-scans of 71 patients diagnosed with stage 4 neuroblastoma >1 year of age were retrospectively scored using both scores (modified Curie: 9 skeletal regions plus soft tissue, score 0-30, SIOPEN: 12 skeletal regions (assessing left and right humeri, forearms, femora, and lower legs separately) score 0-72). For the separate body regions correlation to other body regions and impact on prognosis was analyzed.
Results: Results of both scoring methods were highly correlated (Pearson's correlation coefficient: r=0.97, p<0.001). The regions most frequently involved were femora (84%), spine (74%), pelvis (74%), and humeri (73%) while forearms (32%) and soft tissue (13%) were rarely affected.
There was a high correlation between involvement of right and left humeri (r=0.903, p<0.001), forearms (r=0.881, p<0.001), femora (r=0.838, p<0.001), and lower legs (r=0.861, p<0.001) as well as between cervico-thoracal and lumbo-sacral spine (r=0.916, p<0.001).
Using the modified Curie-score, EFS and OS were inferior if humeri (EFS p=0.009; OS p=0.007) or femora (EFS p=0.014; OS p=0.004) were involved, while involvement of the lower legs was only associated with worse OS (p=0.030). Using the SIOPEN-score this held true for EFS and OS for involvement of the humeri (right: EFS p=0.014; OS p=0.013; left: EFS p=0.037; OS p=0.017) and for OS only for involvement of femora (right: p=0.006; left: p=0.046) and tibiae (right: p=0.019; left: p=0.018).
Conclusions: Regions with prognostic impact (humeri, femora, tibiae) could be identified, while differentiation between left and right limbs did not reveal additional prognostic value and involvement of left and right limbs was highly correlated to each other. However, the subtler subdivision in the SIOPEN-score might allow a more sensitive assessment of the course of disease.
Iodine-123 metaiodobenzylguanidine scintigraphy scoring allows prediction of outcome in patients with stage 4 neuroblastoma: results of the Cologne interscore comparison study, Decarolis et al., J Clin Oncol. 2013 Mar 1;31(7):944-51

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Prognostic significance of imbalanced chromosomal alterations in primary and recurrent neuroblastoma

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139 primary and 22 relapsed neuroblastoma samples were studied for copy number variations (CNVs) using MLPA. All primary and 14 relapsed tumors were obtained during core biopsy. In 8 cases of recurrence liquid biopsy of involved bone marrow has been performed. Prognostic significance was estimated by overall (OS) and event-free survival (EFS) with median of follow-up time 36 months (range 1-190 months).
In 32 patients (23.0%) 1p deletion was revealed and had negative prognostic impact (EFS 0.38SE0.09 vs. 0.63SE0.05, p=0.010, OS 0.49SE0.09 vs. 0.71SE0.05, p=0.008). 17q gain was detected in 60 patients (43.2%), EFS 0.51SE0.07 vs. 0.63SE0.06, p=0.041, OS 0.51SE0.08 vs. 0.74SE0.06, p=0.021. Trisomy 7 discovered in 12 patients (8.6%) decreased survival: EFS 0.41SE0.16 vs. 0.59SE0.05, p=0.032; OS 0.46SE0.18 vs. 0.65SE0.05, p=0.045. Aberrations listed above retained prognostic significance in *MYCN* non-amplified patients.
2p23-24 gain below the *MYCN* amplification (MNA) level was detected in 13 patients and had negative significance in group of patients below 18 moths: both EFS and OS 0.56SE0.20 vs. 0.82SE0.06, p=0.048 and 0.92SE0.04, p=0.024.
9p deletion with haploinsufficiency of *CDKN2A* gene was revealed in 9 patients (6.5%) and resulted in low OS 0.38SE0.17 vs. 0.65SE0.05, p=0.032. *MDM2* gene gain had negative influence on EFS in favorable groups: in infants (0.55SE0.13 vs. 0.86SE0.06, p=0.011), in patients with localized disease (0.61SE0.11 vs. 0.79SE0.06, p=0.057) and in 4S patients (0.20SE0.18 vs. 0.86SE0.13, p=0.043).
In multivariate analysis of OS stage 4 (p=0.042), MNA (p=0.049) and 9p deletion (p=0.041) demonstrated independent prognostic significance.
Investigation of CNVs in relapsed neuroblastomas revealed appearance of new alterations in 9, stable spectrum of aberrations in 3 and lack of original CNVs in 5 cases. Patients harboring new CNVs had significantly worse outcome after the recurrence comparing with those who had identical or lack of CNVs in relapse: EFS 0.00, OS 0.14SE0.13 vs. both 0.73SE0.16, p=0.014, p=0.045.

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^{18}F-Meta Fluorobenzyl Guanidine (MFBG) Positron Emission Tomography(PET) imaging in patients with Neuroblastoma and other Neuroendocrine Malignancies

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Background: ^{123}I-MIBG imaging has several disadvantages: need for second-day imaging schedule, low resolution and imprecise, non-quantitative uptake measurements. ^{18}F-MFBG, a positron-emitting MIBG analog can potentially overcome these limitations. We present preliminary results of a first-in-human pilot study (Clinicaltrials.gov NCT02348749) evaluating pharmacokinetics and biodistribution of ^{18}F-MFBG.
Methods: Six patients (1 neuroblastoma; 5 paraganglioma/pheochromocytoma) received 10mCi/m^{2} ^{18}F-MFBG intravenously followed by whole body (WB) PET imaging at three time points: ~30 min-1h, ~1-2 h and 3-4h post-injection (PI). Serial blood samples were collected 5 minutes-4 hours PI. WB and blood clearance rates, lesion uptake and radiation absorbed dose (RAD) for normal organs was analyzed using OLINDA software.
Results: No adverse events were encountered. WB clearance was mono-exponential with mean effective half-life (T1/2) of 1.8h. Blood clearance was bi-exponential with 0.2h (57 %) T1/2e for the rapid phase and 4.4h (43 %) for the slower phase. Renal excretion was rapid. Prominent activity noted in blood pool, liver and salivary glands, and mild activity in kidneys and spleen decreased with time. Median RAD estimates for urinary bladder, kidney and liver were 0.475, 0.125 and 0.139 cGy/mCi respectively with minimal activity noted in other organs. Mean±SD WB dose was 0.0311± 0.0202rad/mCi . Both skeletal and soft tissue lesions were visualized with high contrast as early as 0.5h PI, and best seen 3-4h PI. Mean±SD SUV_{MAX} at 3-4h pi was 10.8±12.5 (range 1.1-80.7). Tumor to background ratios ranged from 1.35-36.2.
Conclusions: Preliminary data from this ongoing study shows that ^{18}F-MFBG imaging is safe, achievable on day of injection, has favorable biodistribution and kinetics with good targeting of lesions. ^{18}F-MFBG PET has the potential to be a better imaging modality than ^{123}I-MIBG gamma scan. Comparison with ^{123}I-MIBG scans is ongoing and further neuroblastoma patients are being studied.

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^{124}I-hu3F8 radioimmuno-positron emission tomography (PET) in patients with neuroblastoma and other GD2-positive malignancies: preliminary results on biodistribution, pharmacokinetics and tumor targeting

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Objectives: Anti-GD2 monoclonal antibodies (MoAbs) are currently an integral part of therapy for high-risk neuroblastoma. However, biodistribution in patients has not been fully explored. We radio-iodinated the humanized anti-GD2 MoAb hu3F8 with the positron-emitting radioisotope ^{124}I and investigated the biodistribution, pharmacokinetics and tumor targeting of ^{124}I-hu3F8 in patients with neuroblastoma and other GD2-positive tumors in a first-in-human study. (Clinicaltrials.gov NCT02307630). We present preliminary results from this ongoing trial.
Methods: Patients received 3 mCi/m^{2} ^{124}I-hu3F8 intravenously followed by whole body (WB) PET imaging at 3-4 time points 2 to 96 hours post-injection (PI). Serial blood samples were drawn over the same time period. WB and blood clearance rates, lesion uptake and radiation absorbed dose for normal organs were analyzed using OLINDA software.
Results: Three patients (2 neuroblastoma and 1 osteosarcoma) thus far have received ^{124}I -hu3F8 and completed study observations. No adverse events were noted. In the two neuroblastoma patients, there was highly accurate and specific targeting to metastatic disease with 100% concordance of lesions detected compared to MIBG scan. The patient with osteosarcoma underwent ^{124}I-hu3F8 radioimmuno-PET followed by resection of a 7mm pulmonary metastasis. Autoradiography showed specific targeting of ^{124}I-hu3F8 to areas of viable osteosarcoma cells correlating with immunohistochemical staining for GD2, but not to acellular calcified areas. Median bloo half life (T1/2) of ^{124}I-hu3F8 bi-exponential with an early rapid T1/2 of 1.24±2.7h and delayed phase with T1/2 of 28±13.7h. Mean organ radiation absorbed dose (rad/mCi injected) was 2.96±1.58, 2.82±1.42, 2.14±1.4, and 2.22±1.11 for blood, kidney, liver and lung respectively. Whole body effective dose (rem/mCi) averaged 3.55±3.21.
Conclusions: Preliminary data from this ongoing study show that ^{124}I-hu3F8 imaging is safe, pain-free, highly specific and sensitive. Quantitative Information could be obtained for dosimetry calculations. Radio-iodinated hu3F8 has the potential to be used for

radioimmunodetection and radioimmunotherapy of neuroblastoma and other GD2-positive tumors.

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A High ALK expression is associated with an unfavorable histology in Neuroblastoma.

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Background
A high ALK protein expression, unfavorable histology (UH) according to the International Neuroblastoma Pathology Classification (INPC)
and *MYCN* amplification are associated with a poor outcome in neuroblastoma (NB). Correlations between a high ALK expression and histology are inconclusive.

Method
Immunohistochemistry for ALK and INPC was evaluated in 195 NBs analyzed
for *MYCN* amplification, 1p/11q LOH, 17q gain, and *ALK* mutations. Over 50% positivity of tumor cells indicated a high ALK expression. Correlations between the ALK expression and INPC and genomic alteration(s) were analyzed for prognostic significance.

Results
Eighty-six NBs with high ALK expression levels and 109 NBs with low ALK expression levels were identified. High ALK expressors significantly demonstrated UH (70/86, p<0.0001). *MYCN* amplification and 11q LOH were not associated with high ALK expression levels. High ALK expressors with a favorable histology (FH) were younger than those with UH; median age at diagnosis was 6.5 months and 36.5 months, respectively. Among ALK high expressors, *ALK* mutations were detected in 12/70 (17%) in the UH group, and 4/16 (25%) in the FH group. In the FH group, high ALK expressors showed a significantly inferior overall survival rate (OS)(58%) than low ALK expressors (100%) (p=0.001), while ALK expression was not associated with the prognosis in UH (OS was 41% and 50%, respectively). FH and *MYCN* nonamplified (NA) tumors showed less ALK high expression (14/65; 22%) and 11q LOH (9/65; 14%)(p<0.0001). UH and *MYCN*-NA tumors tended to exhibit 11q LOH (43/70; 61%)(p=0.056), but not a high ALK expression. UH and *MYCN* amplified tumors were associated with high ALK expression levels (36/56; 64%)(p=0.033) and less 11q LOH (9/56; 16%)(p<0.0001).

Conclusion
A high expression of ALK correlated with UH, but not *MYCN* amplification or 11q LOH. In the FH group, but not in UH, high ALK expressors had an inferior survival.

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Feasibility of applying F^{18}-DOPA hybrid MR-PET to follow-up of neuroblastoma patients

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Patients with neuroblastoma have to receive regular follow-up examinations including F^{18}-DOPA PET and MR imaging to monitor recurrence of tumors. The conventional CT-PET is about twice the radiation dosage of CT. It is reasonable to replace CT-PET by MR-PET with decrease of ionizing radiation and let patients receive MR and PET examinations simultaneously. F^{18}-DOPA is manufactured by NTUH and already on the market. Ten cooperative neuroblastoma patients were recruited to receive F^{18}-DOPA MR-PET examinations immediately after regular CT-PET examinations without use of additional isotopes. The residual proton density from F^{18}, contrast-enhanced MR images were collected and correlated with the regression or progression in the follow-up images of neuroblastoma. F^{18}-DOPA MR-PET detected 41 neuroblastoma lesions but 2 of the bone marrow lesions were not shown on F^{18}-DOPA PET-CT. No additional lesions were only detected by F^{18}-DOPA PET-CT images. The SUV contrast ratios of 39 neuroblastoma lesions were not significantly different between F^{18}-DOPA MR-PET and F^{18}-DOPA PET-CT images. The results of the study will be applied to evaluate the treatment efficacy and prognosis.

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Co-expression network analysis reveals long non-coding RNA *SNHG1* as a novel biomarker in neuroblastoma

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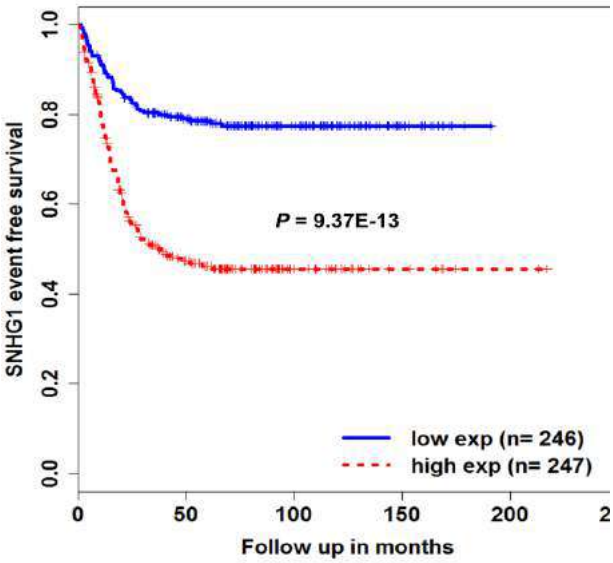
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Background:
Currently, several long non-coding RNAs (lncRNAs) observed to be aberrantly expressed in cancer and modulate the fundamental biological processes. However, few of them have been established as prognostic biomarker. Here, we aim to find differentially expressed lncRNAs and its co-expressed coding genes regulated by N-Myc in the *MYCN* amplified neuroblastoma.

Methods:
Publicly available microarray expression profile was analyzed to identify differentially expressed transcripts in the *MYCN* amplified compared to *MYCN* non-amplified condition. Biological functions of the differential coding genes were identified by ClueGO. Association between lncRNA and coding gene was determined through Spearman correlation coefficient (SCC) and Fischer's Z transformation. MYCN Chip-Seq data set was used to find binding sites of MYCN in the promoter of the regulated genes. Relative expression of common lncRNAs identified in microarray and RNA-Seq differential expression study were validated by RT-qPCR. Moreover, survival analysis and cox regression analysis was performed in the neuroblastoma cohort (n= 493).

Results:
We found 591 coding genes and 13 lncRNAs to be differentially expressed (fold change >= 2 and p <= 0.05). Functional enrichment analysis reveals cell cycle progression for the up-regulated genes and neuron development, synapse assembly for the down-regulated genes. The co-expression data with a z-score threshold ≥ 3.0 and further a SCC cut off >= 0.8, filtered 39 co-expressed pairs. Among which, *SNHG1* and *TAF1D* were found to be highly correlated. Next, patients with high risk neuroblastoma express significant high *SNHG1* than those with low risk disease (p < 2.2E-16), and high expression of *SNHG1* is critical to patient event free survival (p < 9.37E-13). Moreover, multivariate Cox regression analysis reveals hazard ratio of 1.58 (n= 493, p = 2.36E-02).

Conclusion:
SNHG1 and *TAF1D* are significantly correlated and regulated by N-Myc. Moreover, lncRNA *SNHG1* might serve as a novel biomarker for high risk neuroblastoma intervention.



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Post surgical 123I-MIBG SPECT/CT in neuroblastoma

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Objectives: Post-surgical scintigraphy is of importance to assess residual tissue at surgical site in neuroblastoma. The objectives were (i) to to assess post-surgical residual tissue with 123I-MIBG scintigraphy and morphological imaging (ii) to compare 123I-MIBG SPECT/CT acquisition to planar scintigraphy (iii) to assess the impact of MIBG uptake in residual tissue on survival.

Methods: 30 patients consecutively operated for MIBG-positive neuroblastoma were included (INRG stage L1 n=4, L2 n=10, M n=12, Ms n=4). On operative report, surgery was considered as complete in 23 and incomplete in 7 patients, respectively.

Postoperative imaging included 123I-MIBG scintigraphy with 123I-MIBG SPECT/CT and planar acquisitions and morphological exams (MRI or CT or US).

Quantification of 123I-MIBG SPECT/CT was performed with tumor to mediastinum count rate ratio (TMCRR).

Results: Mean delay between surgery and post-operative MIBG or morphological imaging was 39 and 49 days, respectively. Mean additional radiation exposure induced by 123I-MIBG SPECT/CT was 149mGy.cm.

At surgical site, postoperative morphological exam was positive in 14 patients, negative in 16; 123I MIBG SPECT/CT positive in 6 patients (3 for MIBG planar imaging), negative in 24.

In case of reported complete surgery, morphological imaging was positive in 7/23 and MIBG SPECT in 2/23 patients.

With a median follow-up of 25 months (range 6-83), 10 pts had relapse or progression (local in 5 pts including 2 pts with slow local progression; metastatic in 4 pts, local and metastatic in 1 pt).

Median progression-free survival (Kaplan-Meier) was 57 weeks (42-72.5 CI95).

Using a TMCRR cutoff at 2.6 on 123I-MIBG SPECT/CT, we found a significant difference in progression-free survival (p=0.004), patients with a higher ratio having worst prognosis.

No significant difference was found in progression free survival in case of residual tissue presence on morphological exam (p=0.22).

Conclusion: Post-surgical 123I-MIBG SPECT/CT appears a key tool to assess residual tissue in neuroblastoma. Further studies are needed to assess impact for patient's management.

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High-risk neuroblastoma without *MYCN* amplification in patients between 12 and 18 months: Is there a hidden low-risk patient group?

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Background: The HR-NBL-1/SIOPEN trial accrues stage M neuroblastoma patients ≥ 12 months at diagnosis, irrespective of *MYCN* amplification (MNA) status. The International Neuroblastoma Risk Grouping (INRG) has established a cut-off age of ≤18m for low-risk metastatic neuroblastoma (stage Ms) given clinical and genomic criteria (no MNA, no 11q-) are met. The aim of this SIOPEN Biology committee analysis is to genomically identify possible stage Ms patients in the 12-18m HR-NBL1/SIOPEN study.

Methods: Information on segmental chromosome aberrations (SCA) and ploidy was available for 36 patients aged 12-18m (no-MNA tumors). NB samples were analyzed by FISH and/or multi-locus/pangenomic techniques (MLPA/array-CGH/SNP-arrays). Genomic

data were centrally reviewed by the SIOPEN Biology members and SCAs subdivided into typical (typSCAs: 1p/3p/4p/11q losses; 1q/2p/17q gains) and atypical ones (atypSCAs). Ploidy was determined by FCM/ICM or by evaluation of numeric aberrations (near-diploid vs. aneuploid).
Results: Five of 7 patients without skeletal metastases had no 11q-. One died after relapse (incomplete genomic data); another one died on treatment. The other three remaining patients had no relapse; their tumors showed aneuploidy without SCAs (for one of them there is no atypSCA information). Altogether, typSCAs occurred in 33/36 tumors (80% 17q gain, 69% 11q loss), atypSCAs in 17/22. Near-diploid tumors (n=22) showed 1-6 typSCA, aneuploid neuroblastomas (n=12) 0-3 typSCAs (two typSCA tumors without ploidy information). Of 16 patients without or with up to 2 typSCAs (10 aneuploid), one patient relapsed, 2 died of NRM (non-relapse mortality; altogether 5/36 patients with NRM) but none died of disease. However, of 16 patients with >2 typSCA (1 aneuploid), 4 relapsed, 2 are DOD and 3 had NRM. 3/9 patients with 1p- tumors relapsed and died (plus 3 NRM), whereas 0/24 patients without 1p- died of disease, despite 3 relapses (2 NRM). 2/4 tumors with +1q relapsed.
Conclusion: In the 12-18m age group, a small number of stage M patients without skeletal metastases and with favorable genomic features could represent stage Ms patients.

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The detection and quantification of neuroblastoma metastases in bone marrow using plasmids-targets as standards in QRT-PCR

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BACKGROUNG: Bone marrow (BM) evaluation is one of the crucial steps of the clinical staging in neuroblastoma (NB) patients. Additionally, it is believed that the most likely cause of the treatment failure is the presence of NB cells persistent after therapy. Unfortunately, a limit level of tumor cells in BM or their molecular markers that would be clinically significant have not been yet well established.
AIMS: The detection of NB cells in BM samples before and during therapy, and the evaluation of diagnostic accuracy of designed method.
MATERIALS AND METHODS: We employed study samples: 145 BM (bilateral, before and during treatment) from 40 NB patients in all stage and control samples: 32 BM from non-NB cancer patients, 10 peripheral bloods (PB) from healthy volunteers, 3 NB cell lines. BM samples were obtained from patients treated at the Department[3] between 2010 and 2015 year. The QRT-PCR was used to evaluate transcripts of *TH* and *PHOX2B* as marker genes and *B2M* as a control gene. The relative quantification was used to indicate metastases ($\Delta Ct_{meancontrol}-\Delta Ct_{sample}\geq 3$). The absolute quantification was measured with plasmid cDNA templates for *TH*, *PHOX2B* and *B2M* genes for establishing standard curves. The absolute quantification was also used to trace the ROC curve with anti-GD2 immunostaining as a reference. The specificity and the sensitivity of selected marker genes were adequately checked by relative evaluation of its expression in control samples and in serial dilutions of NB cell lines in PB samples.
RESULTS: Positive results were obtained for metastatic disease. The ROC curves determined cut-points for true-positive samples (*TH*=1,32 *PHOX2B*=3,67 transcripts per 10^5*B2M*). The *PHOX2B* gene was the most specific NB cells marker. The sensitivity of QRT-PCR was confirmed at 10^{-6}.
CONCLUSION: It was showed the utility and the high sensitivity of designed method for the detection and monitoring metastases in BM of NB patients. Longitudinal studies are needed to definitely establish its clinical usefulness.

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Validation of image-defined risk factor (IDRF) assignment in patients with intermediate-risk neuroblastoma: a report from the Children's Oncology Group study ANBL0531

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Background: The International Neuroblastoma Risk Group (INRG) classifier utilizes a staging system based on pretreatment imaging criteria, with the extent of locoregional disease determined by the presence or absence of IDRF. An objective of the Phase III study ANBL0531 was to prospectively validate the prognostic value of the INRG IDRF system and to compare the institutional determination of IDRF with that of a central review committee.
Methods: Between 9/2009 and 6/2011, 211 newly diagnosed eligible intermediate risk patients enrolled on ANBL0531 had assessment of primary tumor IDRF performed by their local institution. Risk-stratification and therapy assignment utilized age, INSS stage, INPC, *MYCN*, and tumor ploidy, but not IDRF status or INRG staging. Therapy reduction was prescribed for tumors without loss of heterozygosity at 1p36 and/or 11q23. Following study closure, blinded central review of initial diagnostic imaging was performed by a panel of pediatric surgeons and radiologists to validate the local institutional assessment of IDRF.
Results: The cohort of 211 patients included 12 INSS stage 2A, 16 stage 2B, 83 stage 3, 74 stage 4, and 26 stage 4S neuroblastoma. The 3-year overall survival rate for patients with localized tumors, regardless of tumor biology, was 100%. The accuracy of IDRF assignment by local institutions will be compared to central review assignment and will be reported at the meeting. The potential impact of primary tumor location and number or type of IDRF on choice of initial diagnostic procedures and surgical morbidity will also be presented.
Conclusions: These data will inform the process of transition from INSS to INRG staging, including the impact of IDRF on the choice of the initial diagnostic and surgical procedures, the risk of surgical complications, and survival rates for patients with localized tumors, whether defined as INSS stage 2A/2B, 3 or INRG L2.

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Epithelial to mesenchymal transition and minimal residual disease monitoring in neuroblastoma

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Introduction Not all neuroblastoma patients that relapse can be recognized by PCR-based monitoring of bone marrow (BM) and peripheral blood (PB). Because epithelial to mesenchymal transition (EMT) is shown to be involved in tumor progression and therapy resistance and it also has recently been demonstrated that neuroblastoma consists of neuroepithelial (NE) and mesenchymal (MES) cells, the aim of this study was to identify MRD markers that can specifically detect MES-neuroblastoma cells and to study the dynamics of these markers during treatment.
Methods Microarray data were used to identify genes differentially expressed between NE and MES neuroblastoma cell lines. This was followed by extensive RQ-PCR testing in cell lines, control BM, PB, PBSCs and cell subsets. After selecting a specific panel of markers several serial PB, PBSC and BM samples from high risk neuroblastoma patients were tested. Detection of MES RNA markers was compared with NE RNA markers. For a cohort of patients PBSC samples were tested and survival analyses were performed.
Results PRRX1, POSTN and FMO3 were selected as marker panel for the detection of MES NB cells. MES mRNA was not frequently detected in PB samples. However, MES mRNA was frequently detected in PBSC samples and was associated with a poor event free survival. In 95 serial BM samples from 13 patients in complete remission and 16 relapse patients MES RNA markers showed different dynamics during treatment compared to NE RNA markers. Furthermore, MES mRNA was more frequently detected in BM samples from relapse patients (53%) than in BM from patients in CR (32%) (p=0.03). **Conclusion** We propose to use POSTN, PRRX1 and FMO3 as marker panel for the detection of MES neuroblastoma cells in PB, BM and PBSCs in neuroblastoma patients. MES markers show different dynamics during treatment and are more frequently positive in patients with adverse outcome.

To study the clinical significance these markers should be used alongside the current MRD markers in large prospective studies.

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Computer-assisted Curie scoring for Metaiodobenzylguanidine (mIBG) Scans in Patients with Neuroblastoma

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Background: Curie scores have shown prognostic significance in high risk neuroblastoma. However, the potential for significant inter and intra-observer variability exists when determining (and recording) individual patient scores. We have created a deployable and scalable system for recording and tracking MIBG Curie scores that is comparable to current practice, while allowing lesion tracking.
Methods: We designed three computer-assisted methods for recording Curie scores. Method A recapitulates current practice, allowing the radiologist to record a score of 0-3 for each anatomic region. Method B facilitates the marking of lesions and the assignment of a region from a schematic. Method C permits a user-driven segmentation of the image, computer-assisted marking of lesions, and automatic determination of score. For testing, 38 MIBG scans representing a range of ages and extent of disease were selected. Five experienced radiologists from four academic medical centers scored the 38 scans using each method and were surveyed for feedback.
Results: There was no significant difference in total scores among the three methods (p=0.91) or among the observers (p=0.14). Methods B and C took an average of 58 and 111 seconds longer than Method A, respectively, due to marking each lesion. Overall, radiologists felt that tracking lesions would be very useful for oncologists. Of the three methods, marking the lesions and selecting the region (Method B) was preferred.
Conclusions: A computer-assisted approach to determining Curie scores for MIBG scans successfully provides clinicians with a mechanism to track neuroblastoma lesions over time without loss of score reliability or consistency. The software is built on an easily-deployable platform for use at any medical center as both a research tool and clinical instrument. Given the growing necessity to Curie score MIBG scans, longitudinal lesion tracking and score recording could have an important impact on the care and treatment of children with neuroblastoma.

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Lack of adaptive immunity markers is associated with early death amongst high-risk neuroblastomas.

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Since the development of high-throughput techniques, many studies have focused on defining gene expression-based prognostic signatures in neuroblastoma. The majority of these studies have defined signatures that identify differentially expressed genes between the currently defined clinical subgroups of neuroblastoma, such as high-risk vs. low-risk, alive vs. deceased, MYCN-amplified vs. non-amplified etc. Although these signatures do indeed provide important prognostic information to the current neuroblastoma stratification schemes, they are often closely associated with the pre-defined clinical subgroups. In this study, we use gene expression arrays from 3 independent cohorts consisting of over 800 tumors to identify gene expression patterns associated with early-death events amongst patients who succumb to the disease.
Gene Set Enrichment analyses on differentially expressed genes between early-death vs. late-death patients (death within or after 1 year from diagnosis, respectively) identified immunological processes amongst the main differences between the groups. Making use of previously defined immune cell-specific markers, we further examined expression patterns amongst high-risk neuroblastomas. Firstly, we find that expression of immune cell markers successfully separates high-risk patients into distinct subgroups. Interestingly, patients who succumb to the disease within 1 year from diagnosis displayed a significantly lower expression of markers associated with adaptive immune cells (specifically cytotoxic and helper T-cells). Furthermore, MYCN-amplified patients could be divided into two subgroups: one associated with low expression of adaptive immune makers and high expression of innate immune markers, and the second associated with a general lack of both adaptive and innate immunity markers. In this case, the latter group with a general lack of immune-cell markers was associated with early-death events.
Here we describe a subgroup of high-risk neuroblastoma patients who succumb to the disease within 1 year from diagnosis. This subgroup is characterized by a lack of adaptive immune-cell markers, suggesting immune-repression in these patients. Further investigation into potential underlying immodeficiencies or tumor-dependent immunosuppression in these patients will deepen our understanding of the aggressive factors associated with high-risk neuroblastoma patients.

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High-MKI neuroblastomas - MYC-family-driven tumors with augmented expression of MYCN/MYC protein behaves more aggressively than Non-MYC-family-driven tumors: a report from the Children's Oncology Group

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Background: In neuroblastomas (NBs), high mitotic-karyorrhectic index (High-MKI) defined by the International Neuroblastoma Pathology Classification is often associated with *MYCN* genomic amplification. However, little is known about MYCN and/or MYC protein expression by High-MKI NBs especially when *MYCN* oncogene is not amplified.

Materials and Methods: A total of 241 High-MKI NBs [120 *MYCN* amplified (28 undifferentiated, 92 poorly differentiated subtype) and 121 *MYCN* non-amplified (4 undifferentiated, 117 poorly differentiated subtype)] available at the Children's Oncology Groups Neuroblastoma Pathology Reference Laboratory were studied immunohistochemically for expression of MYCN and MYC protein. Prognostic effects by these protein expressions [(+): high, (+/-): low, (-): none] were analyzed with conventional prognostic factors and prominent nucleolar (PN) formation.

Results: *MYCN* amplified High-MKI NBs were predominantly composed of tumors overexpressing MYCN protein: they were 101(84.2%) MYCN protein (+), one (0.8%) MYC protein (+), 2(1.7%) both proteins (+), and 16(13.3%) both proteins (-)/(+/-). In contrast, *MYCN* non-amplified High-MKI NBs were heterogeneous and included tumors of 7(5.8%) MYCN protein (+), 36(29.8%) MYC protein (+), 3(2.5%) both proteins (+), and 75(62.0%) both proteins (-)/(+/-). MYC-family-driven NBs defined by augmented expression of MYCN/MYC protein were more likely to show PN formation (putative site of RNA synthesis and accumulation). High-MKI NBs linked to a poor prognosis (4-year EFS 56.2+/-5.5%, 4-year OS 64.5+/-5.4%) irrespective of *MYCN* genomic status (amplified or non-amplified). However, patients with MYC-family-driven High-MKI NBs had significantly lower survivals (4-year EFS 50.2+/-6.2%, 4-year OS 56.1+/-6.1%) than those with Non-MYC-family-driven High MKI NBs (4-year EFS 66.3+/-11.1%, 4-year OS 80.1+/-9.5%) (p=0.0131 and p=0.0064, respectively). MYCN protein (+) and PN (+), in addition to clinical stage, were independently predictive of poor survivals in this cohort of patients.

Conclusions: Among High-MKI NBs, MYC-family-driven tumors with augmented MYCN/MYC protein expression were associated with PN formation and behaved significantly more aggressively than Non-MYC-family-driven tumors.

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XPO1 is overabundant in patients with neuroblastoma at ultra-high-risk for treatment failure: rationale for refined diagnostic risk stratification and targeted therapy

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Background: Approximately 15% of patients with newly diagnosed neuroblastoma are at ultra-high-risk for treatment failure and therefore have the most dismal outcomes. Currently utilized clinicobiological variables are insufficient to identify this cohort at diagnosis, and new strategies are needed to refine risk stratification and improve therapy. It is feasible to use computerized image analysis and proteomic profiling on single slides from diagnostic tissue. The nuclear export protein Exportin-1 (XPO1) is more abundant in those at ultra-high-risk for treatment failure, and this druggable target has been implicated in tumorigenesis of multiple cancers.

Methods: Diagnostic tumor samples were obtained from COG and included high-risk neuroblastoma patients defined as INSS stage 4, greater than 18 months. We compared digital histologic features and proteomic profiles from patients at ultra-high-risk for treatment failure, which was defined as death from disease in less than 18 months, with profiles from those with high-risk disease that was successfully treated (survival without recurrence for greater than 5

years). Top targets of interest were further characterized in neuroblastoma cell lines, and the mechanism of action of Selinexor, an XPO1 inhibitor, was evaluated.

Results: Comparative digital histologic analysis identifies distinct features present in those at ultra-high-risk for treatment failure. Comparative proteomics reveals high XPO1 as a most differentially abundant protein associated with inferior outcomes. Correlative studies using immunohistochemical staining intensity are ongoing. XPO1 is variably expressed across cell lines, with proliferative defects and apoptosis observed after treatment with Selinexor. Selinexor results in downregulation of XPO1 and anti-apoptotic proteins BARD1 and Survivin, correlated with a decrease in acetylated STAT3.

Conclusions: Computerized image analysis and proteomics may complement pathologist review and refine contemporary neuroblastoma risk stratification. Identification of patients at ultra-high-risk for treatment failure at diagnosis has potential to improve outcomes. Continued evaluation of XPO1, in addition to other protein targets, will offer insight into the pathogenesis of the most highly aggressive disease and rational combination approaches to therapy.

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Incidence of stage IV neuroblastoma patients 2-5 years of age was increased after the cessation of mass screening in Japan

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Background: Since 2004 mass screening (MS) for neuroblastoma at 6 months of age in Japan has been halted. The clinical features of neuroblastoma before and after the cessation of MS were compared to evaluate the significance of MS.

Methods: Patients with neuroblastoma under the age of 6 were divided into 2 groups. The group A (post-MS cohort) was consisted with 402 patients enrolled into the registry of the Japanese Society of Pediatric Surgeons (JSPS) between 2005 and 2009, and the group B (MS cohort; Hiyama et al. Lancet, 2008) with 1,797 patients registered with the Japanese MS study group between 1990 and 1998. The age at diagnosis, clinical stage defined according to the JSPS staging system that were identical to Evans' staging system and cumulative incidence of neuroblastoma indicated by the number of patients per 100,000 births were compared between the 2 groups.

Results: There was a significant decrease in the incidence of patients 6-11 months of age (9.99, group A; 128.53, group B) and a significant increase in the incidence of patients 2-5 years of age (27.01, group A; 8.00, group B) after the cessation of MS. The number of the patients with stage IV disease increased significantly after the cessation of MS (32.00, group A; 20.24, group B). In stage IV patients, there was a significant decrease in the incidence of patients 6-11 months of age (3.33, group A; 7.73, group B) and a significant increase in the incidence of patients 2-5 years of age (18.31, group A; 5.24, group B) after the cessation of MS.

Conclusion: The incidence of neuroblastoma patients between the age of two and five-years old with stage IV high-risk disease significantly increased after the cessation of MS.

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Molecular Karyotyping in Neuroblastoma – time to stop G-banding.

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Background: Copy number aberrations (CNA) in Neuroblastoma contribute to prognosis and treatment stratification. Single nucleotide polymorphism (SNP) arrays can identify all clinically important CNAs in Neuroblastoma with the exception of balanced translocations, and can detect important rare genomic abnormalities. We compared SNP arrays from 99 neuroblastoma samples and 13 neuroblastoma cell lines with matching karyotypes and clinical data where available. Our goals were to characterise the typical array features of Neuroblastoma, including MYCN amplification, 11q deletion and other segmental CNAs, and define the clinical utility of molecular karyotyping.

Results: Classical karyotyping had a poor diagnostic yield, including false negative results (48%). In all instances, significant CNAs were detected in these same samples by SNP array. The typical features of MYCN amplification on the array platform were alleleic imbalance and saturation of the smoothed logR values. We defined the relationship between absolute copy numbers of MYCN and these array features using orthogonal data, including a digital droplet PCR (ddPCR) assay we developed. No case with true MYCN amplification was missed by array. In serial dilutions of MYCN amplified cell line DNA, ddPCR MYCN assay was an extremely sensitive means to detect MYCN amplification.

Features usually undetected by karyotyping, segmental deletions or copy number gains, are frequently observed in arrays, and the number of segmental anomalies detected by SNP array was a strong predictor of poor outcome ($p = 0.000007$, $n = 42$). The minimal overlapping 11q deletion region of our cohort matches those previously reported. Interestingly, several rare but likely pathogenic amplification events at loci other than MYCN were identified.

Conclusion: SNP arrays significantly improve the accuracy of genomic risk assessment in Neuroblastoma and expand the range of CNAs detected. They obviate the need for conventional karyotype. There are novel, recurrent gene amplifications that may be important driver events in Neuroblastoma.

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Neural stem cell-mediated enzyme/prodrug therapy for neuroblastoma: translation to the clinic

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Although survival for neuroblastoma (NB) in newly diagnosed high-risk children has improved, recurrent disease remains a significant problem with treatment options limited by both anti-tumor efficacy and patient tolerance. We previously demonstrated that neural stem cells (NSCs), engineered to secrete a modified rabbit carboxylesterase (rCE-NSCs) can distribute to metastatic neuroblastoma tumor foci in multiple organs, and convert the prodrug CPT-11 (Irinotecan; IRN) to the 1000 fold more potent topoisomerase-1 inhibitor, anti-cancer agent SN-38, resulting in significant therapeutic efficacy. The goal of our current biodistribution, efficacy and safety/toxicity IND-enabling studies is to identify the optimal dose and schedule of intravenously administered NSCs adenovirally transduced to secrete a modified human CE (hCE1m6), followed by human equivalent doses of IRN. This would ideally provide a tumor selective, more effective and potentially less toxic delivery of treatment for children with recurrent high-risk NB. We have now determined the in vitro IC50 values of 4 human derived NB lines to SN-38, IRN only and IRN + hCE1m6-NSC conditioned media. IC50 values of IRN were decreased by 500 to 6000-fold when IRN was used in combination with the hCE1m6 conditioned media for all NB cell lines (KCNR, SKNAS, CHLA-136 and CHLA-255). In subcutaneous models of human NB we demonstrated tumor-specific conversion of IRN to SN-38. We have also shown clearance of hCE1m6-NSCs through peripheral organs and circulation in non-tumor bearing Es1e/SCID mice by quantitative PCR. Repeated treatments with hCE1m6-NSCs in combination with intravenous IRN (15 mg/kg x 3 days) had a significant decrease of tumor burden (as measured by bioluminescent imaging) for CHLA-136 (1.6-fold /p = 0.003) and CHLA-255 (0.6-fold/p= 0.04) vs. IRN only group in metastatic tumor models. hCE1m6-NSCs with IRN increased long-term survival of mice bearing CHLA-136 and CHLA-255 NB tumors. These studies suggest NSC-mediated enzyme/prodrug therapy may have potential benefit for NB patients.

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A comprehensive preclinical study of ALK inhibitors for the efficacious treatment of *ALK*^{F1174L}/*MYCN*-driven neuroblastoma

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Introduction

Genetic aberrations of *ALK* are associated with a poor event-free survival in neuroblastoma. In particular, *ALK F1174L* mutations are enriched within *MYCN*-amplified tumours and together are associated with an ultra-poor risk neuroblastoma subtype. We have previously shown in our preclinical mouse model of *ALK*^{F1174L}/*MYCN*-driven neuroblastoma that the first generation ALK inhibitor crizotinib is unable to effectively treat the disease as single agent. This inadequacy was subsequently confirmed in a Phase I/II clinical study of crizotinib in patients. Thus, alternative therapeutic strategies must be found. We present here a study of two next generation compounds, LDK378 (Novartis) and PF-06463922 (Pfizer), using the Th-*ALK*^{F1174L}/*MYCN* mouse model.

Methods

LDK378 and PF-06463922 were compared to crizotinib using *in vitro* assays. Subsequently pharmacodynamic studies were performed in the Th-*ALK*^{F1174L}/*MYCN* mouse model and the phosphorylation status of ALK was compared using a mesoscale scale technology ELISA platform.

Results

PF-06463922 was found to be the superior compound in kinase assays and upon treatment of cell lines. In the transgenic Th-*ALK*^{F1174L}/*MYCN* model PF-06463922 treatment resulted in tumour regression in all animals treated, associated with significant dephosphorylation of ALK according to ELISA and reduction in total MYCN upon immunoblotting. LDK378 treatment resulted in a variable and inadequate tumour response.

Discussion

PF-06463922 is a bona fide, potent, ALK inhibitor that should be considered as targeted therapy for children with neuroblastomas harbouring the *ALK F1174L* mutation, in preference to crizotinib and LDK378.

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The CHK1 inhibitor CCT244747, alone and in combination with gemcitabine, is active against p53 deficient models of neuroblastoma resistant to chemotherapy.

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Background: CHK1 inhibitors exhibit efficacy in neuroblastoma alone and in combination with chemotherapy (Russell et al, 2013). Neuroblastoma at relapse frequently exhibits loss of p53 function (LoF) and acquired resistance to standard treatments.
Apporach and Results: To determine whether p53 LoF altered therapeutic response to chemotherapeutic agents and the CHK1 inhibitor CCT244747, we tested (72h SRB assay) gemcitabine, temozolomide and CCT244747 in the CLBGA neuroblastoma cell line overexpressing HPV16-E6 ubiquitin ligase that completely degraded p53, abrogated G1/S checkpoint control after irradiation, and p53-dependent apoptosis after gemcitabine. We also tested the cytotoxic effects of CCT244747 in a panel of neuroblastoma cell lines varying in p53-dependent G1/S checkpoint integrity. The CLBGA with a p53-LoF showed marked resistance to gemcitabine and temozolomide while CCT244747 was equally cytotoxic in p53-functioning and non-functioning cells. A panel of neuroblastoma cell lines showed equal sensitivity to the CHK1 inhibitor independent of the p53 functional status. Interestingly, the combination of CCT244747 with gemcitabine was strongly synergistic in either functional or non-functional p53 LoF CLBGA isogenic variants.
To assess the impact of these drugs in the setting of p53 dysfunction *in vivo*, we generated a derivative of the Th-*MYCN* genetically engineered mouse (GEM) model in a p53 deficient background using a tamoxifen-sensitive allele of p53 (*Trp53ER*^{TAM}) knocked in to the endogenous *Trp53* locus (Th-*MYCN/Trp53*^{KI}) (Christophorou et al. 2005). Th-*MYCN/Trp53*$^{KI/ki}$ mice displayed increased tumour penetrance, shorter latency and reduced overall survival compared to Th-*MYCN* mice, and comparative resistance to cyclophosphamide, gemcitabine and irradiation. CCT244747 was equally effective as single agent and in combination with gemcitabine in both models.
Conclusions: The preclinical candidate CHK1 inhibitor CCT244747 is effective alone or combined with gemcitabine in neuroblastoma cell lines and in in-vivo models regardless of p53 status. This combination warrants clinical evaluation in relapsed, chemoresistant neuroblastoma, where inactivation of p53 function may contribute to chemoresistance.

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High efficacy of the BCL-2 inhibitor venetoclax (ABT-199) in neuroblastoma and rational for combination therapy.

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Background
BCL-2 is highly expressed in a large subset of neuroblastoma patients and plays an important oncogenic role. Previous studies with the small-molecule BCL-2 inhibitor navitoclax showed favorable antitumor activity for BCL-2-dependent neuroblastoma cells but unfortunately, navitoclax was associated with dose-limiting thrombocytopenia in patients due to BCL-X_L inhibition. Therefore, the BCL-2 specific inhibitor, venetoclax was developed. In this study, we explored the preclinical therapeutic potential of venetoclax for neuroblastoma treatment and strategies to prevent venetoclax resistance.

Results
We show that venetoclax induces strong apoptotic responses in cell lines with high BCL-2 expression levels, indicated by dose-dependent cytochrome c release from the mitochondria into the cytoplasm and PARP cleavage. The *in vitro* efficacy of the compound was ascertained by BIM displacement from BCL-2. Venetoclax significantly inhibited the growth of high BCL-2 expressing neuroblastoma xenografts in mice. Despite complete displacement of BIM from BCL-2 and increased cleaved caspase 3 levels, complete tumour regression was not observed. We showed that the *in vitro* and *in vivo* resistance to venetoclax results from upregulation of the anti-apoptotic BCL-2 family protein MCL-1 and BIM sequestration by MCL-1. Knockdown of MCL-1 re-sensitized neuroblastoma cell lines to venetoclax, confirming a pivotal role of MCL-1 in venetoclax resistance. To identify potential synergistic combination treatments we are currently performing a large compound combination screen using Venetoclax resistant cell lines.
Conclusion
Taken together, the results presented in this study strongly suggest that children with neuroblastoma tumors expressing high levels of BCL-2 and BIM/BCL-2 complex might benefit from combined treatment with venetoclax and targeted compounds that interfere with MCL1 function.

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Neuroblastoma drug response profiles are associated with gene expression profiles

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Background: High risk neuroblastoma (HRNB) patients have a poor response to chemotherapy, with 20% unable to achieve complete response. Genomic studies identified 6 targeted agents whose pathways are active in HRNB. The low frequency of established driver mutations, along with the variety of molecular mechanisms for cancer pathway dysregulation, suggest development of gene expression-based predictive biomarkers in addition to sequence-based biomarkers. Our platform for developing gene expression-based biomarkers includes primary NB cell lines, high throughput quantification of cell survival, exome sequencing, and genome-wide expression profiling. We apply this platform to six commonly used targeted therapies.

Methods: Cell survival was quantified for each of thirty primary neuroblastoma cell lines in the presence of each of six drugs (bortezomib, crizotinib, dasatinib, lapatinib, sorafenib, and vorinostat) using high throughput cell survival measurements. Titration curves were based on vehicle and sixteen concentrations of each drug (replicated within and between plates). Univariate summaries of the response of cell line/drug were obtained using the four parameter logistic model as well as the area under the curve. Exome sequences were obtained using Illumina HiSeq technology. Genome-wide expression profiles were obtained using Affymetrix GeneChip (U133 Plus 2.0). Exploratory multivariate analysis included hierarchical clustering, principal component analysis, and multidimensional scaling. Inference was based on parametric univariate and non-parametric multivariate tests.

Results: For three of the drugs (bortezomib, dasatinib, and vorinostat) variation in response between cell lines was statistically significant ($p < 0.05$; $F \geq 3$) and assay results are reproducible in the context of the differences between cell lines (the other three drugs the estimated standard deviation of $\log_{10}$ EC50 ≤ 0.2). Genome-wide expression profiles were associated with variation in drug response between cell lines (example: high PDGFRB expression indicates sensitivity to dasatinib, $p < 0.001$).

Conclusion: High throughput cell survival measurements provided for identification of predictive biomarkers of Neuroblastoma drug sensitivity. These biomarkers are currently being studied in the NMTRC PEDS-PLAN (Pediatric Precision Laboratory Advanced Neuroblastoma Therapy) clinical trial.

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A high-throughput drug screening of FDA approved anti-cancer compounds suggests candidate tyrosine kinase inhibitors for repositioning in neuroblastoma therapy

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Recent studies led to the identification of novel druggable targets in neuroblastoma (NB), opening the way for more effective treatments. However, children with high-risk NB still show high mortality rates, warranting efforts to develop novel therapeutic approaches. The repositioning of FDA-approved molecules is a strategy that has already been successfully employed to discover new applications for existing drugs. Therefore, we performed a high-throughput screening of a library of anti-cancer compounds (Selleckchem), consisting of 349 small molecules, either FDA-approved or under clinical trials, in order to repurpose drugs for NB therapy. The library was tested in three-dimensional multicultural spheroids, recognized as a reliable pre-clinical model to recapitulate drug responses in solid tumors. In the primary screening three NB cell lines (CHP134, IMR-32, SK-N-BE(2)) were treated for 72 hours with the library compounds at 10 μM concentration. Viability of 3D spheroids was evaluated using a high-content imaging approach. Images were processed using in-house developed algorithm in Harmony software (PerkinElmer). Finally, the data were analyzed employing the Strictly Standardized Mean Difference (SSMD) metric. We classified a compound as primary hit when it demonstrated SSMD 50 values for each of the 26 tested compounds were supplemented with available data on severe side-effects, therapeutic index, and molecular targets. In conclusion, our survey prioritized four candidate drugs, belonging to a class of multi-target tyrosine kinase inhibitors that potentially might be promising for repositioning in NB.

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ITCC Biology: pre-clinical targeted drug development for high-risk pediatric cancers

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Background

The European consortium *I*nnovative *T*herapies for Children with Cancer (ITCC) has been created in 2003 and aims to develop novel therapies for pediatric cancers. Within the consortium, ITCC Biology endeavours to supply the biological rationale for targeted drug trials in children with cancer. Therefore, a stepwise pipeline for the pre-clinical testing of targeted drugs across high-risk pediatric cancers has been set-up, from drug selection to *in vivo* evaluation. Currently, ITCC Biology focusses on opportunities for further expansion and development of the pre-clinical drug development pipeline.

Results

A robotic platform allowing high-throughput drug testing across multiple cell lines has been set-up. This platform has successfully been used to evaluate the *in vitro* efficacy of venetoclax, cobimetinib, binimetinib, selumetinib, trametinib, alectinib, crizotinib, LDK378, ribociclib, RG7388, TH588 and AT7519 in a panel of 32 cell lines representing 5 types of pediatric cancer, including neuroblastoma. IC_{50} and LC_{50} values were calculated using a novel developed analysis tool that is linked to the bioinformatics platform R2 (http://r2.amc.nl), thereby offering the possibility to directly correlate drug responses to genetic features. We were able to reproduce earlier findings in neuroblastoma cell lines, including correlations between sensitivity to MEK inhibitors and RAS-MAPK pathway mutations and between responses to MDM2 inhibition and p53 status. The same correlations could be found in other pediatric cancer types, such as rhabdomyosarcoma. Next screen will be performed using more compounds, including alectinib, pictilisib, taselisib, vemurafenib, ipatasertib, RG6016, RG7666, RG7741, RG7842, BCT-100, TH1579, TH1827 and JQ1, and will be finished within 6 months.

Conclusion

Proposed improvements in pre-clinical drug testing should eventually lead to more drugs entering clinical trials for neuroblastoma treatment and the faster discovery if drugs might also be useful for other pediatric cancers. The first screen clearly indicates that drug effects observed for neuroblastoma are comparable with effects obtained in other pediatric cancer types.

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Effects on tumor cells and the immune microenvironment may both contribute to the anti-tumor activities of DFMO in neuroblastoma pre-clinical models

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Background: Polyamine synthesis is an oncogenic pathway deregulated by *MYC*. Ornithine decarboxylase (Odc) is a *MYC*-target encoding the rate-limiting enzyme in polyamine synthesis. DFMO is an FDA-approved Odc inhibitor being studied in neuroblastoma trials at doses from 1,000-9,000 mg/m2/day, and as monotherapy in first response or with chemotherapy following relapse. Better understanding its mechanisms of activity may identify testable responder hypotheses and guide subsequent trial design.
Methods: We used qPCR and SNP-arrays to define *MYCN* and *ODC1* gene copy-number, and correlated this with DFMO sensitivity and global protein translation (governed by *MYC*-polyamine signaling). DFMO was tested in tumor xenografts and transgenic *TH-MYCN* models at exposures equivalent to ~7,500 mg/m2/day (high-dose); exposures of ~2,000 and ~4,000 mg/m2/day were tested in *TH-MYCN* mice. As *MYC* influences the tumor microenvironment (TME) via arginine-polyamine signaling we characterized tumor infiltrating leukocytes (TILs) by flow-cytometry.
Results: Of 23 cell lines, 13 were *MYCN*-amplified and 4/13 (31%) had *ODC1* co-amplification. Of 256 *MYCN*-amplified primary tumors, 33 (13%) had *ODC1* co-amplification. Inhibition of global protein translation (>50%) by DFMO correlated with *MYC/ODC1* signaling. In treating *MYCN*, *ALK* and *TP53* mutant xenografts, and in *TH-MYCN* mice, high-dose DFMO extended survival ($p<0.05$) on 5 chemotherapy backbones. *TH-MYCN* tumors treated with high-dose DFMO have significantly increased (up to 3-fold) NK, DC, CD4- NKT and G-MDSC cells defining an inflammatory TME. Lower DFMO exposures (≤4,000 mg/m2/day) did not inhibit tumor progression but elicited intermediate TIL changes.
Conclusions: *ODC1* is transcriptionally activated by *MYCN* and by genomic amplification, and expression has been independently correlated with poor survival. We show DFMO inhibits Odc to deplete tumor polyamines, impede *MYC*-driven protein translation and tumor progression, and antagonize the tumor-permissive TME. Ongoing studies are defining the exposures required for cell autonomous and immune effects, relative sensitivity of *MYCN/ODC1* co-amplified tumors, and testing for synergy with immunotherapy.

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Targeting NEDD8: a novel approach to treating neuroblastoma

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Background: Patients with high-risk and relapsed neuroblastoma have a poor event free survival (EFS). Novel chemotherapy agents, such as the NEDD8-activating enzyme (NAE) inhibitor pevonedistat, provide great possibilities for advancements in cure. Pevonedistat blocks the degradation of proteins that would normally be degraded by the 26S proteasome, the major mechanism of protein removal in the cell. Pevonedistat is more specific than previous proteasome inhibitors because it blocks the degradation of Cullin-RING ligases (CRL), narrowing the targets to only a handful of key regulatory proteins important in cell survival.

Methods: MTT assays were used to test the efficacy of pevonedistat. Flow-cytometry was used to determine cell-cycle arrest, presence of DNA rereplication, and apoptosis after staining with Annexin V/PI. Whole cell extracts were analyzed by immunoblotting from cells treated with pevonedistat for proteins targeted in neddylation. Orthotopic xenografts of human neuroblastoma were generated. Mice were randomized in three treatment groups of ten mice each: vehicle control, pevonedistat 50 mg/kg, and MLN4924 100 mg/kg. All drugs were given i.p.daily 6 days/week for 2 weeks. After 4 weeks tumor weights were compared using Kruskal-Wallis analysis.

Results: The IC50 of pevonedistat in neuroblastoma cell lines was 200-500 nM. and unaffected by N-myc or p53 status. After treatment with pevonedistat, p53 mutant neuroblastoma cell lines undergo rereplication and cell-cycle arrest in G2-M, while p53 wild-type cell lines exhibit cell-cycle arrest in G0-G1. In treated cells proteins involved in DNA rereplication are increased in p53 mutant compared to wild-type cell lines. Control mice had an average tumor weight of 1.6mg+0.8mg versus mice treated with pevonedistat 0.5mg+0.4mg ($p<0.05$).

Conclusion: Neddylation affects proteins involved in DNA replication. The mechanism of action of pevonedistat in neuroblastoma cell lines appears to be p53 dependent. In our neuroblastoma xenograft experiments there was a significant decrease in tumor weight between control and pevonedistat treated mice.

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Targeting the polyamine pathway in combination with conventional chemotherapy for the treatment of childhood neuroblastoma

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Introduction

Polyamines are highly regulated essential cations that are elevated in rapidly proliferating tissues. We have previously shown that the MYCN/MYC transcriptional target ornithine decarboxylase (ODC1), the rate-limiting enzyme for polyamine biosynthesis, is a therapeutic target for neuroblastoma (*Cancer Res* 68:9735, 2008). In addition, we found that each member of the polyamine pathway is regulated by MYCN and that their expression is prognostic of outcome. We have shown that the FDA-approved ODC1 suicide inhibitor, difluoromethylornithine (DFMO), represents a potentially powerful treatment strategy when combined with chemotherapy. Based on our research, a NANT Phase I clinical trial combining DFMO with conventional chemotherapy and an inducer of catabolic SAT1, Celebrex, is currently underway, with early results showing promising responses. We have now investigated combining DFMO with other polyamine depletion agents to further improve this form of therapy.

Methods

Cytotoxicity assays, colony assays, and determination of synergy were performed with combinations of polyamine depletion compounds and chemotherapeutic agents as previously described (*Sci Transl Med* 7(32):312 2015). The TH-MYCN transgenic mouse model was used to study the most effective combinations *in vivo*.

Results

We investigated a range of compounds targeting various components of the polyamine pathway including blocking polyamine synthesis (DFMO, 4MCHA, APCHA), promoting polyamine catabolism (Celebrex), blocking polyamine uptake (AMXT-1501) and polyamine analogues (CGC-11047, CGC-11093, CGC-11144, ANTMHSpd). Amongst these agents, CGC-11093, which has been studied in a phase I clinical trial for the treatment of cancer, was found to be highly synergistic with both DFMO and chemotherapy. Moreover, the combination of DFMO and AMXT-1501 is also proving to be effective at inhibiting tumour progression in TH-MYCN mice, particularly when combined with conventional chemotherapy.

Conclusions

These data highlight the therapeutic potential of treating neuroblastoma by targeting multiple steps in the polyamine pathway in combination with conventional chemotherapy.

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Targeting cell cycle and transcriptional CDKs using Roniciclib leads to significant high cell death in MYCN/MYC-activated neuroblastoma cells

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Background

Poor overall survival of high-risk neuroblastoma patients requires novel molecularly targeted therapies. Since MYCN itself is still undruggable, we identified a group of MYCN direct target genes being strongly activated by MYCN in high-risk neuroblastoma tumors and inhibited them using the small molecule pan-CDK inhibitor, Roniciclib.

Methods

A gene expression-based classifier of 702 neuroblastoma patients revealed high expression of cell cycle genes, especially the group of cyclin-dependent kinases (CDKs) and cyclins, in *MYCN*-amplified high-risk neuroblastoma tumors. The small molecule compound, Roniciclib, was used to simultaneously inhibit the following CDKs and cyclins: CDK1/CycB, CDK2/CycE, CDK3/CycE, CDK4/CycD, CDK5/p35, CDK7/CycH and CDK9/CycT. About 30 neuroblastoma cell lines established from advanced stage disease harboring different characteristic genetic aberrations affecting *MYCN/MYC*, *ALK* and/or members of the p53/pRB pathway (e.g. *TP53*, *MDM2*, *CDK4*, *CCND1* and *CDKN2A*) were treated with different concentrations of Roniciclib and tested for viability reduction, IC50/EC50 values, cell cycle arrest (flow cytometry), cell death induction (nicoletti and BrdU staining, caspase assay), anchorage independent growth inhibition (soft agar assay) and MYCN expression.

Results

Morphologically, Roniciclib treatment induces early cell detachment (~24h) being associated with rapid and strong viability reduction at significant lower nanomolar concentrations in MYCN/MYC-activated neuroblastoma cell lines compared to cells with normal MYCN/MYC expression (IC50/EC50 16.6/16.1 nM MYCN/MYC activated vs 36.1/31.0 nM MYCN/MYC normal). Viability reduction is caused by a prominent G2/M arrest followed by caspase 3/7 activation resulting in cell death induction from all cell cycle phases. Anchorage independent growth was completely inhibited at 10 nM concentration of Roniciclib in 11/24 cell lines and in 23/24 cell lines at 25 nM. Reduction of MYCN/MYC protein expression was observed in 22/24 cell lines 72h after treatment with Roniciclib.

Conclusion

Simultaneous inhibition of cell cycle and transcriptional CDKs using the small molecule compound, Roniciclib, significantly induces high levels of cell death in MYCN/MYC activated neuroblastoma cell cultures opening a novel therapeutic approach for high-risk neuroblastomas.

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Identification of novel pathways and molecules able to down regulate oncogenes expression by *in vitro* drug screening approaches in neuroblastoma cells.

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Despite advances in multimodal treatment, neuroblastoma (NB) is often fatal for children with high-risk disease. There is an urgent need to develop new drugs which can be fast-tracked into frontline therapy to improve cure rates for patients with high-risk disease and to decrease toxicity in long-term survivors. MYCN amplification and anaplastic lymphoma kinase (ALK) activation have been identified as two major oncogenic events in NB pathogenesis, especially in the high-risk group. Our aim was to develop and validate pharmacodynamic (PD) biomarkers to evaluate both proof of mechanism and proof of concept for drugs that block such as PI3K/AKT/mTOR or ALK pathway activity in children with neuroblastoma. In this study, we hypothesized that screening a small-molecule library might identify already existing drugs that are able to modulate the oncogenic activity, specifically found in NB. To identify small molecules with the potential of inhibiting oncogenic activity and, consequently, its downstream genes such as *MYCN* or *ALK*, we implemented a high throughput chemical screen, using a curated library of ~450 compounds from the Scientific Research on Innovative Areas, Scientific Support Programs for Cancer Research, from The Ministry of Education, Culture, Sports, Science and Technology, Japan. In the drug screen, aurora kinase inhibitors (three molecules), JAK-STAT kinase inhibitor (one molecule) and proteasome inhibitor, in particular the proteasome-selective compound, bortezomib, was the most discriminatory with regard to sensitivity for MYCN-amplified cell lines. In an expanded panel of 20 NB cell lines, those with or without MYCN-amplification or 11q loss of heterozygousity were the most sensitive to low nanomolar concentrations of those compounds. We demonstrated that novel candidate compounds were rapidly and effectively identified by an in screening strategy, followed by in vitro assays. Therefore, our studies have uncovered a novel targetable susceptibility in high-risk neuroblastoma with potential clinical application.

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Cell lines (CLs) and patient derived xenografts (PDXs) established from post-mortem neuroblastoma samples display heterogeneity in sensitivity to chemotherapeutic agents commonly utilized in the treatment of high-risk neuroblastoma patients.

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Introduction: Cancer cell lines (CLs) and patient-derived xenografts (PDXs) are essential for biological and preclinical therapeutic studies. CLs and PDXs can be established from post-mortem neuroblastoma (NB) samples. We assessed the activity of a chemotherapy regimen commonly used for re-induction chemotherapy in 72 NB CLs and 9 xenograft models which were established from either pre-treatment (DX) or post-mortem (PM) samples.

Methods: STR-validated, tyrosine hydroxylase-positive, EBV-negative continuous NB CLs (47 DX, 25 PM) were profiled for 4-hydroperoxy-cyclophosphamide (4-HC, 0-30μM) and topotecan (TOPO, 0-1μM) cytotoxicity with the DIMSCAN assay. Response of 9 SQ xenograft models (7 PDXs, 2 CL-xenografts (CLX)) to cyclophosphamide (CYCLO) + topotecan was assessed in nu/nu mice. Cytotoxicity *in vitro* of 4-HC+TOPO was assessed for the PDX models and their matching cell lines.

Results: To date, 25 validated, continuous CLs and 10 PDXs have been established from 40 NB PM samples: 65% blood (50-200mL), 12.5% bone marrow, and 22.5% tumor; 25 of 40 placed in culture (63%) generated a CL, 10 of 16 xenografted (63%) generated a PDX. Success with PM samples was higher than observed for 1838 pre-treatment (CLs=6.6%, PDXs=9.7%) and 246 progressive disease (CLs=17.1%, PDXs=2.9%) samples. Both DX and PM models demonstrated heterogeneity in response to 4-HC+TOPO *in vitro* and CYCLO+TOPO *in vivo*; some were highly resistant while others highly sensitive. Complete tumor responses after 1-2 cycles were observed in 1 of 2 DX and 2 of 4 PM PDXs. PDXs and CLs established from the same samples demonstrated comparable responses *in vitro* and *in vivo*.

Conclusion: CLs and PDXs are readily established post-mortem from heavily pre-treated patients and demonstrated marked heterogeneity in response to CYCLO (4-HC) + TOPO *in vitro* and *in vivo*. PM NB models will enable delineation of molecular mechanisms of drug resistance, provide ideal models for testing new agents, and are available at www.COGcell.org.

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Enhancing ^{131}I-mIBG radiation therapy with oncolytic HSV1716 and NAT gene therapy in high-risk neuroblastoma

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Increasing NAT (noradrenaline transporter) expression to enhance ^{131}I-mIBG therapy is a targeted approach to improving survival and outcomes in children with high-risk neuroblastoma. Seprehvir (HSV1716) is a $\Delta\gamma_1$34.5 attenuated HSV currently in a pediatric phase 1 clinical trial (NCT00931931). HSV1716/NAT is a derivative of Seprehvir constructed to deliver NAT cDNA to cancer cells. Previous studies show increased antitumor effects in glioma and melanoma xenografts exposed to ^{131}I-mIBG and HSV1716/NAT.[1] We sought to determine the antitumor efficacy of the oncolytic virus HSV1716/NAT in combination with ^{131}I-mIBG in preclinical models of neuroblastoma. We determined endogenous NAT expression in 12 neuroblastoma cell lines and in xenograft tumors grown in athymic nude mice by qRT-PCR. Gene transfer, viral production, and cytotoxicity assays demonstrated each cell lines' response to HSV1716/NAT. We exposed neuroblastoma cells and xenografts in mice to HSV1716NAT and/or ^{131}I-mIBG to determine differences in cytotoxicity and ^{131}I-mIBG uptake. We found variation in endogenous NAT mRNA expression, in HSV1716/NAT susceptibility and permissivity, in effective NAT production, and in radiation sensitivity among the neuroblastoma cell lines. Neuroblastoma cells and xenograft tumors infected with HSV1716/NAT showed increased NAT mRNA expression, increased specific uptake of ^{131}I-mIBG via NAT (see Fig 1), and increased cytotoxicity with ^{131}I-mIBG. We are currently evaluating animal survival in

mice with neuroblastoma xenografts exposed to

HSV1716/NAT, ^{131}I-mIBG, and the combination of HSV1716/NAT and ^{131}I-mIBG. We aim to translate this project into a clinical trial with potential to expand to other pediatric tumor types.

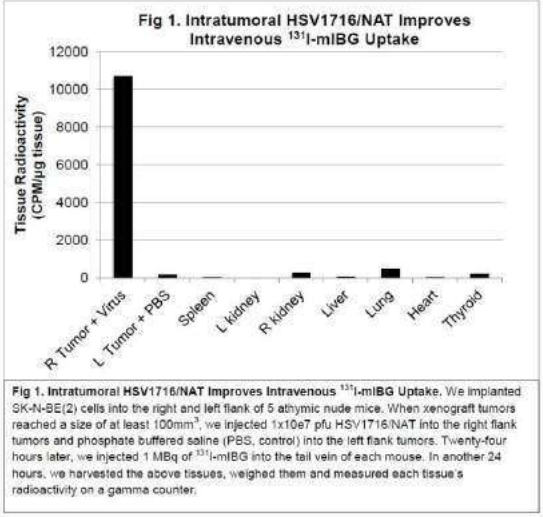


Fig 1. Intratumoral HSV1716/NAT Improves Intravenous ^{131}I-mIBG Uptake. We implanted SK-N-BE(2) cells into the right and left flank of 5 athymic nude mice. When xenograft tumors reached a size of at least 100mm^3, we injected 1x10e7 pfu HSV1716/NAT into the right flank tumors and phosphate buffered saline (PBS, control) into the left flank tumors. Twenty-four hours later, we injected 1 MBq of ^{131}I-mIBG into the tail vein of each mouse. In another 24 hours, we harvested the above tissues, weighed them and measured each tissue's radioactivity on a gamma counter.

Sorensen A, Mairs RJ, Braidwood L, et al., J Nucl Med, 2012 Apr; 53 (4):647-54.

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Common germline variants at *MLF1* and *CPZ* loci associated with neuroblastoma susceptibility

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Background: An ongoing genome-wide association study (GWAS) in neuroblastoma has identified multiple common and rare variants associated with susceptibility and tumor aggressiveness. Sequencing efforts have also revealed rare germline mutations contributing to tumorigenesis. However, together these still explain only a fraction of the heritability, and additional susceptibility variants/mutations remain to be discovered.

Methods: We performed a GWAS in a discovery cohort of 2,101 neuroblastoma cases and 4,202 controls following genotype imputation using 1000 Genomes Phase I Release 3 data. Significant associations were replicated in three independent cohorts: African American (365 cases; 2,491 controls); United Kingdom (371 cases; 1,122 controls), and Italian (427 cases; 783 controls). *In vitro* studies were performed in neuroblastoma cell line models following genetic manipulation of candidate genes to assess biological significance. A GWAS annotation tool incorporating functional genomic, transcription factor binding site, and evolutionary conservation data was created to infer putative causal variants.

Results: We refined previously reported susceptibility loci at 6p22 (*CASC15*), 2q35 (*BARD1*), 11p15 (*LMO1*), 6q16-q21 (*HACE1*, *LIN28B*), and 17p13 (*TP53*), and identified two new genome-wide significant associations at 3q25 and 4p16. Both novel associations replicated robustly in independent cohorts (3q25: rs6441201 combined $P=1.2x10^{-11}$, Odds Ratio 1.23, 95% CI:1.16–1.31; 4p16: rs3796727 combined $P=1.26x10^{-12}$, Odds Ratio 1.30, 95% CI: 1.21-1.40). The 3q25 signal resides within the arginine/serine-rich coiled-coil 1 (*RSRC1*) gene and upstream of the myeloid leukemia factor 1 (*MLF1*) gene. The 4p16 signal maps within the carboxypeptidase Z (*CPZ*) gene. Increased expression of *MLF1* was observed in neuroblastoma cells carrying the rs6441201 risk allele ($P = 0.02$), and significant growth inhibition was observed upon depletion of *MLF1* ($P < 0.0001$). Putative causal variants were identified at multiple susceptibility loci.

Conclusion: We show that common DNA variation at 4p16 within *CPZ* and at 3q25 upstream of *MLF1* influences neuroblastoma susceptibility. *MLF1* likely plays an important role in both initiation and disease progression; ongoing studies will further elucidate the role of this important cancer gene in neuroblastoma.

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Mutational dynamics between primary and relapse neuroblastoma involve the Hippo/YAP1 pathway and genes relevant for epithelial-mesenchymal transition

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The molecular mechanisms behind neruoblastoma relapse are poorly defined. We here used whole exome sequencing, mRNA expression analysis, array CGH and DNA methylation analysis to holistically characterize 16 paired samples from neuroblastoma patients at diagnosis and relapse. Global allele frequencies at relapse indicated clonal mutation selection in addition to de novo mutations during disease progression. Promoter methylation patterns were consistent over disease course and patient specific. No relapse tumor acquired new mutations in previously identified

neuroblastoma driver genes, but MYCN amplification was acquired in one. Inactivating mutations in the putative PTPN14 tumor suppressor and a relapse specific increase in the mRNA expression based pathway activity score for the PTPN14 target gene, YAP and the Hippo-Pathway, were identified, and represent the first hint for Hippo/YAP signaling involvement in neuroblastoma relapse. Recurrant new mutations in HRAS, KRAS, DOCK8, and genes mediating cell-cell interaction in 13 of 16 relapse tumors also point to disturbances in signaling pathways mediating epithelial-mesenchymal transition. Our results indicate a role for epithelial-mesenchymal transition processes and their modulation by genomically altered upstream signaling in neuroblastoma recurrence.

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Comprehensive analysis of neuroblastoma using high depth cancer gene panel sequencing

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Background: Neuroblastoma (NB) is the most common extra-cranial solid tumor of childhood. Recently, several genetic studies have reported oncogenic variations in NB, but the identified mutations were found in a low frequency partly because of the low depth sequencing platforms such as whole exome sequencing. Here, we used high depth targeted sequencing platform (~1000x) covering important 81 oncogenes.
Methods: Genomic DNA from 64 NB patients was sequenced by targeted deep exome sequencing for 81 oncogenes.
Results: Ninety four percent of patients had at least one mutation, and total 256 somatic mutations (186 SNVs and 6 Indels with freq. > 5%) and 107 copy number alterations (53 amplifications and 54 deletions) were identified. However, novel translocation of target genes was not found in this study. The most common mutations were found in the *TP53*, *ROS1*, *ALK*, *ATM*, *EGFR*, *NF1* and *PTCH1* genes. Compared with previous studies, we identified more recurrent variations in *TP53* (25.4% in this study vs. 0.4% in previous study), *ROS1* (15.9% vs. 1.3%), and*ALK* (14.3% vs. 9.2%). Interestingly, a deletion (rs3841650) at chromosome 15 was first discovered in *IGF1R* gene for in our 23 patients. It has been known that the expression of *IGF1R* gene was related with pathogenesis of NB. From the therapeutic point of view, we identified meaningful mutations such as *ALK* R1275Q, *ALK* F1741I, *EGFR* T790M, *HRAS* Q 61R, and *NRAS* F12D when considering the use of targeted agents.
Conclusions: Our study using targeted deep sequencing platform provides useful information about genetic landscape of NB.

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Suppressing the expression of a single novel long noncoding RNA leads to neuroblastoma regression or eradication

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N-Myc gene amplification occurs in one quarter of human neuroblastoma tissues, and is a marker for poor patient prognosis. We performed RNA sequencing experiments, and identified 5 transcripts, including RP1X, which were most considerably differentially expressed between N-Myc gene amplified and non-amplified human neuroblastoma cell lines. Affymetrix microarray studies revealed that DEPDC was one of the few genes considerably down-regulated in neuroblastoma cells after RP1X depletion. Chromatin immunoprecipitation assays showed that knocking-down RP1X expression reduced histone H3 lysine 4 trimethylation, a marker for active gene transcription, at the DEPDC gene promoter. Luciferase assays demonstrated that knocking-down RP1X decreased DEPDC gene promoter activity. Depletion of RP1X or DEPDC with two independent siRNAs or shRNAs significantly reduced ERK protein phosphorylation, N-Myc protein phosphorylation at Serine 62, N-Myc protein stabilization, neuroblastoma cell proliferation and survival. Clonogenic assays showed that knocking-down RP1X with doxycycline completely abolished colony formation capacity of neuroblastoma cells stably transfected with doxycycline-inducible RP1X shRNAs. Importantly, treatment with doxycycline in mice xenografted with neuroblastoma cells stably transfected with doxycycline-inducible RP1X shRNA, led to tumour regression or eradication. In human tumour tissues from 600 neuroblastoma patients, high levels of RP1X gene expression correlated with DEPDC gene expression and poor patient prognosis. In conclusion, this study identifies the novel long noncoding RNA RP1X as an important regulator of N-Myc protein stability and neuroblastoma tumourigenesis.

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Long non-coding RNAs as novel components in the TP53 pathway

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Primary neuroblastoma tumors rarely display mutations in the TP53 gene. However, the TP53 pathway is impaired at different levels, e.g. through amplification of MDM2, cytoplasmic sequestration of TP53, unfavorable conformations for integration in transcriptional complexes, all resulting in reduced transcriptional activity of the protein. Long non-coding RNAs form a novel class of RNA molecules and have not yet been explored as putative components of the TP53 pathway in neuroblastoma. Recent work demonstrated that TP53 induces long non-coding RNA (lncRNA) expression in various cancer types to modulate different aspects of the TP53 tumor suppressor function. Through further dissection of the TP53 pathway in neuroblastoma, we aim to identify lncRNAs acting as downstream effectors of TP53.
Two neuroblastoma model systems were treated with nutlin-3a for 24h to induce TP53 activity followed by lncRNA expression using a custom microarray platform detecting 38 000 genes, including 17 000 lncRNAs. Of these, 46 lncRNAs were differentially expressed upon TP53 activation. Further analysis of a nutlin-3a time-course experiment at 4, 8 and 24 hours resulted in a core set of 8 lncRNAs that had a high, early and sustained response to TP53 activity. Several of these lncRNAs demonstrated TP53 binding events in their promoter region and showed high expression correlation with neighboring protein coding genes implicated in cell cycle control and DNA replication, possible through cis-regulatory activity. Two of these lncRNAs were associated with patient survival, age at diagnosis, tumor stage and MYCN status in a cohort of 80 primary neuroblastomas. Perturbation of these lncRNAs using antisense oligonucleotides is currently ongoing and could provide novel insights in their role in the TP53 pathway in neuroblastoma.
This work was supported by a PhD grant from the Agency for Innovation by Science in Flanders.

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Genomic profiling in low and intermediate risk neuroblastoma to refine treatment stratification and improve patient outcome – LINES: a SIOPEN Trial

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Background: In neuroblastoma (NB), a genomic profile characterized by segmental chromosome alterations is associated with poorer outcome. The SIOPEN LINES trial (Low and Intermediate Neuroblastoma European Study) was designed to maintain or improve the excellent outcome in low risk patients (LR), whilst diminishing overall treatment burden whenever possible. Treatment is stratified according to the genomic profile in addition to clinical parameters. In intermediate risk (IR) patients, the genomic profile is studied prospectively.

Methods: NB samples with >60% tumor cells are analyzed using pangenomic techniques, with central review, entering of data in the SIOPEN-R-NET database, and return of the clinically relevant conclusions to the treating clinician within six weeks after diagnosis. NB without MYCN amplification are classified into two groups: numerical chromosome alterations (NCA) only, versus segmental chromosome alterations (SCA) >2Mb known to occur recurrently in NB, without or with numerical alterations.

Results: For 277 enrolled patients (190 LR, 87 IR), genomic profiling was performed in 230 cases using MLPA (n=7), aCGH (n=151), SNP-arrays (n=61) or other techniques (n=11). A genomic profile could be determined in 203 cases (131 NCA, 72 SCA). In 27 cases, no result was obtained, either because no copy number changes were seen (n=7), or because of alterations for which the prognostic relevance has not been clearly established (n=10), including small

interstitial deletions of chromosomes 8p or 3p, deletions of 5q, 11p, 17p, 19q and 22q, 2 cases with focal amplification of 12q14 encompassing CDK4/MDM2, amplification of 1p34.2, or a gain of 18p. For 10 other cases, no genomic profile could be obtained either due to insufficient material or technical issues. For 47 cases (23 LR, 24 IR), no up-front genomic profiling was performed.

Conclusion: In SIOPEN's LINES trial, genomic profiling in a diagnostic, real-time setting is feasible, with a success rate of 87% in analyzed cases.

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Genomic profiling using circulating free tumor DNA highlights heterogeneity in neuroblastoma

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Background: In neuroblastoma (NB), characterization of the overall tumor genomic copy number profile is important for treatment stratification. We have studied the genomic copy number profile of circulating cell free tumor DNA (ctDNA) and compared this to the aCGH of the primary tumour.
Methods: In a series of 70 NB patients for whom aCGH had been done on tumor tissue and for whom plasma was available for ctDNA extraction, ctDNA copy number profiling was performed using the OncoScan® platform.
Results: Higher ctDNA quantities and qualities were observed in advanced stages of disease. Interpretable ctDNA copy number profiles were obtained in 66/70 cases. An overall identical genomic profile between aCGH of the primary tumor tissue and ctDNA was observed in 47cases, with an additional segmental chromosome alteration seen in ctDNA in 1 case. In 14 other cases, ctDNA analysis did not reveal any copy number changes, 10/14 of these cases having localized disease. Finally, in 4/8 cases with a silent aGCH profile, ctDNA analysis revealed a dynamic profile. A total of 397 breakpoints common to both samples, primary tumor and ctDNA of any given patient, were identified (mean: 10 common breakpoints per case; range 1–37 breakpoints). In addition, 25 breakpoints were only seen by aCGH on the primary tumor, and 33 breakpoints were seen in ctDNA only, including two cases with interstitial gains encompassing IGFR1, and one alteration targeting TERT.
Conclusion: These results demonstrate the feasibility of copy number profiling using ctDNA in NB patients at the time of diagnosis. Given the possibility of evolution of copy number profiles in NB progression, the possibility of performing sequential analysis using ctDNA is of importance. This study further highlights the heterogeneity of NB. Further studies will help to determine the cellular origin of ctDNA and whether it represents genetic alterations from more aggressive tumour cells.

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The chromatin associated protein JARID2 Is a novel LIN28B-Influenced target in neuroblastoma

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Background: Although alterations in the somatic genome are known to influence tumorigenesis, our laboratory has demonstrated that germline variation plays a pivotal role as well, shaping oncogenic signaling to drive neuroblastoma oncogenicity. We showed that common variation at the *LIN28B* locus influences neuroblastoma susceptibility, and acts in cis to drive LIN28B overexpression. LIN28B, an RNA binding protein implicated in Wilms tumor, hepatoblastoma/hepatocellular carcinoma, and colon cancer, inhibits the let-7 family of tumor suppressor microRNAs and directly binds mRNAs. We recently described a LIN28B-mediated oncogenic network within neuroblastoma, linking LIN28B to RAN GTPase and Aurora kinase A signaling and sought to define additional key LIN28B-mediated signaling networks.
Methods: Using pooled siRNAs, we transiently depleted LIN28B in three neuroblastoma cell lines, isolated RNA, and performed RNA sequencing. We used gain and loss of function approaches to manipulate transcripts of interest, and then measured effects on downstream signaling, protein-RNA interactions, and proliferation.
Results and Conclusions: We identified JARID2 as a novel LIN28B target, as it was within the top ten most differentially regulated genes (6/10) across 18 samples and was strongly correlated with *LIN28B* expression in neuroblastoma tumors ($p<0.0005$). JARID2, a member of the Polycomb family, interacts with both chromatin and long non-coding RNAs, and, in embryonic stem cells, balances self-renewal and differentiation. We next demonstrated that LIN28B regulates JARID2 mRNA and protein expression. largely through direct binding of LIN28B to JARID2 mRNA. As the role of JARID2 in tumorigenesis is incompletely understood, we further focused on the

role of JARID2 itself, demonstrating that JARID2 enhances cell proliferation. Currently, we are studying whether JARID2 affects cell cycle progression or apoptosis to influence neuroblastoma proliferation. In summary, our findings link LIN28B to JARID2, suggesting a potential mechanism by which LIN28B might influence the neuroblastoma epigenome.

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Loss of *ATM* function confers risk for advanced stage neuroblastoma but provides a therapeutic target for poly-ADP ribose polymerase inhibitors

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Neuroblastoma (NB) is the most common extracranial solid tumor in children. Advanced NB is one of the most incurable childhood cancers. To improve NB treatment outcomes, risk classification, identification of molecular targets associated with prognosis and development of an optimized therapeutic approach are essential. Loss of 1p, 3p, and 11q are observed in advanced stage of NB, and are associated with an unfavorable prognosis. 11q deletion is observed in 30% of NB; these cases are known to harbor a *MYCN* single copy with distinct genetic subtypes. However, little is known about molecular target located in 11q. Here, we report the genomic alteration of *ATM* and other DNA damage response (DDR)-associated genes *MRE11A*, *H2AFX* and *CHEK1* located in 11q as well as *BRCA1*, *BARD1*, *CHEK2*, *MDM2* and *TP53* in 45 NB-derived cell lines and 237 fresh tumor samples. *ATM* loss or imbalance was detected in 20% of NB, over 90% of which were stage 3 or 4. An additional 10% contained *ATM* mutations. Rare nucleotide variations in DDR-associated genes other than ATM were detected in 26% of samples, and were mutually exclusive. ATM-defective cells exhibited dysfunction in homologous recombination repair, suggesting the potential for synthetic lethality by PARP inhibition. Indeed, most NB-derived cell lines exhibited sensitivity to PARP inhibitor. PARP inhibitor sensitivity was also assessed using *in vivo* xenografts of ATM haploinsufficient SK-N-DZ cells into nude mice, wherein tumor cell growth was markedly attenuated in the group receiving PARP inhibitor treatment. Therefore, PARP inhibitors represent potential candidates for NB therapeutics. Administration of PARP inhibitors to a DDR-defective NB subgroup may contribute to increased survival, especially in advanced stage, poor-prognostic NB

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Investigation on the miRNA signature in retinoic acid-resistant neuroblastoma cells as novel therapeutic targets

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Background/ Aims:
Retinoic acid (RA) is used for the treatment of high-risk neuroblastoma patient, but the prognosis remains unsatisfied for the arising of drug resistance during treatment. MicroRNAs (miRNAs) are small RNAs with 19-23 nt in length and function as a master regulator in cellular signaling pathways. In this research, we aimed to investigate the miRNA signature that specifically exists in RA-resistant (RAR) neuroblastoma cells.

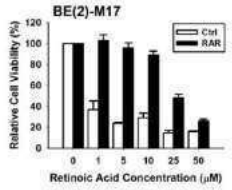
Materials and Methods:
RAR neuroblastoma cells were selected through repeated treatment of RA, with 25 micro-molar as the selection endpoint. The RNA extracted from control and RAR cells were subjected to Affymetrix miRNA 4.0 array. The results were analyzed with software Expression Console and Transcriptome Analysis Console.

Results:
After the selection procedure, RAR cells showed higher tolerance to RA treatment than control cells (Figure 1A). The array data revealed that 114 miRNAs have significantly different expression level (5-fold difference) in RAR cells compared to control (Figure 1B). The potential target of these miRNAs comprises genes essential for regulating cell cycle, proliferation, stress response, and drug metabolism. The significance of these miRNAs and their respective targets in RA resistance are currently under validated.

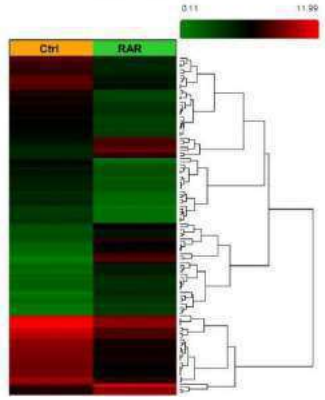
Conclusion:
Our results suggested that aberrant miRNA expression may represent the molecular signature for RA resistance. By targeting these miRNAs we would find novel treatment strategies for RA-resistant neuroblastomas.

Figure 1

(A)



(B) Hierarchical clustering plot of 114 miRNAs with significant difference expression between control and RAR cells.



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Integrated network analysis of G-quadruplex and replicative stress related genes as sources for neuroblastoma genomic instability

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We have provided in vitro and in vivo evidence for BRIP1/FANCJ acting as cooperative driver gene in neuroblastoma and showed strong up regulation of this gene during TH-MYCN driven tumor formation in mice (Vanhauwaert, this meeting). BRIP1/FANCJ is a DNA helicase implicated in unwinding of G-quadruplexes (G4s) and knock down induces replication fork stalling and epigenetic instability. G4s are secondary DNA structures, formed by stable Hoogsteen hydrogen bonding guanines in planes of 4 guanines stacked on top of each other. Several studies have demonstrated that G4 structures can cause genomic instability and are prone to particular genomic rearrangement due to the action of DNA damage tolerance pathways activated to resolve replication stalling at these G4s. Consequently, G4s pose a threat to the rapid cycling and the transcriptionally active state of neuroblastoma cells as replication forks will typically stall at G4s under conditions of replicative stress. We hypothesize that neuroblastoma cells are "replicative stress resistance" addicted. To further elucidate the molecular basis for this presumed addiction, we collected several data sets allowing to perform an integrated network analysis with final aim of identifying a G4 based gene signature reflecting dependence of neuroblastoma cells to alleviating G4 driven replicative stress. First, RNA sequencing was performed on neuroblastoma cells after knock down of BRIP1/FANCJ. Secondly, RNA sequencing was done after exposure of neuroblastoma cells to selected G4-binding ligands. Finally, in vivo drugging experiments are ongoing to provide RNA sequencing on zebrafish neuroblastomas after exposure to G4 ligands in combination with selected small molecules currently under evaluation in clinical trials. In the future, we will further integrate data mining and cross species genomics analyses for the identification of G4 marked genes critically involved in maintenance of the neuroblastoma phenotype.

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The histone demethylase KDM5A regulates p53 function via a translation mechanism

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The tumor suppressor p53 is inactivated in over half of all cancers via genetic mutation or MDM2-mediated degradation. However, these events rarely occur in some cancers such as neuroblastoma, suggesting there may be other ways tumors suppress p53 function. We identify KDM5A, a H3K4me3/me2 histone demethylase, as a novel regulator of p53 signaling through translational regulation of p53 expression via modulation of eukaryotic translation initiation. The expression of KDM5A tends to be higher in p53 wild-type tumors. Importantly, loss of KDM5A significantly reduces $p53^{+/+}$, but not $p53^{-/-}$ or mutant p53, tumor growth. Thus, our findings reveal a novel regulatory mechanism of p53 function and suggest KDM5A as a new target for the treatment of tumors expressing wild-type p53.

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Identification of non-invasive biomarkers for treatment response in neuroblastoma by circulating miRNA profiling

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Introduction An outstanding question in neuroblastoma treatment is whether clinically useful biomarkers of response to therapy can be identified. Biomarkers that allow monitoring of targeted drug pathway activation are highly desirable for the clinical follow-up of patients treated with such drugs. Minimally-invasive methods for patient follow-up, like measuring expression levels of miRNAs in serum, may be an elegant approach to obtain a higher specificity, sensitivity and lower handling time than other currently used biomarkers.

Method MYCN/ALK^{R1279Q} transgenic mice were

treated with crizotinib. Mice carrying orthotopic xenografts of SH-SY5Y cells were treated with RG7388 or temsirolimus. Serum samples of these mice were collected at different time points before, during and after treatment as well as matching end-point tumor material. Small RNA sequencing was optimized for low input and depleted for abundant non-relevant RNA sequences. Small RNA sequencing and whole miRNome RT-qPCR was performed to identify circulating miRNAs that are responsive to treatment or tumor cell engraftment.
Results We were able to observe expression changes for dozens up to one hundred circulating miRNAs, some of which had a >10 fold change in expression value. miRNAs showed differential expression both as a result of treatment and tumor cell engraftment. These include miRNAs with known roles in neuroblastoma tumor biology like the oncogenic miR-17-92 cluster. Moreover, for some miRNAs expression changes were found to be possibly drug-specific.
Conclusion These data encourage further investigation of circulating miRNAs as biomarkers for treatment response, also in a clinical setting.

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Busulfan and melphalan pharmacokinetics in high-risk neuroblastoma patients treated on the HR-NBL1/SIOPEN trial

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St Anna Children's Hospital and Children's Cancer Research Institute, Vienna, Austria

Background Busulfan and melphalan (BuMel) are established as the high-dose chemotherapy regimen of choice in the HR-NBL1/SIOPEN trial. Previous studies have reported marked inter-patient variability in the pharmacokinetics of both drugs in various tumour types. The current study reports on BuMel pharmacokinetics in patients treated on the HR-NBL1/SIOPEN trial.
Methods Busulfan was administered 4xdaily for 4 days, either orally (doses of 1.45-1.55mg/kg) or IV (doses of 0.8-1.2 mg/kg according to body weight strata). Blood samples were obtained at 2-6h after the start of infusion or oral administration for dose 1. Melphalan was administered as an IV short infusion of 140mg/m^2 (4mg/kg for patients <12kg) with samples collected at 5, 23 and 90min. Busulfan and melphalan levels were quantified by GCMS and HPLC analysis, respectively. Data were analysed using NONMEM population pharmacokinetic approaches.
Results Busulfan plasma concentrations obtained from 82 patients and melphalan data from 55 patients were fitted using one- and two-compartment pharmacokinetic models, respectively. A mean busulfan AUC value of 1059±261µM.min (range:434-1954) was observed, with 71% of children achieving AUC values within the defined target of 900-1500µM.min (56% following oral dosing, 78% with IV dosing). Busulfan AUC values in patients who experienced grade 3/4 non-haematological toxicity (n=47) were significantly higher than in patients with no grade 3/4 non-haematological toxicity (n=30) (1107±237 vs 960±251µM.min; *P*=0.027). A mean melphalan AUC of 10.9±6.0µg/ml.h (range:3.9-44.4) was observed, with a higher AUC in patients experiencing grade 3/4 VOD (n=7) than those with no VOD (n=31) (16.5±12.8 vs 9.7±3.9µg/ml.h; *P*=0.05). A significant difference in melphalan exposure was observed in children 2 (n=45) (7.4±2.6 vs 11.7±6.3µg/ml.h).
Conclusions The data confirm higher and more consistent busulfan exposures with IV versus oral dosing and indicate a relationship between BuMel AUC and toxicity. Melphalan dosing based on body weight in children.

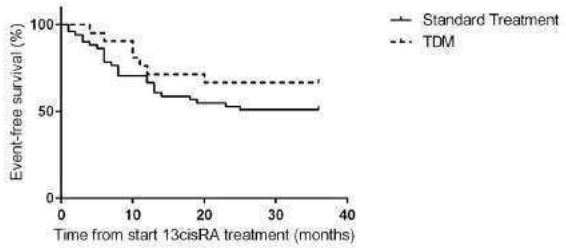
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Clinical follow-up of high-risk neuroblastoma patients receiving individualised 13-cis-retinoic acid based on pharmacological exposure as part of a national UK study

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Background Due to concerns over extensive 13cisRA pharmacokinetic variability, we previously utilised a therapeutic drug monitoring (TDM) approach to target a defined drug exposure in high-risk neuroblastoma patients (*Clin Cancer Res* 2013;19:469-479). Findings suggested that current dosing regimens may be pharmacologically suboptimal in patients receiving reduced body weight–based 13cisRA dosing and children unable to swallow 13-cisRA capsules, with drug extracted before administration. We now have 3 year clinical follow-up for the patients recruited to this trial.
Methods Patients received 13cisRA alongside ch14.18/CHO as maintenance therapy on the HR-NBL1/SIOPEN trial. 13cisRA (160mg/m^2 or 5.33mg/kg/day for patients <12kg) was administered to 72 children and dose increases of 25-50% were implemented on consecutive courses to achieve peak plasma concentrations ≥2µM as required. EFS was calculated from the start of 13cisRA treatment. An event was defined as relapse, disease progression or death.
Results 13cisRA dose increases were implemented in 21 patients who experienced plasma concentrations <2µM. 3 year EFS rates for patients receiving standard versus TDM 13cisRA were 51% and 67%, respectively. With the limited patient numbers, there was no statistically significant difference between these groups (*P* = 0.1113).
Conclusions The current study involved selecting patients experiencing the lowest 13cisRA exposures and utilising a TDM approach to individualise treatment. Despite small numbers of patients, data generated raise the question as to whether high-risk neuroblastoma patients may benefit from increased

doses of 13cisRA, either based on a TDM approach or more practically, involving dose increases to achieve a maximally tolerated dose in individual patients.



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Providing information on clinical trials to parents of children with neuroblastoma: a novel liaison in a clinical nurse specialist role.

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Objective: Parents of children with refractory or relapsed high-risk neuroblastoma (RR NB) require thorough and objective guidance in understanding the complex rationale for treatment and evaluating clinical trials that may be considered for their child. Approximately 40 children per year in the United Kingdom have RR NB and are potentially eligible for enrollment in clinical trials. Despite available clinical trials within the UK, a significant percentage of parents seek access to clinical trials abroad for their children.
Methods: A novel nursing position has recently been established as a central contact and service for all patients with RR NB in the UK. This new post is based at Great Ormond Street Hospital (GOSH) and aims to liaise between the National Health Service (NHS) physicians and the parents considering clinical trials open in the UK or abroad. The programme effectiveness is evaluated by tracking cases and soliciting ongoing feedback on parent and physician satisfaction.
Results: Jointly supported by the charity Solving Kids' Cancer and the NHS staff at GOSH, this new position provides an objective service to families requiring sensitive guidance in a critical situation. The nurse will facilitate the creation and operation of a national advisory panel for children with RR NB which will serve as an unbiased platform for parents to obtain second opinions. The position will also provide an opportunity to collect data on the different processes of neuroblastoma care around the UK, with a view of improving best practices. Successes and challenges of this unique service will inform and further define the role, and set new standards for parent education in paediatric cancer.
Conclusions: This clinical nurse specialist role serves as a successful model for all paediatric cancers. Objective, evidence-based information helps parents navigate the complex landscape of clinical trials for children with a poor-prognosis cancer.

Wendy Landier, Marcia Leonard and Kathleen S. Ruccione on behalf of the COG Nursing Discipline Committee. Children's Oncology Group's 2013 Blueprint for Research: Nursing Discipline. Pediatr Blood Cancer. 2013 Jun; 60(6): 1031–1036. PMCID: PMC3645887

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Premature physeal closure following prolonged fenretinide administration in patients with neuroblastoma

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Background: Despite advances in multimodal therapy for high risk (HR) neuroblastoma, survival remains suboptimal. Retinoid therapy with 13-*cis*-retinoic acid (13*cis*RA) has contributed to improved outcomes. The synthetic retinoid fenretinide has demonstrated *in vitro* toxicity against neuroblastoma cells that have developed resistance to other retinoids, and has a favorable toxicity profile in Phase I trials. Skeletal effects, including premature physeal closure, have been described with other retinoids, but have not been described in children treated with fenretinide to date.

Methods: Retrospective chart review identified two patients with HR neuroblastoma who developed premature physeal closure following protracted oral fenretinide therapy.

Results: Patient 1 (Caucasian girl), and Patient 2 (African American boy) were diagnosed at ages 6 and 5 years respectively with HR stage 4, single copy *MYCN* neuroblastoma. Both failed induction therapy and received investigational ^{131}I-MIBG followed by consolidation chemotherapy and stem cell rescue, local radiation therapy, and 13*cis*RA. Due to persistent metastatic disease, both received oral fenretinide (capsular formulation, 800 mg PO TID) for a total of 70 and 61 cycles, respectively, and ultimately achieved complete remission. During the prolonged administration of oral fenretinide over at least 5 years, both patients developed asymmetric premature physeal closure of multiple long bones, resulting in limb length discrepancies and angular deformities. Nearly a decade off therapy, both are alive and in remission from neuroblastoma.

Conclusion: Phase I and II studies of fenretinide in children have not reported skeletal toxicity as a late effect of therapy. The unusual and strikingly similar radiographic and clinical findings strongly suggest that the skeletal abnormalities may be a consequence of protracted fenretinide exposure. Further study of potential skeletal toxicity in neuroblastoma patients receiving retinoid therapy is indicated to better understand the mechanism and risk factors of retinoid bone toxicity.

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Establishment of a reproducible methodology and results for molecular radiotherapy dosimetric assessment of ^{177}Lu-DOTATATE in neuroblastoma

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Introduction

Molecular radiotherapy (MRT) using ^{177}Lu-DOTATATE is increasingly utilized in the treatment regime of primary refractory or poorly responding patients.

A phase 2 trial is in progress to evaluate the efficacy and safety of this treatment in neuroblastoma. It incorporates patient-specific whole body, tumour and normal organ dosimetry. Avoidance of unacceptable bone marrow and renal toxicities is essential for safety, and knowledge of tumour dose is desirable for correlation with response.

We present here a methodology for molecular radiotherapy dosimetry for this formal phase 2 clinical trial, in which accurate whole-body, tumour and organ-sensitive doses are a key component for improving the therapeutic index and minimisation of toxicity. This methodology involves accurate determination of time-activity curves derived from the correlation of probe counting methods with planar and SPECT/CT imaging.

Methods

Children with relapsed or refractory high-risk neuroblastoma are assessed for suitability for in-patient peptide receptor radionuclide therapy (PRRT) with ^{177}Lu-DOTATATE by ^{68}Ga-DOTATATE PET/CT. The administered activity of the first fraction of ^{177}Lu DOTATATE is weight-based (75MBq/Kg). Activity for subsequent administrations at 8 to 12 week intervals – 4 fractions in total - depends on the whole-body radiation dose received and the haematological toxicity from previous administrations. Whole-body dose is determined by ceiling-mounted scintillation probe monitoring, performed regularly by carers over 4 days; and gamma camera imaging at 6 time points with whole body and SPECT/CT imaging for critical organ and tumour dose distribution/dosimetry.

Results

To date, 26 fractions of therapy have been administered to 16 patients in total. A complete set of probe and imaging data has been successfully acquired for every fraction delivered.

Mean whole-body dose for each patient, from fraction 1 (75MBq/kg) was 0.28Gy (range 0.19 – 0.39), which allowed dose escalation to 100MBq/kg for fraction 2.

Conclusions

Use of accurate dosimetry enables effective monitoring of organ sensitivity and enables safe dose-escalation for ^{177}Lu-DOTATATE in Neuroblastoma.

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Withdrawn

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An anti-O-acetylated GD2 ganglioside antibody for the immunotherapy of High – Grade Diffuse Glioma in children

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High – Grade Diffuse Gliomas (HGGs) represents between 15 to 20 % of all central nervous system (CNS) tumor in children and have a 2 year survival rate of only 10 to 30 % [(1)]. The impact of current treatments is unfortunately small, highlighting the need for new and novel therapies. Membranes glycosphingolipids (GSL), including gangliosides, represent an unexplored source of tumor-associated antigens. In our laboratory, we have generated a mouse monoclonal antibody that is specific for the O-acetylated derivative of the neuroblastoma-associated tumor antigen ganglioside GD2 (OAcGD2). Although prior reports suggest that OAcGD2 is expressed by glioma cells, the expression of OAcGD2 on the cell surface of malignant gliomas has not been fully evaluated. Thus, we explored the usefulness of OAcGD2 as a candidate target antigen for antibody-mediated therapy in patients with malignant glioma. We first demonstrated that OAcGD2 is expressed on surgically resected human glioma tissues and glioma cell lines. Next, we studied the anti-HGGs properties of mAb and showed that mAb 8B6 inhibits HGGs cell proliferation. Our findings suggest that HGGs are suitable for specific targeting by mAbs specific for O-AcGD2. In addition, they provided the preclinical support for the use of anti-O-acetylated GD2 mAbs as a valuable addition to current therapeutics.

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Immunohistochemical evaluation of target expression in high-risk neuroblastoma samples to facilitate optimisation of molecular radiotherapy

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Introduction

Molecular radiotherapy targeting the noradrenaline transporter molecule (NAT) with ^{131}I-meta-iodobenylguanidine (mIBG) and the somatostatin

receptor subtype-2 (SSTR-2) with ^{177}Lu-DOTATATE are used for the treatment of relapsed and refractory neuroblastoma. Currently, patient selection for treatment is based on ^{123}I-mIBG and ^{68}Ga-DOTATATE imaging. This study aimed to quantify the degree and intensity of expression of both NAT and SSTR-2 in archival neuroblastoma tissue using immunohistochemistry and to correlate these with clinical factors.

Methods

Archived formalin fixed paraffin embedded neuroblastoma tissue was received from the Children's Cancer and Leukaemia Group Tissue Bank. Each sample underwent immunohistochemistry for both NAT and SSTR-2 using commercially available monoclonal antibodies. Each sample was then scored for 1) intensity and 2) percentage of cells staining positively, for both NAT and SSTR2. Scores were correlated with known matched clinical data on age, stage, MYCN status and outcome.

Results

75 tissue samples were available with matched clinical data at the time of analysis. The median age of the cohort studies was 18 months and 68% had INSS stage 4 disease. MYCN was amplified in 23%, not amplified in 61% and unknown in 16%. 83% of samples were poorly differentiated, 14% were differentiating and 4% were undifferentiated. There was considerable heterogeneity of expression of NAT and SSTR-2 amongst the samples. In stage 4 patients, 56% had high intensity and 47% high percentage expression of SSTR-2 whereas 8% had high intensity and 39% high percentage expression of NAT.

Conclusion

Immunohistochemistry may possibly be used to facilitate the appropriate selection of molecular radiotherapy in neuroblastoma. Immunohistochemical assessment of target expression will be correlated with results of nuclear imaging in a current trial of molecular radiotherapy in neuroblastoma. These results suggest that molecular radiotherapy targeting both NAT and SSTR2 simultaneously or sequentially may have clinical advantages.

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Primary tumor resection after high dose chemotherapy with autologous hematopoietic stem cell transplantation is a safe and feasible option. A report from the Japanese neuroblastoma study group (JNBSG)

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Background In the current high-risk neuroblastoma (NB) clinical trial JN-H-11, primary tumor resection is scheduled after the completion of the entire chemotherapy regimen, including myeloablative chemotherapy with autologous hematopoietic stem cell transplantation (HDC). We assessed the safeness and feasibility of this approach by comparing the surgical outcome and complications of resections after HDC to those of resections performed at conventional timings.

Methods Case report forms and surgical records of 30 cases from the JN-H-07 study in which tumor resection was performed after 3 or 4 courses of induction chemotherapy (conventional group), and 39 cases from the JN-H-11 study in which surgery took place after HDC (delayed group) were analyzed. Background of patients, operation time, blood loss, blood transfusion requirement, surgical complications were analyzed.

Results Mean operation time (conventional 285 min vs delayed 296 min), blood loss (270g vs 134g), and blood loss / estimated blood volume ratio (23% vs 12%) did not differ between the two groups. There were more procedures in the delayed group that required platelet transfusion (conventional:13%, delayed:36%). No major surgical complications other than bleeding were encountered in both groups. Nephrectomy was carried out in 20% of the conventional group and 7.7% of the delayed group.

Discussion The "delayed local treatment" concept is widely accepted in our country, although there remain concerns regarding safeness of the approach, since surgeries are to be performed on patients under severely immunocompromised and myelosuppressive conditions. Our results suggest that surgery after HDC could be performed safely with acceptable complication rates.

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Opsoclonus-myoclonus syndrome in neuroblastoma: A report from the Japan Neuroblastoma Study Group (JNBSG)

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Background: Opsoclonus-myoclonus syndrome (OMS) is a rare complication of neuroblastoma; however, its epidemiology, effective therapy and long-term outcome remain unclear. We herein investigated the clinical features and long-term outcome of OMS in Japan in order to determine effective therapy.
Methods: We retrospectively reviewed neuroblastoma patients registered in the Japan Neuroblastoma Study Group (JNBSG) between 1998 and 2013.
Results: The total number of registered neuroblastoma cases was 1,682 cases, and 53 cases had OMS (3.2%). The onset age of those 53 neuroblastoma patients ranged from 7-67 months, with a mean age of 21.4 months. These cases were assigned to stage 1, 47.1%; stage 2A/2B, 22.6%; stage 3, 15.1%; stage 4, 9.4%; and stage 4s, 0%. Only one case died. The treatment rates for OMS were oral prednisolone (60.3%), intravenous immunoglobulin (49.1%), methylprednisolone pulse therapy (37.7%), rituximab (11.3%), and adrenocorticotrophic hormone (ACTH) therapy (7.5%). Finally, the symptoms of OMS disappeared in 33 cases (62.3%), but remained in 20 cases (37.7%). There were no significant differences in the onset age of neuroblastoma between cases with disappeared symptoms and cases with remained symptoms (22.6 and 19.5 months, respectively). The rate of therapy, which included chemotherapy for neuroblastoma and therapy for OMS, in cases with disappeared symptoms was higher than that of the remained symptoms cases.
Conclusion: The prognosis of neuroblastoma patients with OMS is good, however, neurological symptoms can remain in a great number of patients. Therefore, chemotherapy and immunological therapy is necessary for improvement in the neurological prognosis.

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Combination therapy of highly activated natural killer cells and anti-disialoganglioside (GD2) antibody for Neuroblastoma: An experimental study

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Background: The anti-gyasileganglioside (GD2) antibody has been clinically shown to mediate tumor killing via conplement-derived cellular toxicity (CDC) and antibody-dependent cellular toxicity (ADCC). Although the combination of anti-GD2 antibody and cytokines has become a novel therapy to prevent recurrence in patients with advanced neuroblastoma, the therapeutic outcome has been insufficient. On the other hand, clinical studies to treat advanced malignancies using NK cells are emerging worldwide; however, overall purity and numbers of NK cells and their antitumor potencies are unsatisfactory as to obtain clinical outcome. Therefore, we established a simple method to generate purified and highly activated NK cells (Zenithal NK=Z-NK) from peripheral blood mononuclear cells (PBMCs). However, it has yet to be shown whether adoptive transfer of Z-NK cells combined with tumor-specific antibody has more therapeutic potential than Z-NK alone.
Purpose: To confirm the synergic efficacy of Z-NK cells and anti-GD2 antibody to human neuroblastoma cells.
Methods: *In vitro*: IMR32 cells are incubated for 1 hour at 4°C with or without anti-GD2 antibodies (1μg/ml). Cells are co-cultured with ZNK in Effector/Target (E:T) = 1:10 for 3 hours at 37°C. Then, percent specific lysis is calculated. *In vivo*: Dermal tumor composed of IMR32 on NOD/SCID mouse is treated with ZNK alone, GD2 antibody alone or ZNK combined with GD2 antibody via tail vein. The growth of tumor is monitored.
Results: A dose-dependent response was apparent, 3hr of co-culture with ZNK cells was sufficient to kill nearly 100% of IMR32. The %lysis values against IMR32 (E:T=1:10) were found to be approximately ～30% (without GD2 antibody), and ～50% (with GD2 antibody) at 3 hr.
Conclusion: These findings indicate that the ZNK cells may be under a near optimally activated state that has never been achieved by preexisting techniques and have an excellent antitumor activity especially with tumor-specific antibody. Thus the combination therapy may contribute to the advance of adoptive immunotherapy against neuroblastoma in a clinical setting.

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Analysis of surgery for Neuroblastoma in The Netherlands

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Background

To evaluate the morbidity and mortality after surgical excision in Dutch neuroblastoma patients.

Methods

A retrospective review was performed of all 187 neuroblastoma patients presented at two pediatric oncology centers (EMC and Radboud UMC) between 1998 and 2014. Morbidity and mortality caused by surgical intervention was documented.

Results

Surgical excision was performed in 146 patients (78.1%): Stage I (18/20), Stage II (16/20), Stage III (23/26), Stage IV (77/99), Stage IVs (3/11).
Peroperative complications occurred in 66 patients (45.2%), varying from minor vessel injury to removal of organs or structures, the majority involving stage IV patients (n=38).
Unplanned removal of organs or structures occurred in 38 patients (26%). In nine children this involved total nephrectomy (6.1%).
Postoperatively, 53 children (36.3%) experienced Clavien-Dindo grade 1 or grade 2 complications, including renal ischemia in 6 children, leading to complete renal atrophia in 2 patients.
Clavien-Dindo grade 3, 4 or 5 complications occurred in 12 children. Secondary surgery was necessary in four patients: subtotal colectomy for intestinal ischemia, surgical drainage of chylo-abdomen, nephrectomy for renal atrophia and surgical reduction of intestinal intussusception. Longterm ICU admission was necessary for one child who endured severe sepsis after aspiration due to tracheal tube dislocation after surgery.
Longterm complications involved, among others, scoliosis, Horners' syndrome and mechanic bowel obstruction (n=6), leading to surgical reintervention in three children.
Peroperative mortality occurred in one patient; a 2-year old child with stage IV disease. Laceration of a liver vein caused an air embolus due to low intrathoracal pressure, leading to irreversible cardiac arrest. No postoperative mortality was reported.

Conclusion

Surgical excision for Neuroblastoma is associated with significant complications and peroperative mortality, especially in Stage IV patients.

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Low-risk neuroblastoma in Russia: therapy results and prognostic factors

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Introduction
The aim of the study was to analyze the prognostic factors in the cohort of patients with low-risk neuroblastoma (NB) treated in Russian Federation.
Methods
157 patients with low-risk NB were treated for the period 01.2012-06.2015 (42 months). The diagnosis has been established on the basis of the international criteria. Patients were stratified and treated according to the German NB2004 protocol. Surgery was the mainstay of therapy. Chemotherapy was reserved for the patients with life-threatening symptoms (LFS) persisted after surgery.
Results
Male: female ratio was – 1.04:1. The median age at the diagnosis was 5.8 months (range 0.5- 205.4). 34 (21.7%) patients had LFS at the time of diagnosis. 1p and 11q deletion was observed in 9 (5.7%) and 6 (3.8%) patients, respectively. Initial therapy consisted of surgery in 115 (73.2%), chemotherapy – 27 (17.2%) and observation – 15 (9.5%). Only 35 (22.3%) patients received chemotherapy as a part of first-line therapy. Median follow-up was 24.4 months (range 0.0-59.4). 3-year event-free survival (EFS) and overall survival (OS) was 80.6±3.2% and 96.1±1.5%, respectively. Progression/relapse was observed in 24 (15.2%) patients with a median time to event 4.1 months (range 0.5-17.3). OS after relapse/progression was 95,4±4,4%. 6 (3.8%) patients died. Median time to death was 2.0 months (range 0.0-6.7). 5 deaths were therapy-related, 1 – tumor-related. Cumulative incidence of treatment-related and disease-related mortality at 12 months was 3,2% and 0,6%. Age < 12 months and stage 3 were associated with inferior EFS; non-abdominal location, presence of LFS and stage 3 were associated with inferior OS in the univariate analyzes. 1p and 11q status didn't affect prognosis. In multivariate analyzes only presence of LFS associated with OS.
Conclusion
The prognosis for patients with low-risk NB is good. Risks associated with therapy should be considered in patients with LFS when choosing treatment strategies.

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Pharmacokinetics (PK) of 13-cis Retinoic Acid in COG Phase III Neuroblastoma Studies

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Background: Isotretinoin (13-cis-retinoic acid; 13-cRA), a differentiation inducer, improves outcome for high-risk neuroblastoma. Published PK studies suggested that many patients achieve 13-cRA levels lower than 5 mM (effective against neuroblastoma *in vitro*). We showed that 4-oxo-13-cRA, a metabolite of 13-cRA was equally active compared to 13-cRA against neuroblastoma *in vitro*. We sought to determine the levels of 13-cRA and 4-oxo-13-cRA achieved in patients and their association with clinical outcome.
Methods: Plasma samples (4 hours post-dose, day 14) from COG phase III clinical trials (A-3973, ANBL0532, ANBL0032, and ANBL0931) were obtained for PK analyses. The relationship of PK to clinical outcome was analyzed, limited to patients treated with ch14.18 antibody.
Results: In 629 patients, 370 (60%) achieved median plasma concentrations over 5 mM for 13-cRA+4-oxo-13-cRA combined levels. Plasma levels were higher in patients taking whole capsules relative to open-capsule takers (13-cRA: 1.74 vs 1.03, 4-oxo-13-cRA: 7.22 vs 3.27mM). Both 13-cRA (r=0.29) and 4-oxo-13-cRA (r=0.46) concentrations positively correlated with age (p<0.001). Relationship of PK to overall survival (OS) was analyzed for 524 patients that received both 13-cRA and immunotherapy. In patients ≥18 months old OS at 5 yrs was higher (73 %) for 13-cis-RA plasma levels > 75th percentile (2.5μM) vs. pts with 13-cRA < 25th percentile (0.6μM, 63%) (p=0.039). OS at 5 yrs was also higher for patients ≥18 months-old with 4-oxo-13cRA >5μM vs. <1μM: 76 vs. 66% (p=0.032) and 13-cis-RA + 4-oxo-13-cis-RA >5μM vs. <1μM: 73 vs. 61% (p=0.019).
Conclusion: Combined levels of 13-cRA and the 4-oxo-13-cRA active metabolite were >5 mM in the majority of patients. Age and route of administration influence plasma levels of 13-cRA and 4-oxo-13-cRA. In patients ≥18 months old low plasma levels of 13-cis-RA + 4-oxo-13-cRA were associated with a lower overall survival.

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Long term survival after KIR ligand incompatible allogeneic cord blood transplantation as a salvage therapy for relapsed stage IV neuroblastoma

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Background: Relapsed stage IV neuroblastoma patients have rare chance for a long-term survival. Although benefits of allogeneic stem cell transplantation (SCT) from HLA matched sibling for relapsed neuroblastoma has been denied, SCT from HLA mismatched donor is still explored. We evaluated effects of HLA mismatched SCT as a salvage therapy in patients with relapsed neuroblastoma, especially in terms of Killer immunoglobulin like receptor (KIR) ligand compatibility. **Methods:** Seven patients with relapsed stage IV neuroblastoma received allogenic SCT from HLA mismatched donor in our hospital from 2007 to 2014. The median age was 6 (range: 2-9) years old at relapse, consisting of 4 boys and 3 girls. Three patients received allogenic SCT from KIR compatible donor (KIR match group). Two were from HLA haploidentical related family and one from cord blood. Other four patients received KIR ligand incompatible cord blood transplantation (KIR mismatch group). **Results:** All patients except for one could achieve the second complete response prior to SCT due to several different salvage chemotherapies. All three patients in KIR match group developed disease progression after SCT resulting in death. The time interval between transplantation and death were 4, 7 and 20 months, respectively. On the other hand, three out of four patients in KIR mismatch group were alive without any chemotherapies after transplantation for 13, 26 and 63 months, respectively. One patient in KIR mismatch group developed a local recurrence in his right femur bone, 9 months after CBT, but he received artificial bone displacement and alive without any further treatment. Finally, both progression-free and overall survival were significantly better in KIR mismatch group than in KIR match group (p=0.01, 0.04, respectively). **Conclusions:** CBT from KIR ligand incompatible donor could be an efficient salvage therapy for relapsed stage IV neuroblastoma if a patient achieves the 2nd remission prior to CBT.

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Treatment high-risk neuroblastoma

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OBJECTIVE: To improve the survival of patients with high-risk neuroblastoma.

METHODS: From November 2013 to April 2015 in our hospital treated 13 patients on the protocol of treatment of patients with high-risk neuroblastoma. 4 patients with stage 3 disease, 9 patients with stage 4. The median age at diagnosis was 35.5 (20-92) months. N-myc amplification was detected in 3 patients, 11q23 deletion in 9 patients, 1p36 deletion in 6 patients. 12 patients had metastases to the regional lymph nodes. All 9 patients with stage 4 disease had metastasized into the bone marrow, 8 patients had bone metastases, 2 patients - soft tissue metastases orbits 1 - liver 1 - lungs and 1 - pleural metastases. All patients received induction chemotherapy, surgery, high-dose chemotherapy and radiation therapy. 2 patients with stage 4 disease got MIBG-therapy. Patients with stage 3 disease receiving biological treatment with retinoic acid (Roaccutane) in the consolidation phase. Patients with stage 4 disease, received immunotherapy with dendritic tumor vaccines in the consolidation phase.
RESULTS: 4 patients with stage 3 are alive without evidence of recurrence and disease progression. The observation period of 6 to 21 months. In 4 of the 9 patients with stage IV in the period of 3-6 months from the end of treatment was a relapse of the disease. All of them died after 2-4 months after the detection of recurrent disease (3 from the progression of the disease, one of the complications of antirelapse therapy). 5 of 9 patients with stage IV are alive without evidence of recurrence or progression of the disease, the time of observation of 6-9 months.
CONCLUSIONS: Complex treatment including intensive chemotherapy, surgery, high-dose chemotherapy, radiation therapy, and biotherapy, provides excellent results of treatment of patients with stage III neuroblastoma high risk. Results of treatment of patients with neuroblastoma stage IV high risk remain poor. Research aimed at improving the survival of patients with stage IV neuroblastoma, is a priority.

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Prognostic Factors in stage 4 neuroblastoma patients treated with Busulphan-Melphalan. Report from the European High Risk Neuroblastoma HR-NBL1/SIOPEN Trial.

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Aim: Evaluation of prognostic factors in high risk stage 4 neuroblastoma(HRNBL) patients(pts) treated with busulfan-melphalan(BUMEL) within HR-NBL1/SIOPEN.
Methods: Between 2002-2009 (prior antiGD2 immunotherapy) BUMEL was given to 475pts(275males) in 149 centres/20 countries. Pts<1year(yr) had MYCN amplification(MNA). After induction (COJEC ± 2-4 TVD,) BUMEL/SCR was given once pts achieved at least PR. Local control aimed at gross surgical resection (achieved in 76%) and for radiotherapy (21Gy) to the primary tumour site. Maintenance was 13cis retinoid acid only. The median age at diagnosis was 3yrs (1month-19yrs). The median observation time is 7.4yrs. Outcomes are reported as 5-yrs EFS rates.
Results: EFS was 0.64±0.12 for age<1yr (17pts), 0.62±0.08 for 1-1.5yrs (42pts), 0.40±0.03 for 1.5-5yrs (317pts) and 0.20±0.04 for pts>5yrs (99pts)(p=0.0001). EFS was better with BUMEL/SCR given ≤240days (421pts) after diagnosis (0.41±0.02) than for 54pts taking longer (0.16±0.05;p<0.0001). Outcome was not different in 221pts randomised for BUMEL (221/475). Pts with TVD have a significantly different EFS (0.41±0.03(214pts)

vs.106pts+TVD:0.30±0.05) (p=0.026). CR-pts prior to BUMEL (172pts; 36pts+TVD) had an EFS of 0.45±0.04, whilst EFS was 0.38±0.04 for VGPR-pts (165pts; 36pts+TVD) and 0.31 ±0.04 for PR-pts (138 pts; 34pts+TVD) (p=0.004). Earlier CR of pts without TVD appears superior (0.48±0.04 vs. 0.33±0.08 for CR after TVD(NS). EFS in PR-pts prior BUMEL with ≤3 mIBG avid skeletal spots was better (0.38±0.05) than with more spots (0.13±0.06; p=0.014). Also involvement of only one metastatic compartment results in significantly better EFS (0.50±0.07 vs. 0.37±0.02 for multiple compartments, p=0.037). Severe toxicity rate was 7% (ICU, toxic deaths), the VOD rate 24% (grade 3:4%).
Conclusion: Multivariable analysis (stage 4 patients >1yr and BUMEL ≤240days) reveals as independent unfavourable prognostic factors: age group 5-10yrs (Hazard-Ratio 2.77, p<0.0001), age group 1.5-5yrs (Hazard-Ratio 1.73;p=0.043) and PR prior BUMEL (HR=1.46;p= 0.008). These findings enable guidance to future altered phase II approaches.

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Primary tumour response to busulfan/melphalan high-dose chemotherapy in patients with high-risk neuroblastoma: a pilot study.

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Background: Patients with high-risk neuroblastoma (HRNB) treated on the HR-NBL-1/SIOPEN trial typically undergo surgical resection of the primary tumour after induction chemotherapy and before high-dose chemotherapy (HDC) with busulfan/melphalan (BuMel). Occasionally surgery may be delayed until after HDC because the tumour is unresectable or has unacceptable surgical risk. Whether treatment with BuMel can lead to further shrinkage of the primary tumour and improvement of surgical risk following induction chemotherapy is currently unknown.
Methods: This was a retrospective review of all patients treated at Great Ormond Street Hospital and enrolled on HR-NBL-1/SIOPEN trial between 2002-2015. In patients in whom primary tumour (whole or part) was in situ during HDC response by International Neuroblastoma Response Criteria (INRC) and Response Evaluation Criteria in Solid Tumour (RECIST), and surgical risk and resectability, was assessed by comparison of pre- and post-HDC CT/MR imaging. Tumour volume was calculated as the product of three dimensions x0.52.
Results: Of 84 patients enrolled on HR-NBL-1/SIOPEN, 16 (19%) had primary tumour in situ during HDC. Surgery had been attempted but abandoned in 5 cases; 3 had bilateral or two-compartment disease with partial resection only; the remaining 8 had surgery deferred until post-HDC. BuMel was given in all but one case, who received treosulfan/melphalan due to an underlying cardiomyopathy. Tumour measurements could be made in 13 of 16 cases (no imaging was available in one case and 2 had ultrasounds only): 8 had stable disease; 4 (31%) had partial responses (by INRC and RECIST criteria) and 1 was stable by RECIST, but progressed by INRC criteria. Mean tumour volume before HDC was 61.4cm^3 versus 43.1cm^3 after (p=0.0044, paired t-test), with a median overall volume reduction of 37%. 9 of 16 (56%) patients ultimately underwent complete/partial resection.
Conclusion: These preliminary data suggest that HDC can lead to useful further shrinkage of primary tumours in patients with HRNB and that delaying surgery until after HDC may make complete surgical resection easier to achieve.

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Engraftment following busulfan/melphalan (BuMel) high-dose chemotherapy for high-risk neuroblastoma. A report from the HR-NBL-1/SIOPEN trial.

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Background: The HR-NBL-1/SIOPEN trial for patients with high-risk neuroblastoma includes consolidation with high-dose chemotherapy (HDC) for patients achieving an adequate response to induction chemotherapy. Bone marrow reconstitution after HDC is with reinfusion of autologous peripheral blood stem cells (PBSCs) or, less commonly, bone marrow-derived cells (BMSCs). Data relating to engraftment have not previously been reported in detail, but are important to establish a baseline for quality assurance of autografts performed at individual centres and to correlate with outcome.
Methods: Data were obtained from the SIOPEN-R-NET trial database. Only patients who received BuMel MAT were included. Neutrophil engraftment was determined as the time to ANC>0.5x10^9/L (irrespective of G-CSF administration) and platelet recovery to >20 or >50x10^9/L (transfusion independent). Kaplan-Meier estimates of event-free and overall survival were calculated from day of cell reinfusion and curves compared using log-rank test.

Results: Data were available for 1226 patients. Overall median time to ANC recovery was 12 days, and 20 or 35 days for platelets >20x10^9/L or >50x10^9/L respectively. 1164 (95%) patients had ANC recovery by day 30. 17 (1.4%) died within 150 days without ANC engraftment. ANC and platelet engraftment did not vary depending on year of procedure, route of busulfan administration (oral/IV), or body weight (12kg cut-off). Engraftment was significantly delayed in patients in whom fewer than 3x10^8/kg PBSC/BMSC had been harvested (D30 ANC engraftment in 90±3% of patients vs 96±1%, p=0.005). Patients without ANC engraftment by D30 (n=59, 5%) had poorer 5-year survival (EFS 38±7% vs 44±2%, p=0.042; OS 42±7% vs 52±2%, p=0.006), with a significantly increased risk of early non-relapse mortality (5-year NRM 10±4% vs 3±1%, p<0.0001). These effects will be further explored in a multivariable analysis.
Conclusion: These data confirm a clear baseline for successful engraftment post-BuMel HDC. Delayed engraftment is associated with poorer survival emphasising the importance of ongoing quality assurance of autograft programmes and cautioning against proceeding with HDC with inadequate PBSC/BMSC harvest.

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Phase I study of anti-GD2 antibody ch14.18/CHO long term infusion in recurrent or refractory neuroblastoma patients in Japan

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Background: The primary objective of this study was to assess safety and tolerability of single agent monoclonal anti-GD2 antibody ch14.18/CHO continuous long-term-infusion (LTI) in Japanese neuroblastoma patients. Secondary objectives were to evaluate pharmacokinetic (PK) and pharmacodynamics (PD) parameters and anti-tumor activity in patients with evaluable diseases.

Methods: Continuous infusion of 100 mg/m^2/cycle (3.3mg/kg/cycle if body weight ≤12 kg) ch14.18/CHO was administered over 10 consecutive days every 5 weeks. Patients could receive up to five cycles. Blood samples were collected for assessment of PK, HACA response, immunophenotype, ADCC, CDC, biochemistry, Fc gamma receptor polymorphism, and MRD assessment.
Results: Ten patients with recurrent/refractory neuroblastoma were enrolled between September 2014 and July 2015. Nine patients (5 boys and 4 girls) were eligible and evaluable for toxicity and response assessments in this study. The median age was 4 (range 2-9) years old. Grade 3 or higher toxicities included anemia, leukopenia, neutropenia, elevation of hepatic transaminases, hyponatremia, and urticalia, but all these toxicities were reversible until the next scheduled cycle. The supportive care guidelines allowed all patients to continue the treatment at the same dose level. No unexpected Grade 4 toxicities were observed. PK parameters of ch14.18/CHO in Japanese patients were not different from Caucasian patients. All evaluable patients revealed an increased ADCC response over base line against neuroblastoma cells. Three of nine patients had partial responses evaluated by MIBG scans or tumor marker analysis. One patient discontinued after 2 cycles because of progressive disease in central nervous system. Eight patients are alive with stable disease without further chemotherapy (median follow-up 10 months from enrollment; range 7-17).
Conclusion: Japanese patients receiving ch14.18/CHO for high risk neuroblastoma had similar PK, tolerability and objective response rate compared to Caucasian patients. These results bridge data of ch14.18/CHO between Asian and Caucasian patients.

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Complication of surgery for abdominal neuroblastoma: Chyle Leak

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Background: The incidence and optimal management of postoperative chylous leak of neuroblastoma is inadequately documented. We analyzed the risk factors, management, and the implication of chyle leak following surgery for abdominal neuroblastoma.
Methods: One hundred sixty patients who underwent surgery for abdominal neuroblastoma between September 2004 to August 2014 were evaluated. To find the oncological implication we evaluated the delay in starting further treatment, local control, event free and overall survival.
Results: Chyle leak was the most common complication (20%). The median measure of leakage was 100 ml/day and it persisted for a median of 12 days. All patients were managed conservatively except

one who needed exploration for wound dehiscence. Number of lymph nodes resected was the only factor associated with the risk of chyle leaks (p=0.013). Adjuvant chemotherapy was not delayed in any patient because of chyle leaks per se and the local control, event free and overall survival was not different for patients with and without chyle leak. Conclusion: Chylous leakage is a common postoperative complication of abdominal neuroblastoma, predisposed by the number of lymph nodes resected. It responds to conservative management and does not compromise the further oncological treatment and outcome hence; it should not be a deterrent to complete surgery.

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Expression, trafficking and biological significance of mitochondrial MRP1 in neuroblastoma.

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Purpose: Over-expression of multi-drug resistance protein 1 (MRP1) predicts poor outcome for children with neuroblastoma (NB) (Haber et al, 2006, JCO, 24, 1546-53). MRP1 is expressed in a number of different subcellular organelles and is a reported client protein of the heat shock protein (HSP) family (Roundhill et al, 2016, FASEB, Epub, PMID:26722004). We have investigated the expression, transport and activity of MRP1 in NB.
Methods: MRP1 expression was examined by subcellular fractionation and western blotting (WB), electron microscopy (EM) and immunofluorescent (IF) microscopy. Expression was increased following infection with an MRP1 expressing retrovirus. MRP1 efflux activity was evaluated using calcein-AM and disrupted using valinomycin. Viable cell number was measured by trypan blue exclusion and cell migration examined in a wound healing and 3D assay. Interactions between MRP1 and HSPs were investigated by immunoprecipitation, and inhibition of HSP90 using NVPAUY and 17-AAG.
Results: MRP1 protein and efflux activity were detected in NB cell lines (12/12). High MRP1 expression was associated with *MYCN* amplification (4/6). MRP1 was detected in the plasma membrane, nucleus and mitochondria of NB cells and tumours. Disruption of mitochondrial MRP1 activity did not enhance vincristine (MRP1 substrate) cytotoxicity in the *MYCN* and *MYCC* amplified cells (CI >0.9), although it did increase vincristine-induced cell death in the non-amplified cells (CI <0.7). Resistance to MRP1 substrates (p>0.05), cell doubling time (p>0.05) and migration (p>0.05) were unchanged after increased expression of MRP1.
NB cells expressed the chaperone proteins HSP70, HSP90α and HSP90β. MRP1 co-precipitated with HSP90β. NVPAUY (20-1280nM) significantly decreased viable cell number by 44-59% (>160nM; p<0.05) in NB cells, whereas 17-AAG (30-200nM), a less potent HSP90 inhibitor, had no effect on NB cell number (p>0.05 at all concentrations examined).
Conclusions: MRP1 is expressed in the mitochondria of NB cells and may be a client protein for HSP90β. The clinical significance of mitochondrial MRP1 in NB requires further investigation.

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OMS/DES 2011 : a Multinational European Trial for Children with Opsoclonus Myoclonus Syndrome

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Background : Opsoclonus myoclonus syndrome (OMS) is a rare paraneoplastic syndrome characterized by opsoclonus, myoclonus and ataxia, as well as behavioural abnormalities, and is frequently associated with neuroblastome (NB) in children. Although oncological outcome is often favorable, neurological outcome remains poor.
Methods: SIOPEN (SIOP Europe Neuroblastoma), GPOH (Gesellschaft für Pädiatrische Hämatologie und Onkologie), and EPNS (European Paediatric Neurology Society) have launched a collaborative trial, using an escalating 3-step therapeutic design based on neurological response using standardized neurological evaluations. Neurological and oncological outcomes will be determined
Treatment steps:

dexamethasone (20 mg/m2 x 3 days), monthly bolus (x12 months);

if no neurological improvement after 3 months: cyclophosphamide 750 mg/m2, monthly bolus (x 6 months);

-if no neurological improvement after 3 months: rituximab 375 mg/m2 x2.

Results: Since opening April 2013, the trial has recruited 31 of 100 planned patients. Enrolment rate has increased progressively: 6 patients in 2013, 9 in 2014 and 16 in 2015 due to successive trial opening in participating countries (n=7 to date). For the first 25 patients for whom complete datasets are available, mean age at diagnosis was 24 months (range 6-59m). NB was evidenced in 15 children (60%), of whom 7 required chemotherapy as oncological treatment. The

protocol indications for tissue banking and neurological treatment have been adhered to, and to date 12 SAEs and no SUSARS have been reported.

Conclusion: The final opening (after 15 years) of this collaborative academic international paediatric trial involving neurologists and oncologists highlights administrative difficulties encountered prior to launching such a study, compliant with the European Clinical Trial Directive 2001/20/EC. The current lengthy timelines for trial activation across Europe must be improved with the new Clinical Trial regulation, and it is hoped that implementation of the new clinical trial regulation will improve this situation in Europe. This trial presents a unique opportunity for collaborative research across borders and specialties including biological research on mechanisms involved in OMS and OMS-associated NB.

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Phase I trial of perifosine monotherapy in patients with relapsed or refractory neuroblastoma

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Background: Perifosine is a synthetic alkylphospholipid which inhibits Akt phosphorylation and affects JNK and MAPK signaling pathways. A phase I/Ib trial on neuroblastoma (NB) suggested the efficacy of perifosine monotherapy (Kushner, BH; ANR 2012). Therefore, we conducted a phase I trial to evaluate the tolerability, safety, efficacy, and pharmacokinetics of perifosine in Japanese patients (pts) with NB.
Methods: Pts with relapsed or refractory NB after receiving standard chemotherapies were eligible. Pts received 100-300 mg/body on day 1, followed by daily maintenance doses (50-150 mg/body) until progressive disease. We assessed dose-limiting toxicities (DLTs) and pharmacokinetics in the first 10 pts, as well as safety and efficacy in all pts.
Results: A total of 19 pts were enrolled between June 2013 and March 2014. Median age was 6 years (2-22) and the median number of prior chemotherapy regimens was 3 (1-10). No DLTs were observed. The most common adverse drug reactions were vomiting (63.2%), nausea (52.6%), and diarrhea (36.8%). The frequent grade 3 or 4 adverse drug reaction was ALT increased (10.5%). According to INRC, the response rate (RR) and disease control rate (DCR) were 0% and 55.6%, respectively, and median progression-free survival (PFS) was 122 days (1-344+). According to RECIST, RR and DCR were 9.0% and 54.5%, respectively, and median PFS was not reached. The median overall survival was not also reached at 12 months after the last patient was enrolled. Plasma concentrations of perifosine on day 15 and day 29 were 27.5±9.8 μM and 27.3±11.5 μM, respectively.
Conclusion: Perifosine monotherapy was tolerable in Japanese NB pts. Although DCR was more than 50% and some pts achieved long PFS, RR did not show encouraging efficacy. Further investigation of combination therapy with other drugs is warranted.

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Generation of a new bicistronic DNA vaccine encoding for tyrosine hydroxylase and IL-15 to induce an active immune response against neuroblastoma

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Introduction: Tyrosine hydroxylase (TH) is highly expressed in neuroblastoma (NB) and therefore a promising target for active immunity approaches. Interleukin-15 (IL-15) represents an attractive adjuvant for DNA vaccination due to its ability to induce cytotoxic but not regulatory T cells. Here, we report generation and characterization of a new bicistronic DNA vaccine encoding for TH and IL-15 for active immunotherapy against NB.
Methods and Results: The bicistronic mammalian vector system pIRES allows simultaneous expression of two genes of interest from the same bicistronic mRNA transcript due to an internal ribosome entry site (IRES) flanked by two multiple cloning sites (MCS). To generate the new DNA vaccine, a previously described DNA sequence encoding for TH combined with upstream Ubiquitin (Ub) was cloned into the first MSC of pIRES using standard molecular biology techniques. Ub ensures proteasomal degradation of TH in antigen-presenting cells and its MHC class I presentation to cytotoxic T lymphocytes (CTL) followed by their activation. Synthesis and insertion of murine splenocyte IL-15 cDNA-sequence into the second MSC of pIRES allows secretion of the bioactive cytokine into the micro milieu stimulating TH-specific CTL activation. In order to prove cytokine secretion *in vitro*, an IL-15 specific ELISA was performed using supernatants of CHO cells transfected with the TH and IL-15 containing plasmid. Correct insertion of both gene sequences TH and IL-15 was verified by gene-specific PCR as well as restriction- and sequence analysis. To evaluate anti-tumour immunity *in vivo*, a syngenic NB mouse model will be used. Thereby, A/J mice will be vaccinated with the new vaccine by oral application of plasmid-bearing attenuated *Salmonella typhimurium* SL7207, which are known as an effective transport vehicle for oral immunization.
Conclusion: We generated and partly characterized a

new bicistronic DNA vaccine encoding for TH and IL-15 to induce a long lasting immunity against NB.

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Spinal canal invasion in peripheral neuroblastic tumors. Study design and preliminary results of a prospective SIOPEN Study Registry.

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Background. Spinal canal invasion (SCI) occurs in 10-15% of children with newly diagnosed peripheral neuroblastic tumors (PNTs). These subjects tend to have favorable clinical and biologic characteristics at onset (low stage, thoracic primary site, "normal" biologic features) and, consequently, have a better survival probability than the general PNT population. However, the question regarding the optimal treatment of this condition (in particular, if and when neurosurgery should be applied) has not been solved yet. This is a relevant issue, since a high rate of late neurologic and orthopedic sequelae has been reported in these patients, and the treatment modalities could influence their frequency and severity.
Methods. A prospective registry to collect clinical, therapeutic and follow-up data on symptomatic and asymptomatic patients with PNT and imaging evidence of SCI was set up by the SIOPEN NB-SCI Study Group. The type and severity of symptoms were to be reported according to *CTCAE (version 4.0)* and *ASIA scale*, except for pain (*FLACC scale*) for children younger than 36 months. A minimum of 150 patients is expected to be recruited.
Results. Patient enrolment started on July 2014. Up to January 2016, data regarding the clinical presentation, imaging studies, biology, treatment and related response, as well as neurological and orthopedic outcome, have been collected on 24 patients from 8 countries. In the 19/24 presently evaluable, 10 are infant and 9 are older children, the thoracic primary site prevails (10/19), 11 are symptomatic and 8 are asymptomatic, 12 have localized and 7 disseminated disease (six stage 4 and one stage 4s). Patient recruitment is expected to increase after clearance from Ethics Committees of other national groups.
Conclusion. We encourage all physicians who diagnose/treat patients with PNT and SCI to participate in this prospective international cooperative study.

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Creating Three-Dimensional full size model based on preoperative CT images for laparoscopic adrenalectomy and liver biopsy in a case demonstrating adrenal neuroblastoma with liver metastasis

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<Background>A 3-dimensional (3D) printing model based on preoperative computerized tomography (CT) images facilitates the visualization of complex structures, such as vessels and tumors, and it is also useful for understanding the anatomy of various organs preoperatively. We made a full size 3D model using a 3D printer for a case with adrenal neuroblastoma and liver metastasis. We compared the findings between the laparoscopic view of a 3D Model based on preoperative CT images and the real laparoscopic view during laparoscopic adrenalectomy and liver biopsy.
<Materials and Methods> Multi-detector CT images were transferred to a 3D workstation and then 3D volume data were obtained by reconstructing the sections. The adrenal tumor and tumor vessels, liver with multiple metastases, kidney, renal vein and artery, inferior vena cava, aorta and outer body were fabricated using different color resins to create a 3D printed model. Pneumoperitoneum, the insertion of trocars through the outer body and a laparoscopic view were all attainable using this model.
<Results> A 1-year-old girl was diagnosed with right adrenal neuroblastoma with multiple liver metastases. After administering neoadjuvant chemotherapy, both the primary tumor and liver metastases decreased. We thereafter performed laparoscopic adrenalectomy and a liver biopsy.
The laparoscopic view in the 3D model for the retroperitoneum organs, such as the adrenal tumor and tumor vessels closely matched the real laparoscopic view during surgery. However, regarding the metastatic liver mass, the laparoscopic view in the 3D model did not accurately match the real laparoscopic view during surgery. This difference was due to liver deformation caused by pneumoperitoneum pressure during surgery.
<Conclusion> 3D printing models based on preoperative CT images are useful for simulating laparoscopic surgery for adrenal and retroperitoneum neuroblastoma and is useful for understanding the patient's anatomy.

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131Iodine-metaiodobenzylguanidine (131I-MIBG) and autologous stem cell transplantation harvesting and hematological reconstitution in high-risk neuroblastoma patients.

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Aim: To evaluate feasibility of stem cell (SC) apheresis and hematological reconstitution after autologous stem cell transplantation (ASCT) in high-risk neuroblastoma (HR NBL) patients treated with upfront 131Iodine- metaiodobenzylguanidine (131I-MIBG) therapy.
Methods: In two prospective, multi-centre (AMC, Erasmus Medical Centre, University Medical Centre of Groningen, PMC) cohort studies (January 2005-2011 and 2011-October 2015), newly diagnosed HR NBL patients, 0-19 years, were treated with 2 courses of upfront 131I-MIBG therapy (fixed dose GBq (mCi): 1st 7.4 (200), 2nd 5.5 (150)), followed by HR GPOH NBL protocol. Harvest SC yield, number of sessions needed and time to neutrophil (0.5x109/L) and platelet reconstitution (>20x109/L) after ASCT were analysed (Kaplan Meier methodology).
Results: Eighty-two children were included (median age 3.2 years): thirty-eight (46.3%) treated with 131I-MIBG therapy, forty-four (53.7%) received only chemotherapy, because of poor clinical condition(n=27), MIBG non-avid tumors(n=11) and logistic failure(n=6). Median cumulative 131I-MIBG dose/kg was 0.76 gBQ (20.6 mCi). Median SC-apheresis yield (range): 5.4 x106/kg CD34+ cells (0.9-32.3) in 131I-MIBG+ patients(n=35) and 5.6 x106/kg (0.5-44.5) in chemotherapy-only patients(n=36). Median cumulative apheresis days: 2 days (range 1-8). One apheresis day was sufficient in 44.1% of 131I-MIBG+ and 62.2% of chemotherapy-only patients, two days in respectively 75.5% and 75.7%. Total number of apheresis sessions and -days were comparable between groups. Failure to harvest PB (peripheral blood) SC: 131I-MIBG therapy one patient and chemotherapy group two patients. A multivariate regression model for SC harvest yield showed, after adjusting for age/ gender/ MycN amplification/ LOH1p /Cisplatin dose, a significant association with bone marrow infiltration at diagnosis, (p=0.002). Median time to platelet reconstitution (95% CI) was 29 (11-47) and 15 days (12-18) (p=0.037) respectively for I-MIBG+ and chemotherapy-only group, neutrophil reconstitution (95% CI) respectively 11 (10-12) and 10 (9-11) days.
Conclusion: Stem cell harvesting is feasible after upfront 131I-MIBG in HR NBL patients with comparable neutrophil, but delayed platelets reconstitution compared with chemotherapy-only patients.

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BEACON-2: design of a SIOPEN/ITCC multi-arm multi-stage (MAMS) trial for relapsed neuroblastoma

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Background: Running clinical trials in relapsed neuroblastoma is challenging due to its rarity and involve a long process from concept to publication. Given the paucity of reliable evidence, there remains a need to identify optimal conventional therapies as well as evaluating novel agents quickly as they become available.
Design: Most randomised controlled trials (RCT) consist of two arms, while there have been many single arm studies in neuroblastoma. If several treatments are of interest, rather than just selecting one (possibly an ineffective one) for evaluation against the standard, a MAMS design permits several treatments to be assessed in a single trial; insufficiently active treatments in terms of intermediate outcome (e.g. response) are dropped after Phase II; recruitment continues in the remaining experimental arms for Phase III evaluation of event-free (EFS) and overall survival. Such adaptive designs are very efficient and, being randomised and comparative, avoid the biases of single-arm Phase II trials and historical controls.
Results: A prospective MAMS design is planned for the next RCT in relapsed neuroblastoma (BEACON-2). Once through Phase 1 dose-finding in children, agents given top priority by the Neuroblastoma New Drug Development Strategy project will be evaluated, with stratification by molecular profile where appropriate. Potential agents may target: ALK, BET, TORC, MDM2, MEK, CHK1, BIRC5, PD-1/PD-L1, CDK4/6. Optimal chemotherapy will continue to be identified. Ancillary biological studies will enable biomarker analyses. Preliminary design parameters and patient numbers per arm (80% power) are: Phase II, one-sided alpha=0.2, n=70 assuming baseline response rate 25% and 15% improvement; Phase III, two-sided alpha=0.05, n=130 assuming baseline 6-month EFS 50% and 17.5% difference.
Conclusion:

The adaptive design of BEACON-2 will allow multiple agents/regimens to be evaluated quickly and efficiently, with ineffective ones being dropped and new ones brought in as they become available, in a rolling long-term programme of research.

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The role of image defined risk factor (IDRF) in evaluation of the risk of post-surgical kidneys dysfunction in children with neuroblastoma

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The aim of the study was to define the role of presence of IDRF related to kidneys in evaluation of the risk of kidneys function disturbance after surgery in children with neuroblastoma.
From 2002–2015, 95 patients with neuroblastoma stage 2-4 were treated in the Department of Pediatric Oncology and Hematology, Institute of Pediatrics in Krakow; 71 with tumors in abdomen had tumors removed in the Department of Pediatric Surgery. In all patients with abdomen tumor, imaging performed before surgery was analyzed for the presence of IDRF. In 21 children (30%), IDRF defined as renal pedicle infiltration and/or infiltration of one or both kidneys were present at presurgical imaging. In this group, kidneys in post-operative imaging as well as GFR were evaluated and compared with the GFR results in children with other tumor localization or abdominal tumors without IDRF related to kidneys.
In 5/21 (24%) children the postoperative kidneys dysfunction was found. Ischemic lesions were found on control post-operative imaging, described as partial (n=3) or total (n=2) kidneys dysfunction. In 1 child it was necessary to remove the kidney after chemotherapy because of uncontrolled hypertension with the loss of kidney function. In this group, if kidneys dysfunction was described in post-operative imaging, GFR was lower than in children without any pathologies at this time. Present IDRF related to kidneys may predict the increased risk of increased GFR after surgery.
Conclusions: IDRF evaluation before surgery is helpful in predicting the risk and avoiding of post-surgical kidneys dysfunction, also in patients without obvious kidneys injury during surgery. It is especially important in high risk patient, in whom the kidney function has the crucial meaning for the course of further therapy.

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Targeting activating transcription factor 5 (ATF5) in neuroblastoma with a novel dominant negative inhibitor

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Background: Activating transcription factor 5 (ATF5), a member of the ATF/CREB family of transcription factors, is a potential target for neuroblastoma (NB) therapeutics. ATF5 mediates resistance to apoptotic NB cell death. ATF5 has significantly higher expression in Stage 4 MYCN-amplified tumors compared to Stage 1 tumors, and inversely correlates with survival. We utilized a recently developed novel drug CP-d/n-ATF5, a cell penetrating, dominant negative inhibitor that targets ATF5.
Methods: A panel of nine NB cell lines was treated with 50uM, 100uM and 200uM of CP-d/n-ATF5 and cell viability was assessed by WST-8 assay. To investigate the anti-tumor effects of CP-d/n-ATF5 in vivo, the human NB cell line, SK-N-Be2c, was implanted into the left kidney of nude mice to generate xenograft tumors. Tumors were then treated with CP-d/n-ATF5 or vehicle at Day 8, with a dose of 50mg/kg IP daily for 3 days, then twice per week. Tumor growth was monitored by bioluminescence. Mice were sacrificed when tumors reached a target flux. Kaplan-Meier survival analysis was performed. Ex vivo bioluminescence imaging was performed on liver, spleen, kidney to assess the metastatic burden. Bone marrow metastasis was evaluated by harvesting cells and measuring bioluminescence with a lumiometer. TUNEL immunostaining was performed to assess the apoptosis.
Results: CP-d/n-ATF5-S1 significantly decreased cell viability in vitro in a of panel nine NBL cell lines in a dose dependent manner. CP-d/n-ATF5 significantly prolonged survival of SK-N-Be2c tumor-bearing mice compared to vehicle treated mice (P=0.0013). Most notably, metastasis to bone marrow, liver, spleen, and kidney were all significantly suppressed by CP-d/nATF5 treatment. TUNEL staining showed a significant increase (P<0.05) of apoptotic cells in the tumors treated with drug.
Conclusion: Our data shows ATF5 is a novel target in NB and the drug CP-d/n-ATF5 is a potential anti-NB therapeutic agent in the clinic. Our data also shows CP-d/n-ATF5 exerts anti-tumor effects by inducing apoptosis and reducing organ metastasis.

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A research of the induction and differentiation therapy for neuroblastioma in children

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Purpose: To report the efficacy of low dose chemotherapy in the treatment for neuroblastoma (NB) in children. According to our laboratory research, using low dose chemotherapy is able to induce and differentiate the immature neuroblastic cells into mature cells. Materials and Methods: From 1986 to 2010, 155 NB patients enrolled into this study. All the 155 cases were treated by low dose chemotherapy and traditional Chinese medicine, and/or low dose chemo+surgery (mass only partially resected)+low dose chemo continuously+ second. surgery. Results: In the 155 NB cases, there were 8 cases who were treated by low dose chemotherapy and traditional Chinese medicine only, the tumor disappeared completely. In these 8 cases, five were Stage IVs, 2 were Stage IV and one was Stage III. There were 3 cases the treatment was low dose chemo+ surgery (only partial resection) after surgery low dose chemo continued, finally, a second surgery was performed, the mass was completely removed which was confirmed by pathology to be a GN. There were 2 cases the patients were treated by low dose chemo + traditional Chinese medicine only, the huge tumor rapidly decreased in size and the mass became nodular-like with calcifications which remained stable for 2 and 4 years respectively. In one of which the cranial metastases was also decreased obviously. Conclusion: According to our experience the low dose chemotherapy is efficient in the treatment of NB in children it can raise the survival rate of this malignant disease.

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Generation of new DNA- and protein vaccines for active immunotherapy against *MYCN*-expressing neuroblastoma

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Introduction: *MYCN* oncogene is overexpressed in high-risk neuroblastoma and associated with aggressive and refractory disease. Interleukin-21 (IL-21) is known to promote anti-tumor effects by stimulating cytotoxic but not regulatory T cells. Therefore, we developed protein and IL-21-based DNA vaccines with *MYCN* minigenes encoding for highly antigenic T-cell epitopes of MYCN. To increase proteasomal degradation and MHC class I presentation of MYCN epitopes to cytotoxic T lymphocytes, an upstream ubiquitin sequence was additionally integrated in the DNA vaccine. For evaluation of anti-NB effects *in vivo*, syngeneic mice will be immunized with protein vaccine in combination with the adjuvant aluminum hydroxide and DNA vaccine using attenuated *Salmonella typhimurium* SL7207 as vehicle.

Methods and results: For protein vaccine, a DNA sequence containing *MYCN* minigenes combined with an upstream leader sequence for protein secretion *in vitro* was synthesized and inserted into a plasmid encoding for human IgG1 constant heavy chain using standard molecular biology techniques. To enable permanent production of the fusion protein, CHO cells will be stably transfected with the generated plasmid. Finally, the fusion protein will be isolated from supernatant for *in vivo* vaccination.

For DNA vaccine, previously designed *MYCN* minigenes combined with ubiquitin sequence and IL-21 DNA fragment synthesized from murine spleen RNA were inserted into the respective multiple cloning site of the bicistronic expression vector pIRES allowing simultaneous expression of both inserts.

Correct plasmid assembly of both vaccines was confirmed by gene-specific PCR, restriction and sequence analysis. MYCN epitope expression and IL-21 production was approved *in vitro* by ELISA and Western blot after transfection of CHO cells with the respective plasmid.

Conclusion: We generated and partly characterized two new MYCN-based DNA and protein vaccines for active immunotherapy against MYCN-expressing neuroblastoma.

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Boolean modeling identifies Greatwall/*MASTL* as an important regulator in the AURKA network of neuroblastoma

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Aurora Kinase A (AURKA) is often overexpressed in neuroblastoma (NB) with poor outcome. The causes of AURKA overexpression in NB are unknown. Here, we describe a gene regulatory network consisting of core regulators of AURKA protein expression and activation during mitosis to identify potential causes. This network was transformed to a dynamic Boolean model. Simulated activation of the serine/threonine protein kinase Greatwall (GWL, encoded by *MASTL*) that attenuates the pivotal AURKA inhibitor PP2A, predicted stabilization of AURKA. Consistent with this notion, gene set enrichment analysis showed enrichment of mitotic spindle assembly genes and MYCN target genes in NB with high GWL/*MASTL* expression. In line with the prediction of GWL/*MASTL* enhancing AURKA, elevated expression of GWL/*MASTL* was associated with NB risk factors and poor survival of patients. These results establish Boolean network modeling of oncogenic pathways in NB as a useful means for guided discovering in this enigmatic cancer.

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Growth advantage and oncogene addiction of neuroblastoma cells bearing an ALK mutation

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Activating ALK mutations have been observed at clonal or sub-clonal levels in patients affected by neuroblastoma, either at diagnosis or at relapse. To further decipher the specific role of ALK mutation in disease aggressiveness and progression, we sought to compare the properties of isogenic cell lines bearing the ALK mutation at different levels.
First, we characterized two neuroblastoma cell lines derived from a stage 4 patient at diagnosis, either from the primary abdominal tumor (PT) or from the bone marrow (BM). SNP array analysis showed that both cell lines exhibited similar genomic profiles with 1p deletion, 17q gain and MYCN amplification. Both samples presented with 2 copies of the ALK locus. Interestingly, by deep sequencing and digital PCR, we measured an ALK mutation rate (F1174L) of 2.4% in the PT-derived cell line, which likely corresponds to 5% of the cells bearing an heterozygous mutation. In contrast, a mutation rate of 50% was observed for cells from the BM-derived cell line indicating that all cells exhibited the mutation. The ALK mutation rate in the PT-derived cell line increased with successive passages. *In vitro* growth analysis showed that the BM-derived cell line had higher proliferation rate compared to the PT-derived cell line. Orthotopic injection of both cell lines in Nude mice resulted in tumor formation only for the BM-derived cell line.
Second, we sought to knock-out the *ALK* gene in a neuroblastoma cell line bearing a R1275Q mutation. Among the clones that grew following CRISPR/Cas9 induced rearrangements in the first exon, we observed either in-frame rearrangements or knock-out of the wild-type ALK allele. No clone with an invalidation of the mutated allele could be obtained, suggesting an oncogene addiction of the cells to mutated ALK.
Altogether, our results suggest that the ALK mutation significantly contributes to disease aggressiveness and progression.

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Impact of Neuroblastoma recurrent mutations on embryonic sympatho-adrenal development

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Neuroblastoma (NB) is the most common solid extracranial cancer of childhood and accounts 15% of all pediatric cancer deaths. Little is known about NB cancerogenesis, whereas the pathogenesis has been enigmatic for a long time. Nowadays only few gene defects were identified and associated with this lethal tumor, such as MYCN amplification, ALK activation, ATRX and PHOX2b downregulation. It is widely assumed that primary tumors originate from sympatho-adrenal (SA) lineage development, indeed the majority of cancers arise in the adrenal medulla and in the sympathetic trunk.
The availability of valid preclinical in vivo models is a prerequisite to develop and study novel targeted therapies. Unfortunately the existing murine models, expressing MYCN or ALK oncogenes, resemble only to the MYCN-amplified group of human NB.
Our project aims to shed light on the impact of genetic modifications recurrent in NB along embryonic development of SA lineage. Thus, we want to express oncogenes and to silence tumor suppressors in neural crest stem cells (NCSCs) of murine models. To address our questions we are using Sox10-Cre mouse line, where Cre expression is leaded by Sox10 promoter, marker of NCSCs. Through recombination it is allowed population-specific transcription of proto-oncogenes or knocks out of tumor suppressors.
In our work, we are showing that independent expression of MYCN or ALK in Sox10 expressing cells gives embryo lethality *in vivo* ($P<0.001$; $P<0.001$) blocking neural crest differentiation and tissue maturation. Moreover, in order to explore the oncogenic mechanisms driven by others genetic mutations we are now assessing the impact of ATRX and PHOX2b depletion on NCSCs.

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Natural antibody against neuroblastoma of the TH-MYCN transgenic mice has CDC activity

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Background: The mechanism of spontaneous regression of neuroblastoma has been unclear. There are four major pathways that are reported to explain the spontaneous regression. That is: immune mediated cell killing (1), neurotrophin deprivation(2), loss of telomerase activity(3) and alterations in epigenetic regulation(4). However, there is no clinical evidence, and no animal models have been developed to investigate the involvement of immune systems, especially natural antibodies, against neuroblastoma.
Methods: We performed an immunological analysis of homo- and heterozygous TH-MYCN transgenic mice as a model of aggressive neuroblastoma. The tumor cells obtained from TH-MYCN mice were incubated with mouse plasma obtained from TH-MYCN mice and stained by anti-mouse IgG/ IgM, and the fluorescene was measured by flow cytometry. The tumors were detected by ultrasound.
Results: Approximately half of the heterozygous mice developed intraabdominal neuroblastoma and died within 20-week-age. On the other hand, microscopic findings of the superior mesenteric ganglion in 2 to 3-week-age mice revealed that the ganglion of all individuals contains solitary or multiple tumor foci, and then the frequency of tumorigenesis decreases, indicating the spontaneous regression might be occurred in half of this model. Mice with no or small (<5mm) tumors showed higher antibody titers in plasma than mice with large (>5mm) tumors. A significant negative correlation was observed between the tumor diameter and the titer of anti-tumor antibody. Surprisingly, wild type mice had similar amount of anti-tumor IgG/IgM. And, the plasma obtained from wild type mice has higher complement dependent cytotoxicity against neuroblastoma cells comparing to the plasma obtained from tumor developing mice.
Conclusion: We revealed that TH-MYCN transgenic mice have a functional antibody against neuroblastoma that influences the disease prognosis. The antibody strongly correlates to the spontaneous regression of neuroblastoma. TH-MYCN transgenic mice will be an important tool for elucidating the mechanism of spontaneous regression of neuroblastoma.

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MYCN Amplicon in Neuroblastoma (NB) Cell Lines

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Background: *MYCN* amplification is frequently found in high-risk NBs. Amplification is usually manifested cytogenetically by extrachromosomal double minutes (DMs) or by chromosomally integrated homogenously-staining regions (HSRs). *MYCN* is the only gene that is consistently amplified in NB tumors and cell lines. However, little is known about the structure of *MYCN* amplicons or the mechanisms that give rise to *MYCN* amplification. Here, we analyzed the NB lines Kelly, BE2C and LA-N-5 to determine the DNA sequence of amplicon junctions.
Methods: We performed SNP-Arrays on 27 NB cell lines and used this information to construct a map of amplicons. Real-time PCR was employed to identify the boundaries of each amplified region. Once the boundaries of each amplicon end were narrowed to <3 kb, PCR and DNA sequencing were performed to determine the exact sequences of amplication junctions to determine the orientation of single or adjacent amplicons (head-tail, head-head, tail-tail).
Results: All amplicons analyzed were composed of a simple repeat of genomic DNA fragments: Kelly (chr2:15,834,141-16,840,864 ~1Mbp-HSR), SK-N-BE2C (chr2:16,076,406-16,602,741 ~0.53Mbp/HSR), LA-N-5 (chr2:15,489,976-17,114,868 ~1.7Mbp/DMs), and a small fragment (241 bp) in the same orientation (head to tail). The *MYCN* gene (chr2:16,082,046-16,087,129) was included in all the amplicons studied. None of the amplicon junctions defined at the nucleotide level had either novel, repeat or rearranged sequences. However, we found imperfect hairpin structures around the amplicon junctions in two NB lines.
Conclusion: Our results suggest that the junctional sequences of *MYCN* amplicons are a perfect match to the genomic DNA sequence, with no gain, loss or rearrangement of sequences at the junctions. This indicates that there may be a different mechanism responsible for DNA amplification in human NBs other than the well accepted break-bridge repair model.

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Identification of CASZ1 nuclear export signal (NES) reveals potential mechanism for loss of CASZ1 tumor suppressor activity in neuroblastoma (NB)

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Although a number of chromosome 1p36 loss of heterozygosity (LOH) tumor suppressor genes have been identified in NB, the classic mechanism of mutational inactivation is rarely found to play a role in the loss of function of their remaining allele. How these are inactivated is important for understanding NB pathogenesis. Here we have identified cytosolic sequestration as a novel mechanism for loss of function for the 1p36 NB tumor suppressor CASZ1b in a subset of poor prognosis NB tumors. We performed an analysis of CASZ1b protein expression in a tissue array of 112 primary NB tumors. The results show that low nuclear CASZ1 expression and cytoplasmic-restricted CASZ1 expression is associated with MYCN amplification, unfavorable Shimada histology and high risk (all $p<0.05$). Remarkably, for the samples with CASZ1 restricted to the cytoplasm, 90% showed unfavorable Shimada histology. To understand potential mechanisms that contribute to CASZ1 cytosolic expression, we performed a structure and function study by generating a series of CASZ1b mutant constructs and assessing their localization in 293T cells or SK-N-AS NB cells. We identified CASZ1b N-terminus AA23-40 as a nuclear localization signal that mediates both nuclear localization and nucleosome remodeling and histone deacetylase (NuRD) complex interaction. Mutations of CASZ1b that result in cytosolic localization or lose its ability to bind NuRD complex abrogate transcriptional activity compared to wild-type CASZ1b ($p<0.01$). *In silico* bioinformatic analysis of CASZ1b identified a nuclear export signal (NES) at AA176-192. Structure and function mutational analyses showed that the predicted NES contributes to CASZ1 nuclear-cytoplasmic shuttling in an exportin 1 (XPO1/CRM1)-dependent manner. The finding that treatment of different types of cancer with exportin inhibitors such as KPT-330 results in nuclear accumulation of tumor suppressor genes and growth inhibition, suggests a therapeutic mechanism to restore CASZ1 tumor suppressor properties to a subset of poor prognosis NB tumors that have high cytosolic localization of CASZ1

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PPM1D/Wip1, the candidate gene on 17q contributing to neuroblastoma development

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Background: In neuroblastoma gain of 17q is the most powerful genetic predictor of adverse clinical outcome. 17q+ correlates with poor survival in our population-based material where we found aberrations of chromosome 17 in 85% of primary neuroblastomas, specifically, gain of PPM1D/Wip1 at 17q23. Wip1 is a serine/threonine phosphatase encoded by the gene *PPM1D*, described as a gatekeeper in the Mdm2-p53 regulatory loop involved in genetic stability, inflammation and a potential oncogene contributing to carcinogenesis.
Methods: Comparative genomic hybridization (CGH), immunostaining, mRNA arrays, qPCR, exome- and RNA-sequencing was used to examine PPM1D/Wip1 in neuroblastoma. Genetical and pharmacological inhibition was used to analyse the function of Wip1 in preclinical neuroblastoma models.
Results: CGH-array analysis detected PPM1D/Wip1 extra copies in all tumors and cell lines containing 17q-gain. Expression arrays and immunostaining showed high expression of Wip1 in neuroblastoma corresponding to poor survival. RNA-sequencing confirmed PPM1D-gain and revealed truncated isoforms with oncogenic potential. Exome-sequencing detected a mutation leading to constitutive PPM1D/Wip1 activation in an aggressive metastatic infant neuroblastoma. Wip1 knockdown experiments showed significant decrease of cell viability, proliferation and colony formation as well as substantial increase of DNA-damage response in neuroblastoma cells. Tumor xenograft development was significantly delayed showing median tumor development (0.10 mL) to be more than doubled (median 15 days, vs. 33 days, $p<0.001$) after Wip1 downregulation compared to scrambled controls. A novel Wip1 inhibitor was highly potent in cytotoxic/cytostatic effect in neuroblastoma cell lines (median IC50 0.8 mM). Furthermore, this Wip1 inhibitor significantly inhibited growth of established human neuroblastomas in nude mice after 12 days of treatment ($P<0.01$).
Conclusions: Our results show that PPM1D/Wip1 is oncogenic in neuroblastoma development activated due to chromosomal gain, alternative RNA-isoforms and/or DNA-mutation. PPM1D/Wip1 provides a novel therapeutic target in high-risk neuroblastoma.

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Modulation of immune responses and radioresistance by neuroblastoma-derived and host-derived TrkB-target Galectin-1

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Chemotherapy and external beam radiation constitute the backbone therapy of high-risk neuroblastoma, suggesting that analyses of response modulators could be useful. TrkB expression has been linked to chemoresistance before, but response to radiotherapy has been less well explored. We previously identified the multifunctional protein Galectin-1 (Gal-1) as a target of TrkB to promote angiogenesis and invasiveness. In order to elucidate the contribution of Gal-1 to tumour formation and tumour-host interaction, we crossbred TH-MYCN mice with Gal-1 knock-out mice. Gal-1 gene dosage did not significantly alter tumour incidence, but reduced Gal-1 gene dosage correlated with impaired tumour angiogenesis, splenomegaly and impaired T cell tumour infiltration.
Interestingly, Gal-1 deficient $CD4^+$ T cells presented with a lower migratory capacity towards tumour cells *in vitro*. As TrkB and Gal-1 have also been described as targets of ionizing radiation, we further explored their role in neuroblastoma cells *in vitro*. Upregulation of TrkB and Gal-1 was observed upon ionizing irradiation by qPCR and Western Blot analyses. However, colony formation assays of irradiated cells did not reveal altered cell survival of murine neuroblastoma cell lines when Gal-1 was down-regulated using Gal-1 specific shRNAs. The effect of abrogating Gal-1 and TrkB functions on the radiation response remains to be explored in vivo by using small molecule inhibitors and shRNAs, respectively. These results are expected to reveal the role for the TrkB/Gal-1 axis in response to radiation and to contribute to a better understanding of the complex tumour-host interaction during chemo- and radiotherapy of neuroblastoma.

patients based on KIF1Bb/DHX9/XAF1 biology.

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A non-canonical tumor suppression pathway identified in neuroblastoma – a new paradigm for personalized treatment and prognosis

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Background
Developmental apoptosis of neural crest precursors is crucial in determining the final number of terminally differentiated sympathetic neurons. An aberrant developmental apoptotic pathway is implicated in neuroblastoma. During development, when NGF becomes limiting, neuroblasts undergo apoptosis via *KIF1Bb*, a gene that resides on chr1p36.2 that is frequently deleted in neuroblastoma.
Methods
In order to understand the mechanism behind KIF1Bb-induced apoptosis in neuroblastoma, we employed various cell and molecular techniques, unbiased screens, next-generation sequencing, animal models and patient studies.
Results
We identified that KIF1Bb-induced apoptosis requires RNA/DNA helicase DHX9. KIF1Bb interacts with DHX9 to enhance translocation and accumulation of cytoplasmic DHX9 in the nucleus, resulting in expression of apoptotic XAF1. Transcription-incompetent DHX9 is unable to potentiate KIF1Bb-induced cell death. Knockdown of DHX9 also protects from KIF1Bb-induced cell death whereas KIF1Bb loss-of-function domains or patient-associated point mutants are unable to translocate and accumulate cytoplasmic DHX9 in the nucleus, impairing XAF1 expression. Furthermore, XAF1 silencing protects from KIF1Bb-induced and NGF withdrawal-dependent apoptosis *in vitro* and *ex vivo* as well as promoting tumor growth *in vivo* whereas XAF1 overexpression is necessary and sufficient to induce apoptosis *in vitro* and delays tumor growth *in vivo*. Conditional knockout of KIF1Bb in the superior cervical ganglia neuroblasts of mouse pups specifically ablates XAF1 expression *in vivo* and *ex vivo*, suggesting that KIF1Bb and XAF1 act along the same pathway. Importantly, analysis of tissue microarray data of neuroblastoma patients in conjunction with clinical correlates revealed that XAF1 expression might have prognostic value in determining survival outcomes for post-treatment and overall neuroblastoma cases.
Conclusion
Our findings provide a mechanistic understanding on neuroblastoma development that opens a window of opportunity for personalized therapeutic intervention and prognostication of 1p36-deleted neuroblastoma

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MYCN-induced miR-18a interferes with estrogen and NGF signaling to maintain an undifferentiated and more aggressive phenotype in neuroblastoma

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Neuroblastoma (NB) is a highly heterogenic childhood tumor, which arises from immature or precursor cells of the developing sympathetic nervous system. 20-30 % of all NB carry a *MYCN*-amplification, which is associated with an undifferentiated and more aggressive phenotype and a poor prognosis. MYCN, as the other members of the MYC network of transcription factors, is a crucial regulator of several important cellular processes, such as proliferation, apoptosis and differentiation.
Our group has previously identified microRNA miR-18a as key mediator of MYCN-inhibited estrogen receptor alpha (ERα) expression, which in turn results in reduced neuronal differentiation (Lovén et al., 2010). This study aims to investigate the effect of ectopic ERα expression on the morphology and functional response of NB cells with MYCN amplification. Here we show that overexpression of ERα was sufficient to induce a neuronal differentiation phenotype and to interfere with processes linked to tumorigenesis, including cell viability, migration and anchorage independent growth. Furthermore, overexpression and activation of ERα resulted in upregulation of the NGF (nerve growth factor) receptors TrKA and $p75^{NTR}$, and NGF treatment induced a more robust neuronal differentiation, indicating a cross talk between NGF and estrogen signaling. Interestingly, both NGF receptors are linked to a better prognosis and correlate negatively with *MYCN*-amplification.
In conclusion, we have identified a novel mechanism for how amplification of *MYCN* contributes to an undifferentiated and thus more aggressive phenotype in neuroblastoma. We demonstrate that MYCN-induced miR-18a inhibits expression of ERα and thereby interferes with estrogen and NGF signaling-mediated neuronal differentiation and promotes processes linked to tumorigenesis. Our findings highlight that restoration and activation of ERα by, e.g. inhibition of miR18a, may be an interesting starting-point for the development of new therapeutic strategies against neuroblastoma.

(Lovén et al., 2010) J. Lovén et al., Proc Natl Acad Sci U S A 107, 1553 (Jan 26, 2010).

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NPY/NPY5R copy number increases in relapsing neuroblastoma

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Neuropeptide Y (NPY) is a sympathetic neurotransmitter highly expressed in neuroblastoma (NB). NPY, acting via its Y5 receptor (Y5R), stimulates NB cell survival and chemoresistance, while NPY/NPY5R expression is elevated in NB cell lines and tissues derived from post-chemotherapy patients. However, the mechanisms underlying NPY/Y5R axis activation in these tumors remain unclear. The aim of this work was to investigate the copy number status of the *NPY* and *NPY5R* in NB, as well as other copy number alterations (CNAs) that may be present in chemoresistant tumors. 85 tissue samples, including specimens from the primary tumors, distant metastases and local relapses, before and after therapy, were collected from the Hospital Pequeno Principe, Parana, Brazil. CNAs for *NPY*, *NPY5R* and *MYCN* were investigated using TaqMan copy number assay. Additionally, wide genome array-CGH analysis was performed in 12 paired high-risk neuroblastoma cases at diagnosis and at relapse. Copy number for the *NPY* gene was significantly increased in the post-chemotherapy cases, when compared to the untreated cases (P=0.03), independently of the *MYCN* status. Among post-treatment samples, there was a significant increase in *NPY* gene copy number in distant metastases as compared to local relapse (P=0.03) in patients with non *MYCN*-amplified tumors. Similarly, significant increase in *NYP5R* gene copy number in post-chemotherapy cases, as compared to untreated cases, was observed only in subpopulation of patients without *MYCN* amplification. Array-CGH analyses demonstrated no increase in the total number of CNAs in NB relapses. Losses at chromosome 10 (10p15.3 and 10q11.2), which were present in 33% of the pre-treatment and absent in all post-treatment cases, were the only significantly different abnormality between these two groups of patients. Altogether, our data demonstrates CNA of the *NPY* and *NPY5R* genes in NB and implicate increase in their copy number as one of the potential mechanisms leading to activation of NPY/Y5R axis in chemoresistant tumors.

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MYCN-dependent regulation of gene expression during the cell cycle in neuroblastoma cells

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Background: Amplified *MYCN* is observed in about 20% of neuroblastoma (NB) patients and strongly predicts poor prognosis. The transcription factor MYCN targets an exceptionally large number of genes; in addition, indirect regulation occurs by microRNAs and epigenetic mechanisms. In *MYCN*-amplified neuroblastoma cell lines the knockdown of MYCN expression induces changes in the cell cycle (G1 arrest), apoptosis and differentiation. However, the details of this process are not yet well understood. We argue that regulatory events may occur in a cell cycle phase-dependent manner and are therefore not visible in a bulk population. In this study, we aimed to precisely characterize how MYCN influences expression of its target genes with the help of a time-resolved synchronisation model.
Methods: We used a *MYCN*-amplified cell line in which the level of MYCN can be regulated by an inducible shRNA vector (IMR5/75 *MYCN* shRNA). These cells were synchronised with a thymidine block and released into the cell cycle. RNA and miRNA expression was analysed at regular intervals during 22 hours.
Results: RNA sequencing revealed that the number of genes which were up- or downregulated due to MYCN was approximately equal. Clustering algorithms detected five different patterns of regulation, both cell cycle-dependent and independent. Interestingly, regarding expression of MYCN itself, we observed two peaks during G1 and S phase. In our miRNA expression analysis, we found 164 miRNAs to be differentially regulated. Among the most prominent results were several members of the mir-17-92 cluster, whose expression profile recapitulated the MYCN peaks described above. This cluster is known to have oncogenic potential by driving proliferation and angiogenesis while inhibiting differentiation.
Conclusion: This study for the first time describes MYCN-controlled gene regulation as cells pass through the cell cycle. We are currently aiming at completing this analysis with data on histone modifications and protein expression.

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The ABC transporter ABCE1 is a therapeutic target in neuroblastoma

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The MYCN transcription factor drives neuroblastoma progression by directly up-regulating downstream genes involved in a variety of metabolic processes including protein translation (1). One such gene, *ABCE1* (2), encodes a translation factor and a member of the ABC superfamily of transporters. In yeast, ABCE1 supports protein synthesis by catalysing the dissociation of the 80S ribosomes, leading to the re-initiation of the translation cycle (3). We have previously shown that high expression of ABCE1 is associated with reduced neuroblastoma patient survival (2); however, the biological role of ABCE1 in neuroblastoma is currently unknown. In order to determine how ABCE1 influences neuroblastoma cell biology, we undertook siRNA-mediated suppression of this gene, migration, invasion and colony formation assays, and polysomal profiling using the *MYCN*-amplified neuroblastoma cell lines, SK-N-BE(2) and CHP134. The results demonstrated that ABCE1 suppression severely impaired migration (SK-N-BE(2) P=0.0007; CHP134 P<0.0001) and the invasion of extracellular matrix by the neuroblastoma cells (SK-N-BE(2) P=0.0002), and significantly reduced the number of colonies (SK-N-BE(2) P=0.0007, CHP134 P<0.0001). At the molecular level, suppression of ABCE1 reduced the proportion of heavy polysomes (i.e. translating ribosomes), leading to impaired global protein synthesis. In addition, ABCE1 suppression was associated with reduced expression of vimentin, an intermediate filament protein involved in neuroblastoma cell migration (4). These results show that by enhancing global protein synthesis, ABCE1 supports survival and motility of *MYCN*-amplified neuroblastoma cells and highlights, together with recent evidence demonstrating the clinical potential of inhibiting mRNA translation in Myc-driven malignance (5), the importance of ABCE1 as a therapeutic target for this disease.

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The Poly (ADP-ribose) polymerase inhibitor olaparib causes mitotic catastrophe in MYCN amplified neuroblastoma by enhancing replication stress

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High-risk neuroblastomas with MYCN amplification (MNA) have a very poor outcome, making the search for new therapeutic approaches for these patients an absolute priority. Despite initial encouraging results obtained with PARP inhibitors in neuroblastoma preclinical models, the expression of PARPs and the biochemical consequences of their inhibition on the DNA damage response (DDR) were not characterized. Analysis of multiple datasets in R2 indicates that high PARP1/2 expression is significantly associated with more aggressive tumors and with poor survival in primary neuroblastomas, suggesting it is a potential and previously unrecognized prognostic factor. In vitro, inhibition of PARP activity via olaparib impaired neuroblastoma proliferation with slightly different effects between MNA and MYCN single copy (MNSC) cells. However, olaparib induced accumulation of DNA double strand breaks, a typical DDR including H2AX and p53 phosphorylation, and appearance of heavily disorganized and fragmented nuclei, suggestive of mitotic catastrophe, in a MYCN-dependent fashion. Time-lapse microscopy studies revealed that PARP inhibition elongates cell cycle and mitosis duration irrespective of MYCN expression, while an unsuccessful mitosis culminating in mitotic catastrophe only occurs in MNA cells. An higher rate of 53BP1 foci, micronuclei and anaphase bridges in olaparib-treated MYCN-overexpressing cells - compared to MNSC- indicated that mitotic catastrophe is most likely due to the attempt to undergo mitosis despite the presence of damaged or incompletely replicated DNA. In fact, despite a strong activation of the ATR-CHK1-CDC25A checkpoint, olaparib-treated MYCN-overexpressing cells transiently delay S-phase completion, but subsequently progress through mitosis and eventually undergo mitotic catastrophe. Consistently, CHK1 inhibition further accelerates progression in G2/M and strongly potentiates this phenotype, while CDK1 inhibition impedes mitotic entry and abrogates mitotic catastrophe.

These data emphasize the pivotal role of PARPs in controlling MYCN-dependent replication stress and support the introduction of PARP and CHK1 inhibitors in therapeutic approaches for neuroblastomas with high MYC activity.

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Alternative lengthening of telomeres in primary neuroblastoma specimens – a genomic, epigenomic & proteomic approach

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Background: In order to gain unlimited replicative potential, every cancer cell has to develop a mechanism to maintain its telomeres. Similar to the majority of all cancer cells, *MYCN*-amplified neuroblastoma (NB) tumors as well as *TERT*-translocated tumors accomplish telomere maintenance by telomerase activation. Interestingly, cancer cells can also elongate telomeres by telomerase-independent mechanisms referred to as alternative lengthening of telomeres (ALT), which is also found in a subgroup of high-risk NB patients.
Methods: ALT-positive patients are identified by screening a NB patient tumor cohort comprising about 800 tumors using the presence of C-Circles, which are a reliable marker to determine ALT activity. Subsequently, ALT tumors will undergo a comprehensive analysis using high coverage whole genome sequencing, whole proteome analysis and epigenetic analysis of DNA methylation and histone modifications.
Results: To date, 19 of 268 tested primary tumors were identified as ALT-positive. 90% of those were high-risk stage 4 tumors and all came from patients being older than 1.5 years. ALT activity was found to be mutually exclusive to amplified *MYCN*. Furthermore, 95% of the ALT tumors had the same genomic subtype harboring segmental and numerical aberrations without an amplified *MYCN* gene. Similar patterns of chromosomal aberrations, including a deletion in chromosome 11q and gains in chromosome 17q and chromosome 7, were observed in these patients.
Conclusion: The combined analysis of high coverage whole genome sequencing data, whole protein expression analysis and epigenetic profiling will enable a unique characterization of the ALT-positive neuroblastoma tumors. This study has the potential to deepen the knowledge about the general mechanisms of ALT activation and to reveal unknown vulnerabilities and potential therapy targets for this subgroup of high-risk NB patients as well as for ALT-positive tumors form other entities.

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Characterisation of neuroblastoma cells isolated from bone marrow aspirates of children with stage 4 disease at diagnosis: an NCRI CCL CSG Neuroblastoma Group Study.

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Purpose
Neuroblastoma (NB) cells in the bone marrow are a hallmark of high-risk disease, selecting children for more intensive treatment. These cells can contribute to disease progression and relapse, their elimination being one of the greatest challenges for cure of some children. We have therefore isolated and characterised these cells, to reveal pathways that might be exploited to develop more effective treatments targeting bone marrow disease.
Methods
NB cells were isolated from bone marrow aspirates (BM; n=52) from children with stage 4 disease using immune-magnetic bead selection for the cell surface disialoganglioside GD_2. Self-renewal was assessed by plating single cells in low and substrate adherent plates, and in soft agar. Migratory capacity was analysed using a 3D gelatin-based assay. Self-renewing cells were characterised using TaqMan® MicroRNA arrays, reverse transcriptase quantitative polymerase chain reaction (RTqPCR), immunocytology and microscopy.
Results
Infiltration of BM with GD_2 positive NB cells was 10% (range 0.02 – 43%). Spheroid forming efficiency from a single cell was 6% (range 0.5-27%) and colony formation efficiency in soft agar 3% (range 0-9%). Migratory cells were identified in all NB cultures; the migration index was highly heterogeneous (126; range 10-428). Cells were isolated that expressed tyrosine hydroxylase and nestin, but not NB84, CD57 or MRP4. Interestingly the ABC transporter protein MRP-1 was expressed in cells from 96% of cultures. Cell cultures could not be established from the GD_2 negative cells. Importantly clonal populations of self-renewing cells have been isolated, and propagated. HIF-1, PI3K-AKT and ErbB signalling pathways are highly expressed in self-renewing NB cells (n=38) isolated from the bone marrow, compared to published pathway profiles in primary tumour.
Conclusion
NB cells with heterogeneous self-renewing capacity

and a migratory phenotype have been successfully isolated and maintained in culture from BM aspirates taken at diagnosis from children with stage 4 disease. Signalling pathways have been identified which might in the future be exploited for the development of treatment targeting bone marrow disease.

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PI3K and MAPK pathways mediate the BDNF/TrkB-increased migration and invasion in Neuroblastoma cells

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Background: Brain-derived neurotrophic factor (BDNF) and its receptor TrkB have been reported to be associated with poor prognosis in NB patients. Our previous studies indicated that BDNF activation of TrkB induces chemo-resistance through activating phosphoinositide-3-kinase (PI3K)/Akt pathway. In the present study, we investigated the role of BDNF/TrkB on cell migration and invasion in NB.
Methods: A tetracycline-regulated TrkB-expressing Neuroblastoma cell line (TB3) was used. Scratch wound healing assay, migration and invasion assays were performed when TB3 cells were treated with BDNF or pretreated with the inhibitors for PI3K, MAPK, AKT, and mTOR before BDNF.
Results: TrkB expression was induced in TB3 cells in the absence of tetracycline (TET). Adding BDNF to TrkB-expressing TB3 cells increased the cell migration and invasion. Similar result was not observed in non-TrkB expressing cells in the presence of TET. Pretreatment of TB3 cells with LY294002 (inhibitor for PI3K pathway) or PD98059 (inhibitor for MAPK pathway) before BDNF administration blocked the BDNF/TrkB-induced increase of cell migration and invasion. Furthermore, we found that either AKT inhibitor perifosine or mTOR inhibitor rapamycin could also block the BDNF/TrkB effect on cell migration and invasion.
Conclusions: BDNF activation of TrkB increased NB cell migration and invasion via PI3K/AKT/mTOR pathway or MAPK pathway. Drugs targeting to these pathways or key molecules may be used as potential treatment in NB patients with metastasis.

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Inhibition of the growth factor midkine in neuroblastoma by an Okinawan agricultural product

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The growth factor midkine(MK) is overexpressed in various cancers, including neuroblastoma (Ikematsu et al,Brit J Cancer,2003;Ikematsu et al,Cancer Sci,2008). MK is involved in anti-apoptosis and cell growth. At Okinawa prefecture in Japan that is located in a subtropical oceanic climate zone, many agricultural products are paid attentions since they have various functions such as anti-oxidative activity, tyrosinase inhibition activity and antitumor activity. In this study, we screened Okinawa natural products for antitumor activity using MK as an indicator by enzyme-linked immunosorbent assay. We obtained 7 candidate materials with suppressive activity of MK expression in human neuroblastoma cells. Among the candidates, the suppression of MK expression of Jabuticaba(*Myricaria cauliflora*) leaf ethanol extract was the most remarkable. The Jabuticaba extract downregulated MK mRNA expression, reduced cell survival, and increased caspase 3/7 activity. Furthermore, the extract inhibited antigen-antibody reaction of MK-ELISA, suggesting that Jabuticaba extracts blocked MK action by masking MK. The results suggested that Jabuticaba leaf ethanol extract exerts apoptosis-inducing activity through suppression of MK expression and prevention of the binding between MK and its receptor(s) on cell surface.

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Neuroblastoma: telomere elongation is responsible for aggressive behavior

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Neuroblastoma (NB) is a malignant pediatric tumor of the sympathetic nervous system with a low mutation frequency.

We searched for new structural rearrangements in a series of 350 NBs of all stages using high density SNP microarrays. Exome sequencing was also performed on 15 tumor/constitutional DNA pairs and 25 additional tumors, mainly high-risk NBs, using paired-end sequencing on Illumina instrument.

Recurrent rearrangements as well as gain of *TERT* gene were observed in 15 of 350 neuroblastomas analyzed by SNP array. 12 out of these 15 tumors with *TERT* rearrangement belong to 11q-deletion subgroup of NB patients marking tumors with a poor prognosis. This remodeling of the genomic context abrogates transcriptional silencing of *TERT* in high-risk neuroblastoma and places telomerase activation in the centre of transformation. These rearrangements occurred in high-risk NBs in an almost mutually exclusive fashion with *MYCN* amplifications. Furthermore, by exome sequencing, we detect extensive structural rearrangements and amplification of chromosome 2, 5 and 7 leading to amplification of *MYCN, TERT and CDK6* respectively in one tumor.

Exome sequencing approach detected at average 14 somatic protein-changing alterations per tumor. Combining structural and mutational data shows recurrent alterations in *ATRX* gene in four non *MYCN*-amplified tumors: two focal deletions, one nonsense mutation and one deleterious missense mutation. *ATRX* inactivation results in alternative lengthening of telomeres (ALT). This means that high-risk neuroblastomas cancer cells can preserve their telomeres by ALT or by activation of telomerase reverse transcriptase (encoded by *TERT*). Alterations in *TERT*and *ATRX* were mutually exclusive, which is in agreement with the independent activation by these genes of telomere lengthening. Moreover three cases had chromothripsis of chromosome 5 with rearrangements affecting the *TERT*.

This study identifies recurrent *TERT and ATRX* rearrangements and telomere lengthening as an important mechanism characterizing high-risk tumors and supports the pharmacological inhibition of these targets.

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Stathmin mediates neuroblastoma metastasis in a tubulin-independent manner via RhoA/ROCK signalling and enhanced transendothelial migration

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Neuroblastoma patients frequently present with aggressive metastatic disease and the 5-year survival rates for these children remains poor. Metastasis involves remodelling of the microtubule and actin cytoskeletons. The microtubule destabilizing protein, stathmin, has recently been shown to mediate neuroblastoma metastasis1, and we sought to investigate how stathmin contributes to the metastatic process and potential mechanism(s) by which it exerts these effects. Stathmin suppression significantly reduced neuroblastoma cell invasion of embedded 3D tumour spheroids into an extracellular matrix. Furthermore, stathmin was shown to play an important role in transendothelial migration in two independent neuroblastoma cell lines in vitro. Stathmin's role in transendothelial migration in neuroblastoma cells was mediated by ROCK signalling, a key regulator of cell migration. Suppression of stathmin expression in neuroblastoma cells significantly increased the activation of the key upstream regulator of ROCK, RhoA small GTPase, suggesting that stathmin regulates this key cytoskeletal signalling pathway. To establish whether the effects on migration was tubulin dependent or independent we re-expressed either wild-type or a phospho-mimetic stathmin mutant (4E) that is defective in tubulin-binding in the stathmin shRNA-expressing cells. Notably, re-expression of either wild-type or a phospho-mimetic stathmin mutant restored cell migration and transendothelial migration back to control levels indicating that stathmin may regulate these processes independent of its role in tubulin binding. Furthermore, effects of stathmin suppression on in vivo transendothelial migration of neuroblastoma significantly reduced whole body, lung, kidney and liver metastases in a tail-vein delivery experimental metastases mouse model. In conclusion, stathmin plays a role in neuroblastoma via regulation of RhoA/ROCK signalling which in turn affects cell migration, invasion, transendothelial migration and consequently, metastasis. These findings highlight the importance of stathmin to the metastatic process and its potential as a therapeutic target for the treatment of neuroblastoma.

1 Byrne FA, *et al* Oncogene 2014; 33: 882-890.

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Combination of HDAC and mitochondrial-targeted metabolism inhibitors exhibits strong therapeutic synergy *in vitro* and *in vivo* against neuroblastoma

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Suberoylanilide hydroxamic acid (SAHA) is a histone deacetylase (HDAC) inhibitor that targets class I and class II HDACs, and has broad cytopathic effects on cancer cells. PENAO (4-(N-(S-penicillaminylacetyl) amino) phenylarsonous acid) is a tumour metabolism inhibitor that targets adenine nucleotide translocase of the inner-mitochondrial membrane. We have now shown that *in vitro* treatment of neuroblastoma cells with the combination of SAHA (1 μM) + PENAO (1.5 μM) synergistically decreased cell viability by up to 90% and induced intrinsic apoptosis by up 50-70%, with little effect on normal fibroblasts. Intrinsic apoptosis was confirmed by the induction of mitochondrial depolarisation. The combination of 20 mg/kg of PENAO and 17.5 mg/kg of SAHA was used to treat two different neuroblastoma tumour cell lines, as BALB/c nude mouse xenograft models. The combination prolonged survival from 9 to 19 days. Expression array analysis of neuroblastoma cells treated with SAHA + PENAO showed significant down-regulation of genes involved in cancer metabolism, oncogene transcription, cancer cell growth, and drug resistance. We confirmed that combination treatment of neuroblastoma cells repressed transcription and translation of the two down-regulated gene candidates, early growth response protein 1 (EGR1) and heme oxygenase 1 (HMOX1), by 5.4 and 4.5 fold, respectively. Further research is necessary to confirm the exact role of these candidate genes in the cytotoxic action of SAHA + PENAO. Since both SAHA and PENAO are currently in human cancer trials, our promising *in vitro* and *in vivo* data on the combination of SAHA + PENAO in neuroblastoma warrant further investigation of the combination for early phase trials in children with relapsed neuroblastoma.

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The involvement of Midkine, a growth factor exacerbating cisplatin-induced nephrotoxicity, in cisplatin resistance of neuroblastoma cells

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Midkine (MK) is a growth factor highly expressed in various cancers including neuroblastoma (NB). The plasma MK level is the reliable poor prognosis factor of NB. In addition, we reported that MK is involved in NB tumorigenesis in model mice, and also that it can be a potent therapeutic target. Here we have suggested that MK is involved in cisplatin resistance of NB cells. The cisplatin resistance of NB cells (TNB1, YT-nu, SH-SY5Y, SK-N-BE) were closely correlated with the expression levels of MK. In addition, because the knockdown of MK in TNB1 cells resulted in sensitization against cisplatin, it is suggested that MK is involved in cisplatin resistance of NB cells. In order to investigate the mechanism, we examined the expression level of cisplatin resistance-related genes including influx pumps, efflux pumps, neutralizing enzymes, DNA repairing enzymes and so on. As a result, some of them showed the correlation with MK expression. Their expressions were high in resistant TNB1 or YT-nu cells, and were decreased in response to knockdown of MK. These results suggest that MK would induce cisplatin resistance by regulating those genes. We previously reported that MK promotes cisplatin-induced nephrotoxicity by recruiting neutrophils, and that targeting MK can protect kidney from cisplatin. Taking this into consideration, the combination of cisplatin and MK targeting therapy could bring ideal effects: 1) cisplatin activity could be enhanced by the sensitization of NB cells, 2) nephrotoxicity could be prevented, 3) MK targeting could also exert antitumor effect independently of cisplatin.

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Identification of new synthetic lethal genes in *MYCN*-amplified neuroblastoma cells

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MYCN gene amplification clearly correlates with poor prognosis in patients with neuroblastoma and some types of cancer. Basically transcriptional factors are thought to be "un-druggable" targets, and therefore, alternative approaches are required to develop new therapeutics for this disease. Synthetic lethal (SL) approaches are emerging as a promising strategy for cancer therapy. For instance, poly ADP-ribose polymerase (PARP) inhibitor "Olaparib" has been approved for treating *BRCA*-deficient ovarian cancer in EU and USA. The double-stranded DNA breaks arising from the inhibition of PARP cannot be repaired in *BRCA*-deficient cells, which leads to the cancer cell-specific cell death. This approach is thought to be effective in *MYCN*-amplified neuroblastoma cells with elevated endogenous DNA damage caused by dysregulated gene expression. In order to identify SL genes in *MYCN*-amplified neuroblastoma cells, we performed a genome-wide shRNA library screening. The commercial library that consists of over 80,000 shRNA constructs targeting about 16,000 human genes was used. 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 is then quantified by next-generation sequencing (MiSeq; Illumina Inc.). shRNA sequences with a false discovery rate <0.1 from an exact test analysis in edgeR software were selected. In addition to already known synthetic lethal genes (e.g. *SMC2*, *CSNK1E*), about 130 genes were identified as new candidates. Based on our experimental validations using siRNA or chemical inhibitors, some mitotic kinases were proposed to be new SL genes in *MYCN*-amplified neuroblastoma cells. The molecular mechanisms underlying the lethality and its potential as a drug target will be discussed.

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Suppression of Multidrug resistance protein 4 inhibits neuroblastoma growth both *in vitro* and *in vivo*

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Introduction

Multidrug resistance protein 4 (MRP4), encoded by the ABCC4 gene, effluxes a broad range of cytotoxic agents and endogenous signalling molecules (eg cyclic nucleotides and eicosanoids) from cells, and has been proposed as an attractive therapeutic target in cancer. We have previously shown in primary untreated neuroblastoma tumours, that high ABCC4 expression is a powerful independent predictor of poor clinical outcome across multiple patient cohorts [1, 2]. We have also shown that ABCC4 is transcriptionally regulated by the Myc and MYCN oncogenes [1]. MYCN is amplified in approximately 20% of all primary neuroblastomas, and is an important driver of neuroblastoma tumorigenesis. We have now investigated the effect of inhibiting MRP4 on neuroblastoma growth both *in vitro* and *in vivo*.

Methods

Human BE(2)-C neuroblastoma cells with doxycycline-inducible expression of MRP4 were developed and characterised for growth and colony forming potential *in vitro*. Tumour growth was assessed following subcutaneous xenograft in Balb/c nude mice, with administration of doxycycline commencing one week after engraftment. Neuroblastoma-prone TH-MYCN mice were crossed with Abcc4 knock-out mice, and incidence and latency of tumour development studied.

Results

Induction of *MRP4* knockdown strongly inhibited BE(2)-C cell growth *in vitro* and significantly slowed the growth of xenografted neuroblastoma cells. In contrast, however, loss of murine Abcc4 in the TH-MYCN transgenic mouse model did not alter either tumour incidence or the growth rate of established tumours, suggesting differences between human and mouse MRP4 in this context. Knock-out allografts from these mice, when transplanted into Balb/c nude mouse recipients, demonstrated sensitivity to the MRP4 substrate, Irinotecan, compared to wild-type Abcc4 allografts.

Conclusions

Our results suggest that there are significant differences between human and murine MRP4 in their ability to transport physiological substrates. Importantly, loss of MRP4 strongly inhibited growth of human neuroblastoma cells in the absence of any chemotherapeutic drug treatment, highlighting its potential as a therapeutic target in this disease.

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MYCN mediates metabolic plasticity in childhood neuroblastoma

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Neuroblastoma, which arises from the developing sympathetic nervous system, is one of the most aggressive solid tumors of early childhood. Amplification of the *MYCN* oncogene is found in around 30% of NB patients and is associated with rapid tumor progression and poor prognosis. Our recent findings show 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 MYCN results in metabolic changes including mitochondrial dysfunction leading to accumulation of lipid droplets in NB cells. Similarly, treatment with the bromodomain inhibitor JQ1 leads to MYCN downregulation followed by lipid accumulation.
To investigate downstream effects of MYCN targeting we have performed quantitative proteomics of *MYCN*-amplified NB cells treated with 10058F4 or JQ1. For comparison, downregulation of MYCN expression using short hairpin RNA against MYCN followed by proteomic analysis was performed. We identified over 7000 proteins of which 6500 have been used for identification of novel pathways involved in NB pathogenesis. We found that primary metabolic processes including protein, lipid and nucleic acid metabolic processes were the most significantly affected activities upon MYCN downregulation. For analysis of the impact of MYCN expression on glycolysis and mitochondrial capacity we performed metabolic flux measurements using a Seahorse XF analyser. These results show that *MYCN*-amplified NB cells have a high metabolic potential and that they primarily use oxidative phosphorylation for their energy consumption. We also found that MYCN not only positively regulates the respiratory capacity but also significantly enhances glycolysis in NB cells.
Importantly, we demonstrate that MYCN positively regulates the ability of NB cells to oxidize exogenous fatty acids.
Taken together, our findings show that MYCN expression enhances the bioenergetic capabilities and that NB cells can shift their metabolic processes depending on the available nutrition. Importantly, MYCN regulates metabolic plasticity in NB cells, which contributes to their aggressiveness.

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GOLPH3 regulates Golgi shape and is activated by DNA damage in neuroblastoma cell lines

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Background: Golgi phosphoprotein 3 (GOLPH3) has been reported to be involved in the development of several human cancers. The over-expression of GOLPH3 promotes cell transformation by enhancing the activity of the serine/threonine kinase mTOR. The clinical significance and biological role of GOLPH3 in neuroblastoma (NB) remains unclear. GOLPH3 is a membrane protein that binds to Myosin-XVIIIA (MYO18A) protein linking the Golgi to the actin cytoskeleton. The GOLPH3 pathway is essential for vesicular trafficking from the Golgi to the plasma membrane. A side effect of GOLPH3-dependent trafficking is to generate the extended ribbon shape of the Golgi. Perturbation of the pathway results in changes to both Golgi morphology and secretion, with functional consequences for the cell. Golgi dispersal is a common feature of DNA damage response in mammalian cells. Understanding the cellular response to DNA damage is crucial for discerning the mechanisms by which many chemotherapeutic agents kill tumor cells and the mechanisms of escape from killing.
Methods: We examined whether DNA damage affects Golgi morphology, and the role of GOLPH3 in maintaining Golgi shape in NB cells. We induced DNA damage in the human NB cell lines ACN and SH-SY-5Y with curcumin (10 μM). To investigate the relationship between GOLPH3 expression and DNA damage after treatment with curcumin, GOLPH3 and γH2AX expression were examined by immunofluorescence staining.
Results: Exposure of NB cells to curcumin induced: i) up-regulation of GOLPH3$^+$ cells (80.4±3.22% *vs* 30.2± 6.18%, p= 0.0001); ii) augmentation of double-strand breaks (γH2AX$^+$ cells 60.9±3.22% *vs* 25.7± 6.18%, p= 0.0001); iii) fragmentation and dispersal of the Golgi throughout the cytoplasm.
Conclusion: Our findings suggest that GOLPH3 expression levels may represent a clinical marker of NB patient responsiveness to DNA-damaging cancer therapies. Furthermore, novel agents able to interfere with the GOLPH3 pathway may have therapeutic benefit when used in combination with standard DNA-damaging therapeutic agents in NB.

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Patterns of PD-1, PD-L1 and PD-L2 Expression in Neuroblastoma

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Background: Significant anti-tumor effects have been observed in a variety of malignancies via blockade of immune checkpoints, leading to T-cell-mediated killing of cancer cells. Interaction of PD-1 with its ligands PD-L1 and PD-L2 serves to suppress T-cell function and restrict immune-mediated tumor killing. We sought to examine the pattern of expression of these proteins in children with neuroblastoma, as expression of these proteins may serve as biomarkers of response.
Methods: Sections cut from formalin fixed paraffin embedded (FFPE) tissue blocks were processed and evaluated for PD-1, PD-L1, and PD-L2 by immunohistochemistry (IHC) as well as by mRNA expression. A semi-quantitative 0-5 IHC scoring system (0= negative, 1 = rare, 2 = low, 3 = moderate, 4 = high, 5 = very high) was applied, with scores incorporating combined prevalence of tumor cell and non-tumor cell labeling. Expression profiling was performed using the NanoString nCounter™ system according to manufacturers' recommendations. Data analysis was performed using quantile normalization in which relative ranks of genes (across all genes on the Nanostring codeset) within each sample were replaced by values having the same relative rank from the pooled distribution (from all samples and genes in the dataset). All quantile normalized data underwent subsequent $\log_{10}$ transformation.
Results: 31 FFPE blocks were included in the analysis. PD-1 and PD-L1 IHC were evaluable in all samples. 6/31 samples were not evaluable for PD-L2 IHC. PD-1, PD-L1 and PD-L2 expression was negative to moderate by both IHC (range 0-3) and mRNA expression (range 0-2.19). Correlation between IHC score and mRNA expression was poor for all three tested proteins (PD-1 r^2 = 0.07, PD-L1 r^2 = 0.1 and PD-L2 r^2 = 0.1).
Conclusions: Expression of PD-1, PD-L1 and PD-L2 is low in neuroblastoma. At low levels of expression, IHC score and mRNA expression correlate poorly.

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Targeting fatty acid synthesis to induce neuroblastoma differentiation

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MYCN amplification is found in 25% of neuroblastoma patients and it is consistently associated with treatment failure and poor prognosis. Cancer cells undergo metabolic reprogramming that allows them to maintain a high rate of macromolecular synthesis, essential for cell growth and division. Most of the studies in the field of cancer metabolism are focused on aerobic glycolysis (the "Warburg effect") and a high dependence on glutamine. However, other important pathways such as lipid metabolism have been less extensively addressed. c-MYC has been strongly related to tumor metabolism as a potent activator of both glycolysis and glutaminolysis, but not much has been described about the role of MYCN in these processes. Our group performs pioneering research in the area of neuroblastoma metabolism. We previously demonstrated that MYCN downregulation not only triggers neural differentiation but also accumulation of neutral lipids (Zirath *et al* PNAS, 2013). Silencing of *MYCN* or targeting the protein with small molecule inhibitors prompt changes in lipid metabolism due to reduced ß-oxidation and mitochondrial dysfunction. The lipid accumulation is accompanied by morphological changes and expression of neural differentiation markers. Now, we further investigated the relationship between lipid metabolism and neuroblastoma cell differentiation. The chemical inhibition of *de novo* fatty acid synthesis (targeting either acetyl-CoA carboxylase, ACC, or fatty acid synthese, FASN) triggers MYCN protein downregulation and differentiation of both MYCN-amplified and non-amplified neuroblastoma cells. Importantly, the inhibition of ACC, but not of FASN, impairs accumulation of lipid droplets and downregulates mitochondrial respiration depending on MYCN protein levels. Cell membrane-permeable fatty acids do not prevent the phenotypes observed, suggesting that they are mediated by means other than fatty acid depletion. Our work aims to elucidate the impact of lipid metabolism on neuroblastona biology, with potential implications for metabolism-targeted and differentiation-based therapies.

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Blinding the CYCLOPS – Neuroblastoma vulnerabilities unveiled by genomic loss

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Background:
Heterozygous deletions within distal 1p are observed in 30% of neuroblastomas. So far, several potential 1p tumor suppressor genes have been identified. However, in this study we are focussing on 1p genes whose inactivation is not necessarily linked to tumor development but which mediate cell-essential functions, rendering cells with copy number loss vulnerable to further impairment. These genes are candidate therapeutic targets according to the concept of CYCLOPS (**c**opy number alterations **y**ielding **c**ancer **l**iabilities **o**wing to **p**artial **l**oss).
Methods:
To identify a subset of 1p genes for which heterozygous loss may be tolerated but further reduction leads to cell death, we are currently performing siRNA screens mediating the systematic knock-down of distal 1p genes in five 1p-deleted versus five non-1p-deleted neuroblastoma cell lines. Forward liquid transfections are done using three independent siRNAs per gene. Hoechst stained cell nuclei are counted 96h post transfection. Target gene validation is done by viability assays and cell cycle analysis.
Results:
In an initial screen in one neuroblastoma cell line, we identified a potential CYCLOPS candidate gene. Knock-down of the gene impaired cell viability in 1p-deleted cell lines but did not in 1p-non-deleted cells. G_1/G_0 phase arrest with corresponding S phase decrease was observed in both 1p-deleted and 1p-non-deleted cells. Additionally, neurite-like outgrowth could be observed in 1p-non-deleted cells indicating an induction of differentiation.
Conclusion:
This study identified a candidate CYCLOPS gene in neuroblastoma. Heterozygous deletions of chromosome arm 1p are also frequently observed in other cancers including melanoma, colorectal and breast cancer. We hypothesize that this proof-of-principle opens a new therapeutic window for tumors harbouring a heterozygous deletion of our candidate gene or other cell essential genes on chromosome arm 1p.

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Elevated expression of dyskerin is a potential therapeutic target with a telomerase-independent role in Myc/N-Myc-driven neuroblastoma

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In addition to its role in telomere maintenance, the ribonuclear protein dyskerin is also involved in RNA processing and ribosome biogenesis, anabolic processes that are often upregulated in myc-driven cancers. Since a third of neuroblastomas show augmented N-myc or c-myc activity, we hypothesized dyskerin expression would correlate with myc activity in these neuroblastoma subtypes.
This study shows that dyskerin expression is elevated in neuroblastoma cell lines (n=15) compared to normal cells ($P<0.01$). We showed that the *DKC1* gene is a target of both N-myc and c-myc and that the upregulation of N-myc correlates with increased *DKC1* expression. Furthermore, high *DKC1* expression correlated strongly with poor event-free- and overall-survival ($P<0.0001$) in two independent cohorts of neuroblastoma patients (n=477 and n=197, respectively).
RNAi knockdown of *DKC1* in neuroblastoma cells inhibited proliferation *in vitro* as well as tumor growth *in vivo* ($P<0.01$). This inhibitory effect was independent of telomerase suppression since over-expression of hTR (telomerase RNA component) did not rescue the phenotype.
As dyskerin is a nucleolar protein, we hypothesized that dyskerin depletion would induce nucleolar stress resulting in cell cycle arrest. Consistent with this, dyskerin knockdown resulted the depletion of small nucleolar RNAs as well as the nuclear accumulation of ribosomal proteins, the induction of p53 and cell cycle arrest in the G1 phase. p53 mutant or silenced cells also showed cell-cycle arrest on dyskerin knockdown indicating that the contribution of p53 was not essential for proliferative arrest.
Collectively, these data suggest dyskerin may be a potential therapeutic target for myc-dependent neuroblastoma.

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Epigenetic siRNA and chemosensitivity screens identify a vulnerability to SETD8 inhibition through reactivation of p53 canonical pathway in Neuroblastoma

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High-risk Neuroblastoma(NB) is considered a failure of sympathoadrenal differentiation and there are a paucity of druggable mutations. To uncover epigenetic regulators critical for survival of undifferentiated high-risk NBs, we undertook a chromatin-focused siRNA screen. Of the 400 genes analyzed, high-content Opera imaging identified 53 genes whose loss of expression led to significant decreases in NB proliferation with 16 also inducing differentiation. We further screened 21 epigenetic compounds in 8 NB cell lines to prioritize siRNA hits that are in the drug developmental pipeline. Integration of genetic and chemical screen data revealed SETD8 as an important and druggable NB target. SETD8 is the H4K20me1 methyltransferase regulating DNA replication, chromosome condensation and gene expression. Analysis of 3 different datasets in R2 showed high expression of SETD8 is associated with poor prognosis in NB(ex. Kocak;p=1.4e-07) as well as in Stage-4-NB-wildtype-MYCN(p=0.03) but not in Stage-4-NB-amplified-MYCN(p=0.96). To determine mechanisms of SETD8-mediated growth inhibition, we performed RNA-seq transcriptome analyses on NB cells after genetic or pharmacological inhibition of SETD8. These revealed that SETD8 ablation rescued p53 proapoptotic and cell-cycle arrest functions by reactivation of p53 canonical pathway(IPA). Functional studies indicate SETD8 methylates p53(K382) leading to its inactivation. Moreover the levels of p53K382me1 are higher in wildtype-MYCN-NB cell lines compared to amplified-MYCN cells. Tumor progression is marked by inactivation of p53 and multiple mechanisms have been identified in amplified-MYCN NB cells. For the first time, this study reveals that inhibition of SETD8 is a novel mechanism to reactivate p53 particularly in high-risk wildtype-MYCN NBs which account for some 60-70% of high-risk NB tumors. Our *in vivo* xenograft NB models, showed that genetic or pharmacologic(UNC0379) inhibition of SETD8 confers a significant survival advantage. This identifies that SETD8 is a novel therapeutic target and its inhibition may be especially relevant for the subset of high-risk NB tumors with wildtype-MYCN.

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The expression of AT Rich Interactive Domain 1A (ARID1A) in Neuroblastoma

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Purpose: The AT Rich Interactive Domain 1A (ARID1A) has been implicated in diverse cancers as having either gain or loss activities. The present study was undertaken to determine the expression and effects of ARID1A expression in Neuroblastomas (NBs) and to assess the clinico-pathological association by which ARID1A predict the outcome in these NBs.
Experimental Design: The expression of ARID1A was studied NBs obtained from the 54 primary biopsies by qRT-PCR, Western-blot and immunohistochemistry. The NB features based on clinicopathological data, gene expression data, and a combination of the two were produced and used to predict outcome.
Results: Of 54 NBs, 10 (18.5%) did not express ARID1A, 32 (60.1%) expressed low basal ARID1A and 12 (22.2%) expressed 2-fold ARID1A as compared with human dorsal root ganglia. Remarkably, the low ARID1A expression was associated with higher INSS stages (III and IV vs I and II) and MYCN amplification. The loss of ARID1A was significantly associated with 1p loss (P=0.007) and MYCN amplification (P=0.003), while high ARID1A expression was significantly associated with differentiated histology and showed 5-year survival rate of NB patients.
Conclusions: The ARID1A expression changes can be used to refine traditional outcome prediction, providing a rational approach for tailoring treatments to subsets of NB patients.

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Integrative approach to define the cell surface landscape in neuroblastoma

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Background. The cell surface landscape of primary and relapsed neuroblastoma (NB) is currently unknown. An unbiased survey of these proteins and their isoforms would greatly facilitate the identification of candidate immunotherapeutic targets for preclinical validation.

Methods. We are performing plasma membrane protein extraction utilizing an optimized sucrose density gradient methodology followed by nano-liquid chromatography coupled to mass spectrometry (nLC-MS/MS) to identify proteins on the cell surface of 10 NB cells lines and 10 primary/relapse tumor pairs. In addition, we are developing an analysis program in R optimized to take as input the output table from MaxQuant, a software tool for database searching and quantification commonly used in proteomics. Proteomic data will be integrated with RNA-sequencing data to assess the correlation between these data types, determine differential expression between NB (n=2242) and normal tissues (n=1641), and investigate NB-specific isoforms.

Results. To date, we have optimized our method on the NB cell line IMR5. This methodology has yielded a 66% membrane protein enrichment with high reproducibility between biological replicates. This has allowed us to confirm known cell surface proteins that are currently being developed as immunotherapeutic targets (GPC2 and NCAM1). We have also identified other cell surface proteins that are being further evaluated in additional NB cell lines and tumors. We have created an efficient pipeline for data analysis and visualization. After normalization, our program outputs 27 plots and figure legends that assess data quality, regulated or abundant proteins, and Gene Ontology or motif enrichments.

Conclusion: We have developed a software package and robust methodology for cell surface protein isolation and quantification. Parallel proteomic and transcriptomic studies in additional NB cell lines and patient tumors are ongoing to define the cell surface landscape in primary and relapsed NB.

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Calreticulin-dependent VEGF expression promotes neuroblastoma differentiation

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Neuroblastoma (NB) is a childhood cancer with a low survival rate and great metastatic potential. Calreticulin (CRT), an endoplasmic reticulum chaperone protein, has been identified as one of the prognosis factor for NB patients. Our previous study demonstrated that CRT promotes neuronal differentiation through the upregulation of VEGF-A in NB cell lines. Inhibition of VEGFR-1 signaling by blocking antibodies significantly suppressed the expressions of neuronal markers. These results suggested that VEGF-VEGFR signaling may play important roles in the differentiation of NB cells. In this study, we further confirmed our hypothesis through xenograft model and clinical analysis from NB patients. In xenograft experiments, CRT inducible stNB-V1 cells were injected subcutaneously. Doxycycline treated in the drinking water effectively inhibited the tumor growth. In addition, the mRNA and protein levels of VEGF-A and differentiation marker GAP-43 were upregulated by induced CRT expression. However, no significant correlation between the expression levels of VEGF-A and CD31, a marker of endothelial cells, was observed which suggested a novel mechanism of VEGF-A participating in NB tumorigenesis through an angiogenesis-independent pathway. From NB patients samples, the mRNA expression levels of CRT and VEGF-A were positive correlated. Furthermore, positive VEGF-A expression by immunostaining of NB tumors was found to correlate well with histological grade of differentiation and predicted a favorable prognosis. In conclusion, our findings suggested that CRT may promote neuronal differentiation of neuroblastoma through regulating VEGF-A expression. However, the underlying mechanisms still need further investigation.

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MYCN promotes neuroblastoma malignancy by establishing a regulatory circuit with transcription factor AP4

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Amplification of the *MYCN* oncogene, a member of the MYC family of transcriptional regulators, is one of the most powerful prognostic markers identified for poor outcome in neuroblastoma, the most common extracranial solid cancer in childhood. While MYCN has been established as a key driver of malignancy in neuroblastoma, the underlying molecular mechanisms are poorly understood. Transcription factor activating enhancer binding protein-4 (TFAP4) has been reported to be a direct transcriptional target of MYC. We show for the first time that high expression of *TFAP4* in primary neuroblastoma patients is associated with poor clinical outcome. siRNA-mediated suppression of *TFAP4* in MYCN-expressing neuroblastoma cells impaired migration and colony formation, and led to cell cycle arrest in G1/S phase. Chromatin immunoprecipitation assay demonstrated that *TFAP4* expression is positively regulated by MYCN. Furthermore, when *MYCN* was overexpressed in neuroblastoma cells, TFAP4 was required for the observed increase in cell migration. Microarray analysis identified genes regulated by both MYCN and TFAP4 in neuroblastoma cells, including Phosphoribosyl-pyrophosphate synthetase-2 (PRPS2) and Syndecan-1 (SDC1), which are involved in cancer cell proliferation and metastasis. Overall this study unveils a regulatory circuit in which MYCN by elevating TFAP4 expression, cooperates with it to control a specific set of genes involved in tumor progression. These findings highlight the existence of a MYCN-TFAP4 axis in MYCN-driven neuroblastoma as well as identifying potential therapeutic targets for aggressive forms of this disease.

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Human stem cell models for relapse neuroblastoma

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High-risk neuroblastoma is enigmatic among aggressive tumors. Initially treatment-sensitive, these tumors inevitably acquire therapy-resistance. Relapse neuroblastoma demonstrates frequent activation of mitogen activated protein kinase (MAPK) signaling[1]. Relapse tumors mutate *NF1*, a tumor suppressor also frequently silenced epigenetically in neuroblastoma[1,2]. NF1 is a GTPase activating protein that negatively regulates the GTPase RAS. Loss of NF1 activates MAPK in hematopoietic tumors[3], and phosphatidylinositol 3' kinase (PI3K) in malignant neural sheath tumors[4,5]. How NF1 loss affects signaling in relapse neuroblastoma, and how to best treat these patients, remains poorly understood. To model epigenetic silencing of NF1 in neuroblastoma, we used CRISPR interference (CRISPRi), blocking transcription of NF1 reversibly in human induced pluripotent stem cells (iPSC). Human iPSC were then differentiated to trunk neural crest cells (NCC), presumed cells of origin for neuroblastoma[6]. We combined NF1 CRISPRi with misexpression of *MYCN*. Transduced NCC were subsequently introduced orthotopically into mice, to generate MYCN-driven, NF1 silenced tumors. Characterization of these tumors promises to clarify signaling pathways activated in response to silencing of NF1, to determine how silencing of NF1, when combined with misexpression of MYCN, can inform treatment of relapse, high-risk, therapy resistant neuroblastoma *in vivo*. We demonstrate that NF1 CRISPRi leads to decreased levels of NF1 protein. We are currently combining CRISPi and mis-expression of NF1, to generate a "human in mouse" model of recurrent disease. Human-in-mouse models for *MYCN* amplified, NF1 silenced neuroblastoma promise to inform biology and therapy for high-risk relapse neuroblastoma.

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