Molecular and genetic studies in high-risk neuroblastoma

Ángela Martínez-Monleón

Department of Laboratory Medicine

Institute of Biomedicine

Sahlgrenska Academy, University of Gothenburg

UNIVERSITY OF GOTHENBURG

Gothenburg 2021

Cover illustration: “Neuroblastoma, a pediatric disease” (Tumoral genome with genetic alterations is represented in the background of the image).
By Ángela Martínez-Monleón

Molecular and genetic studies in high-risk neuroblastoma

angela.martinez-monleon@gu.se

ISBN 978-91-8009-196-1 (PRINT)
ISBN 978-91-8009-197-8 (PDF)
http://hdl.handle.net/2077/67122

Printed in Borås, Sweden 2021 by Stema Specialtryck AB

To my wonderful family

But science and everyday life cannot and should not be separated.

Rosalind Franklin

Molecular and genetic studies in high-risk neuroblastoma

Ángela Martínez-Monleón

Department of Laboratory Medicine, Institute of Biomedicine, Sahlgrenska Academy at University of Gothenburg, Sweden, 2021.

Abstract

Neuroblastoma is the most common and deadly cancer in the first year of life. Children with high-risk neuroblastoma have a very poor prognosis, despite heavy multimodal treatment, with less than 50% of 5 years of overall survival. Unfortunately, between 50-60% of high-risk neuroblastoma patients will eventually suffer a relapse with a survival rate of less than 10%. For this reason, a better understanding of the interplay between genetic abnormalities within the nervous system context is necessary to improve patient stratification or aid therapeutic strategies that can ultimately lead to increased patient survival.

In order to find molecular profiles that predispose to development of high-risk neuroblastoma or contribute to the relapse, metastatic or non-responsive status of the tumor, we performed comprehensive molecular characterization of neuroblastoma tumors and cell lines by SNP-microarrays and next generation sequencing techniques in combination with functional exploration of novel recurrent somatic aberrations.

Through our large-scale studies, we confirmed the genetic stability of neuroblastoma PDOXs over serial passaging, explored intra-tumoral heterogeneity of neuroblastoma and monitored a set of primary/relapsed neuroblastoma tumors, highlighting the recurrence of alterations of MAPK signaling, cell cycle progression and telomere activity pathways. Furthermore, we detected and investigated a recurrent structural alteration in *LSAMP* which appears to be a tumor suppressor gene in neuroblastoma. We also characterized a highly aggressive subgroup of neuroblastoma tumors, which presented a high-grade amplification of two loci at 12q, and our *in vitro* results indicated possible tumor inhibition routes through CDK4 and MDM2 inhibition.

To conclude, genome-wide analyses with powerful techniques, such as next generation sequencing, are useful not only for research purposes but also as a clinical tool.

Keywords: Cancer, neural crest, neuroblastoma, heterogeneity, relapse, sequencing, CNVs, SVs, SNVs, *LSAMP*, *CDK4*, *MDM2*

Sammanfattning på Svenska

Ángela Martínez-Monleón. Avdelningen för laboratoriemedicin, Institutionen för biomedicin, Sahlgrenska akademin vid Göteborgs universitet, Sverige, 2021.

Neuroblastom (NB) är en cancerform där tumörer uppstår i det sympatiska nervsystemet och som orsakar 12–15% av alla cancerrelaterade dödsfall hos barn. Barn med högriskformen av neuroblastom (HR-NB) har en mycket dålig prognos som trots kraftig multimodal behandling, har lägre än 50% chans till överlevnad 5 år efter diagnos. Tyvärr är återfall hos patienter med HR-NB vanligt och för dessa är överlevnaden lägre än 10 %. Därför är det viktigt med en ökad kunskap kring underliggande genetiska avvikelser och hur dessa samspelar med utvecklingen av nervsystemet för att förstå hur NB uppkommer. Förhoppningen är att detta ska hjälpa till att urskilja vilka behandlingsstrategier som är lämpliga för en specifik patient eller identifiera nya behandlingsmål som i slutändan kan leda till ökad patientöverlevnad.

För att identifiera genetiska förändringar som leder till ökad risk för utveckling av HR-NB eller återfall, metastasering eller behandlingsresistens så har vi i denna avhandling använt oss av storskaliga metoder som SNP-microarrays och den nya tekniken för DNA-sekvensering tillsammans med funktionella studier av återkommande tumörspecifika förändringar.

Genom dessa studier har vi kunnat bekräfta att arvsmassan hos så kallade PDOX-möss (musmodeller som bär mänskliga NB-tumörer) är stabila med liten genetisk heterogenitet inom PDOX-tumörer. PDOX-möss visar också stabilitet över tid med mycket liten tillkomst av ytterligare mutationer. Den nya tekniken för DNA-sekvensering har också använts för att studera genetiska förändringar som uppstår i samband med återfall av NB och visar att dessa tumörer har avvikelser som driver cellcykeln, aktiverar MAPK signalering eller påverkar telomererna och därmed cellernas kapacitet till obegränsad celldelning. Utöver detta så har vi detekterat en återkommande genetisk förändring i genen *LSAMP* som verkar kunna trycka ned tumörtillväxt i NB cellinjer men också identifierat en aggressiv undergrupp av NB-tumörer med amplifiering av två områden på kromosom 12. Studier i NB-cellinjer indikerar att denna undergrupp skulle kunna dra nytta av läkemedel som inhiberar CDK4 och MDM2, två proteiner som stimulerar celldelning.

För att sammanfatta, storskaliga tekniker för genetisk analys, som till exempel den nya DNA-sekvenseringstekniken är mycket användbara, inte bara för forskningsändamål utan också som kliniska verktyg för individanpassad behandling.

List of papers

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Braekeveldt N**,** von Stedink K, Fransson S, **Martinez-Monleon A**, Lindgren D, Axelson H, Levander F, Willforss J, Hansson K, Öra I, Backman T, Börjesson A, Beckman S, Esfandyari J, Berbegall AP, Noguera R, Karlsson J, Koster J, Martinsson T, Gisselsson D, Påhlman S and Bexell D. Patient-derived xenograft models reveal intratumor heterogeneity and temporal stability in neuroblastoma. *Cancer Res. 2018 Oct;78(20):5958–69*.

II. Fransson S, **Martinez-Monleon A**, Johansson M, Sjöberg RM, Björklund C, Ljungman G, Ek T, Kogner P and Martinsson T. Whole-genome sequencing of recurrent neuroblastoma reveals somatic mutations that affect key players in cancer progression and telomere maintenance. *Sci Rep. 2020;10(1):22432.*

III. **Martinez-Monleon A**, Gaarder J, Kogner P, Martinsson T and Fransson S. Identification of recurrent 3q13.31 chromosomal rearrangement implies *LSAMP* as a tumor suppressor gene in neuroblastoma. *Manuscript.*

IV. **Martinez-Monleon A**, Kryh H, Fransson S, Gaarder J, Berbegall AP, Javanmardi N, Djos A, Ussowicz M, Taschner-Mandl S, Ambros IM, Øra I, Sandstedt B, Beiske K, Ladenstein R, Noguera R, Ambros PF, Ljungman G, Kogner P and Martinsson T. Amplification of *CDK4* and *MDM2*: A detailed study of a high-risk neuroblastoma subgroup. *Manuscript.*

Publications not included in this thesis:

Siaw TJ, Javanmardi N, Van den Eynden J, Lind DE, Fransson S, **Martinez-Monleon A**, Djos A, Sjöberg RM, Östensson M, Carén H, Trøen G, Beiske K, P Berbegall A, Noguera R, Lai W, Kogner P, Palmer R, Hallberg B, Martinsson T. 11q deletion or ALK activity curbs DLG2 expression to maintain an undifferentiated state in neuroblastoma*. CellReports. 2020 Sep 22;32(12):108171.*

Content

Abbreviation

Abe	Abemaciclib
amp	Amplification
CDK4/6i	CDK4/6 inhibitor
CNV	Copy number variation
del	Deletion
GN	Ganglioneuroma
GNB	Ganglioneuroblastoma
HighG	High generation
HR-NB	High-risk neuroblastoma
INPC	International Neuroblastoma Pathology Classification
INRG	International Neuroblastoma Risk Group
INSS	International Neuroblastoma Staging System
ITH	Intra-tumoral heterogeneity
LOH	Loss of heterozygosity
LowG	Low generation
MDM2i	MDM2 inhibitor
MKI	Mitosis-karyorrhexis index
mut	Mutated
N3a	Nutlin-3a
NB	Neuroblastoma
NCCs	Neural crest cells
PDOX	Patient-derived orthotopic xenographs
Rib	Ribociclib
SAPs	Sympathoadrenal progenitors
SCPs	Schawnn cell precursors
SNP	Single nucleotide polymorphism
SRO	Shortest region of overlap
SNV	Single nucleotide variant
SV	Structural variant
WES	Whole exome sequencing
WGS	Whole genome sequencing
wt	Wild type

Introduction

Cancer

Cancer is a term used to determine a complex group of diseases that are characterized by uncontrolled cell proliferation and abnormal cell morphology, which produces a tumor mass with the potential to spread and invade other organs of the body.

Approximately 4 million new cases and 1.9 million deaths were diagnosed with cancer in Europe in 2020[1]. Sweden has about 50.000 new cancer cases and 22.000 cancer deaths annually.

Cancer is considered a multistep genetic disease in which key alterations occur in the DNA repair processes or checkpoint systems, producing genetic instability. This makes cancer cells have a higher mutagenic capacity compared to normal cells, producing genetic alterations such as small mutations, amplifications, deletions and translocations. The genetic alterations can cause changes in protein expression and function, which can lead to advantages for proliferation and survival of the cell, inducing the carcinogenesis. These acquired abilities are also known as “the hallmarks of cancer”, essential characteristics shared by all cancer types, which are: sustenance of proliferative signaling, evasion of growth suppressors, evasion of immune destruction, evasion of cell death, acquisition of replicative immortality, tumor-promoting inflammation, activation of tissue invasion and metastasis, induction of angiogenesis, genome instability and mutation, re-program of the energy metabolism and evasion of immune destruction[2].

Despite the fact that different types of cancer share biological characteristics, each cancerous tumor has a combination of unique genetic and epigenetic alterations[3]. Early diagnosis of the disease plays a key role in patient outcome. For this reason, it is crucial to study tumor genetic changes in a personalized way to be able to more accurately predict the prognosis of the disease and its possible treatments.

Oncogenes and tumor suppressor genes

There are two principal types of genes involved in cancer: oncogenes and tumor suppressor genes. Oncogenes are derived from proto-oncogenes, which are genes that have a normal function in cell division, growth and/or survival. A proto-oncogene becomes an oncogene when abnormally activated, as it acquires a gain-of-function point mutation, gene amplification, gene duplication, genomic translocation or epigenetic modification. The genetical change causes a hyperactivation of signaling pathways producing an uncontrolled cell growth.

On the other hand, tumor suppressor genes are generally involved in unrelenting cell growth by inducing senescence and/or apoptosis, as well as promoting DNA repair. In this case, the tumor suppressor gene can lead to tumorigenesis when this is inactivated, mainly due to loss-of-function mutations together with gene deletions or hypermethylation[4].

Pediatric cancer

All cancers that affect children between 0-14 years old are considered as pediatric cancer. Although the incidence of cancer increases with age, showing a high increase from midlife[5], it is one of the most common cause of death in children[6,7].

Cancer types diagnosed in adults and children are different. In 90% of the cases in adults, the cancer originates from epithelial tissue (carcinoma); the tumor arises from differentiated adult tissue. The stem cells of the epithelial tissue have a high risk of accumulating mutations since the tissue is continually self-renewed throughout life[8].

In pediatric cancer, tumors are not usually epithelial; they are originated from precursor cells of non-self-renewing tissue during the development of organs and tissues[9,10]. The most common cancer types diagnosed in childhood are acute lymphoblastic leukemia (26%), brain and central nervous system tumors (21%) and neuroblastoma (7%)[11].

Cancer in adults is a multistep process where mutations accumulate over several years or even decades[12]. However, pediatric cancer develops in a much shorter time period so there are fewer events for the initiation and progression of the tumor[11]. That is why pediatric cancer tends to have less single nucleotide variants and nucleotide insertions/deletions compared to the majority of

cancers in adults[13]. Pediatric cancer usually starts as a consequence of a defect in the signaling and differentiation of precursor cells during embryonic development, such as in neuroblastoma[13,14].

Neuroblastoma

The term neuroblastoma (NB) was introduced for the first time by J. H. Wright in 1910 to describe a group of pediatric tumors with features of neuronal origin. NB is a childhood malignancy of the sympathetic nervous system originated from undifferentiated neural crest precursors, which has heterogeneous features ranging from patients with tumors that show spontaneous regression to patients with very aggressive tumors and fatal outcome.

NB is the most common extracranial cancerous solid tumor in childhood, being the most diagnosed cancer during the first year of life with a prevalence of 20-50 cases per million individuals[15]. Unfortunately, NB is the third most common pediatric cancer and constitutes approximately 12-15% of all pediatric cancer related deaths[15-19]. In Europe, about 1500 cases are diagnosed each year, of which 15-20 cases are diagnosed in Sweden[20].

This malignancy is an early childhood cancer where the median age of diagnosis is around 17-18 months and 90% of the cases are diagnosed before the age of five. NB is rarely diagnosed in children older than 10 years of age[15,17].

NB tumors are located in the sympathetic chain where the primary tumor arises in the adrenal medulla of the adrenal gland for approximately half of all cases or along the paraspinal sympathetic ganglia (abdomen, chest, pelvis and neck nerves) for the other half[18,21,22] (Figure 1). About 50% of the NB cases present a metastatic disease where metastasis usually occurs in regional lymph nodes, bone or bone marrow, although it can also be found in liver and skin[18].

Depending on the prognosis of the disease, this cancer can be divided in 4 major risk groups: very low, low, intermediate and high-risk NB (HR-NB). Based on these tumor stages, a highly divergent response to the treatment is observed where low-risk patients have a 95% survival rate, while patients with HR-NB have a very poor prognosis with less than 50% of 5 years of overall survival, despite heavy multimodal treatment. Between 50-60% of the HR-NB patients will eventually suffer a relapse with a survival rate of less than 10%[23,24]. For this

reason, a better understanding of the interplay between chromosomal abnormalities, somatic and germline variants and other possible alterations within the nervous system context is necessary in order to improve the survival of these patients. Additionally, better biological understanding and clinical stratification is needed to find profiles that predispose to developing a HR-NB or to contribute to relapse, metastatic or non-responsive status of the tumor.

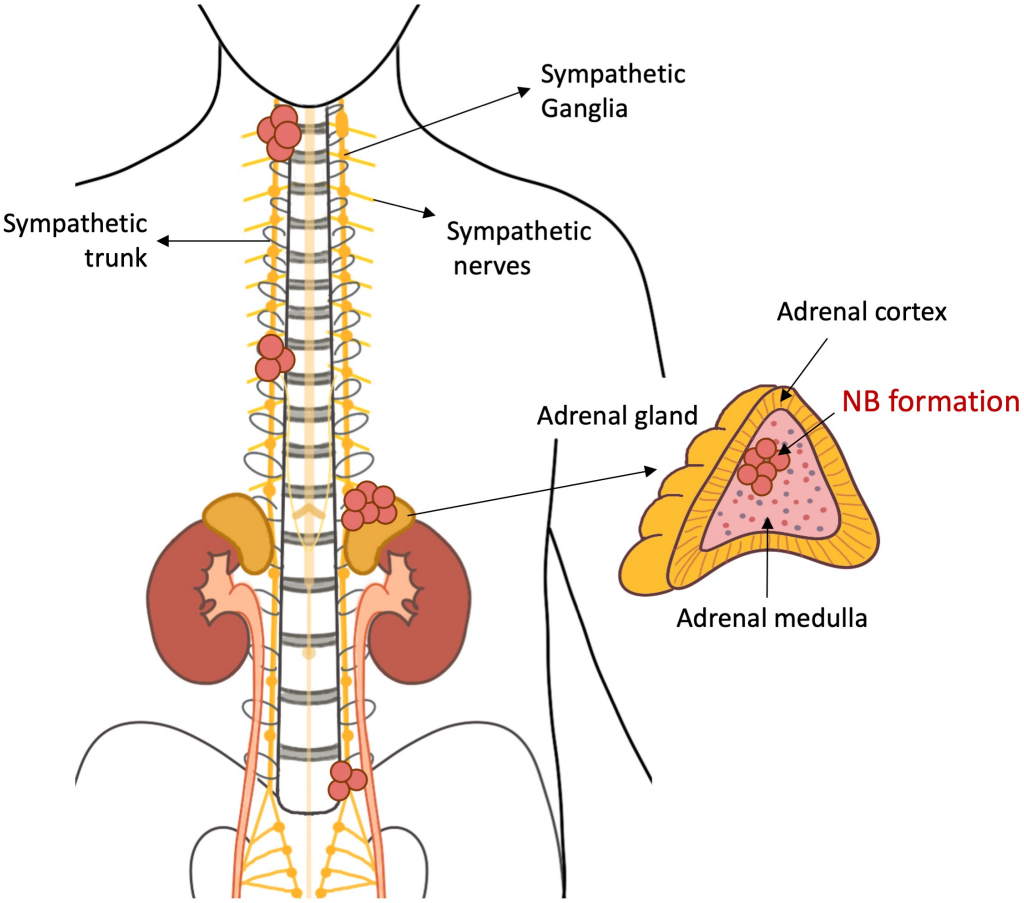

Figure 1. Representation of the sympathetic nervous system showing the primary locations of NB tumors.

Origin of the disease

NB is a disease of the sympathetic nervous system which arises from a cell differentiation failure of the sympathoadrenal linage of the neural crest cells (NCCs) during fetal development[25].

During embryogenesis, the NCCs begin to develop around the neural tube. They then begin to migrate and differentiate into several types of cells. In the case of the multipotent NCCs of the sympathetic linage, they differentiate into neurons, glia, sympathetic ganglia and adrenal medulla[26]. Recently, Furlan *et al.* found that part of the adrenal medulla is originated from peripheral stem cells called Schwann cell precursors (SCPs), which have neural crest origin as well[27].

According to the new finding, the formation process of the adrenal gland medulla is based on two different migration stages: the early migration and the late migration. The early migration of undifferentiated NCCs depends on chemoattractant signals of the dorsal aorta and gives rise to the sympathoadrenal precursor cells (SAPs), which will differentiate into sympathetic ganglion, sympathetic neurons and chromaffin cells. The late migration is nerve-dependent, meaning that NCCs migrate through the sympathetic neurons: in this case, the cells receive the name of SCPs. These cells invade the developing adrenal medulla through the sympathoadrenal neurites and once SCPs reach the medulla, they differentiate into chromaffin cells[26,27] (Figure 2). Linage tracing experiments in mice performed by Furlan *et al.* estimate that from the total amount of chromaffin cells (catecholamine-secreting cells of the adrenal gland), around 80% are originated from SCPs, while 20% arise from SAPs[27].

NB arises due to an alteration in the differentiation process of sympathetic linage cells, halting the development of neurons or chromaffin cells when the precursor cells reach the adrenal medulla[18]. This differentiation failure arises due to aberrations in genes involved in the development of the sympathetic nervous system, such as mutations in *ALK* or *PHOX2B* and *MYCN*-amplifications[25]. Even though the exact origin of NB is still an enigma, these recent discoveries suggest at least two possible origins of NB: alterations in the differentiation of SAPs or of SCPs[28].

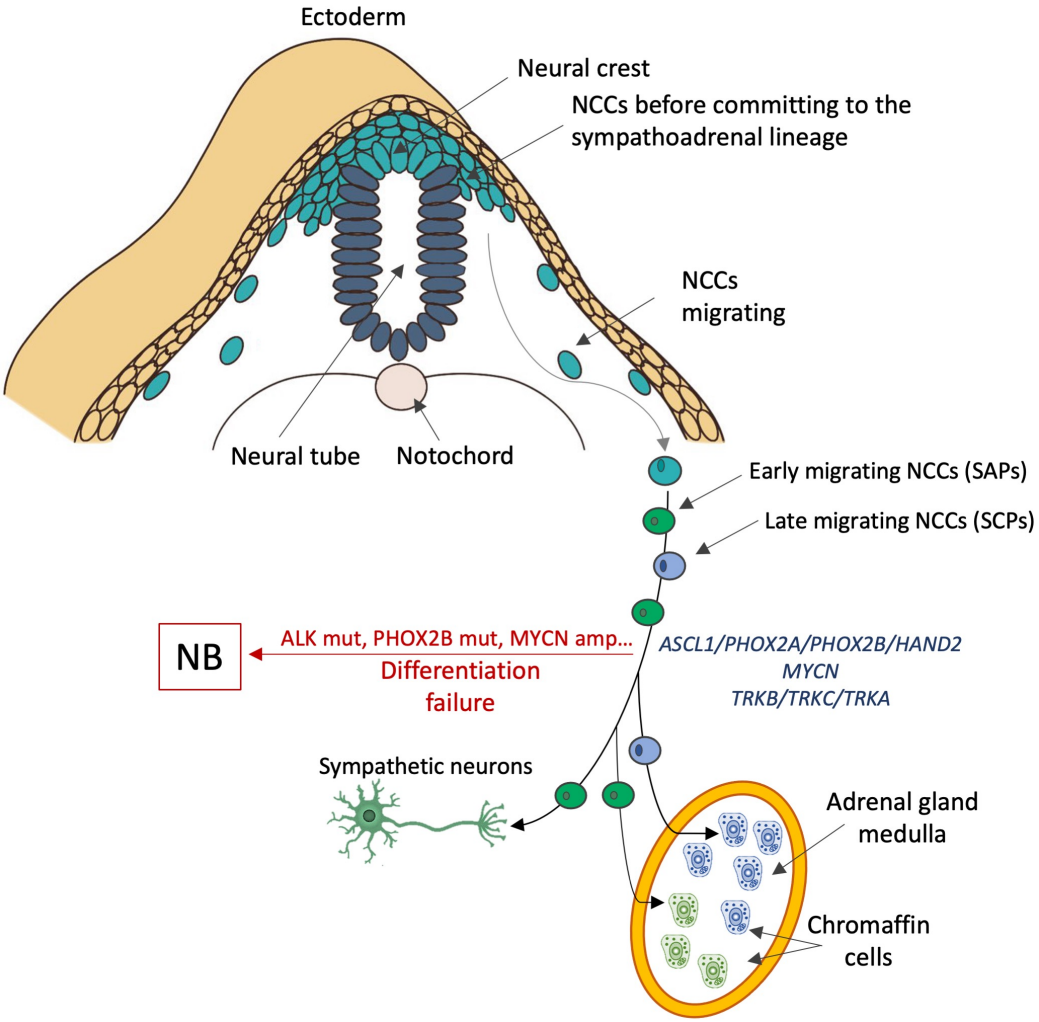

Figure 2. Migration of the neural crest cells after neurulation. The sympathoadrenal precursor cells (SPAs) and Schwann cell precursors (SCPs) are believed to be the origin of neuroblastoma after a failure in differentiation. Adapted from Mohlin *et al*. 2011[29], Cheung *et al.* 2013[30], Furlan *et al.* 2017[27] and Tsubota *et al.* 2017[26].

Constitutional neuroblastoma

Constitutional NB is an uncommon disease that corresponds to 1-2% of all NB cases[23,31], in which the mutations occur in the germline arising *de novo* or being inherited (Familial NB). The segregation of the disease is considered as an autosomal dominant Mendelian trait with incomplete penetrance, since there are frequently unaffected obligate carriers[32-35]. This type of NB is characterized by an early age of diagnosis with the presence of multiple primary tumors in most of the cases. Familial NB is highly heterogenous with a wide variability in

the prognosis of the disease since both high-risk and low-risk tumors have been observed within the same family[35]. Two fundamental genes for which mutations predispose to NB are known to date: *PHOX2B* and *ALK.*

The first gene found that predisposes to NB was the *PHOX2B* homeobox gene, located on chromosome 4p12. This gene encodes a transcription factor with a key role in the regulation of the autonomic nervous system development[17], which is also the reason why its alterations are related with other neural crest-derived disorders that can co-exist with familial NB, such as central congenital hypoventilation syndrome and Hirschprung's disease[31]. Although germline mutations in *PHOX2B* have a clear role in the arise of NB, they are only found in approximately 10% of all familial NB tumors and are rarely found in sporadic NB cases (about 2% of the cases)[35-38].

In contrast, *ALK* activating mutations are found in 80% of the familial NB and in 10% of sporadic NB cases[31,35]. The *ALK* gene, located in 2p23, encodes a receptor tyrosine kinase involved in proliferation and differentiation of the NCCs. The most common *ALK* mutation found in familial NB is R1275Q, which has a high penetrance, but other mutations with lower penetrance have also been reported, such as T1087I, T1151M, G1128A and R1192P[31,39-41].

Despite advances in the knowledge of familial cancer, there is a small fraction of these patients who do not have mutations in *PHOX2B* or *ALK;* for this reason, further investigations are needed to improve the understanding of the disease.

Sporadic neuroblastoma

Sporadic NB represents 98-99% of all NB cases and it is considered to mainly be a copy number variation (CNV) driven disease. These genetic variations include whole or segmental chromosomal gains and losses, amplifications, loss of heterozygosity (LOH) and chromothripsis. CNVs have already been detected in one month old NB patients indicating the arise of CNVs during the embryonal development; pointing out that chromosomal instability is a hallmark and one of the first events in NB tumorigenesis[42] (described in more detail in page 15; Tumor ploidy).

Chromosomal deletion of 1p, 3p, 4p, 11q, chromosomal gain of 1q, 2p, 17q and amplification of the *MYCN* oncogene are the recurrent CNVs with clinical importance in sporadic NB. Both *MYCN*-amplification and 11q deletion are

associated to aggressive HR-NB but they are normally mutually exclusive events[43,44]. The recurrence of segmental alterations indicates the presence of one or several genes with relevance in NB development, but the large size of these rearrangements make it difficult to pinpoint specific driver genes and thereby, possible therapeutic targets. (described in more detail in page 15; genetic segmental abnormalities).

Recurrent somatic acquisition of small genetic alterations is relatively rare. Recent studies show recurrent alterations mainly in *ALK* followed by *PTPN11*, *ATRX* and *TIAM1*[45,46]. Nevertheless, introduction of large scale, high throughput techniques such as next generation sequencing (NGS) is expected to detect additional aberrations that contribute to the etiology of NB[47,48].

Relapsed and refractory neuroblastoma

Despite the efforts in the stratification and treatment of patients, approximately 50% of patients with HR-NB suffer the appearance of a new tumor or relapsed tumor, even if a remission of the initial tumor was observed after treatment. Besides, in 15% of HR-NB cases the tumor does not respond to treatment from the beginning; these tumors are called refractory NB. The prognosis and treatment of patients with relapsed or refractory NB is similar. They have a very poor outcome since they usually develop resistance to the treatment. The average reported survival rate is 12.5% for refractory tumors and 5.7% for relapsed disease in Europe[49].

The genetic aberrations associated with the relapse are still not completely known but recent studies show that relapsed tumors exhibit a higher mutational content compared to primary tumors[50-53]. Moreover, studies of primary/relapsed tumor pairs have shown that the majority of relapsed tumors carried mutations that activate cell proliferation, differentiation and apoptosis through MAPK signaling pathway. These aberrations include activating mutations in *ALK*, *RAS* and *PTPN11* as well as mutations in other genes involved in cell cycle progression and telomere activity[51,53].

Tumor heterogeneity

NB is a disease that has a wide clinical heterogeneity, from a tumor that disappears due to spontaneous maturation to aggressive tumor that is multi-resistant to therapy. Two patients with the same type of tumor and with the same therapy strategy may have a different clinical outcome due to differences in the primary tumor location, the genetic and epigenetic changes and/or the tumor microenvironmental interaction, known as inter-tumor heterogeneity. NB has a high inter-tumor heterogeneity as the tumor can arise in the adrenal gland medulla and also along all the sympathetic chain ganglia; the number of recurrent gene mutations between patients is very limited[3]. The development of personalized therapies is needed as NB inter-tumoral heterogeneity makes the improvement of general therapies very difficult.

NB tumors are also affected by intra-tumoral heterogeneity (ITH), where the tumor of a single patient can present genetic and/or epigenetic aberrations coexisting in the same tumor[54]. ITH is associated to progressive disease leading to refractory/relapsed tumors, probably due to a selection of the resistant clones after treatment[55,56]. ITH can be spatial and/or temporal; ITH spatial takes place when biopsies from different locations of a tumor are genetically different at the same moment in a single tumor and require time to form, while ITH temporal occurs later in time, usually after treatment or another significative event, when a new biopsy is obtained and shows a different genetic profile, normally a more aggressive one. It must be noted that, for ITH temporal to be developed, a previous underlying ITH spatial has to be in place[56].

Symptoms and diagnosis

The symptoms of NB are diffuse and depend on where the primary tumor is placed as well as presence and location of metastases. Patients with a local tumor may have no symptoms and the tumor may be discovered accidentally in the intra-adrenal area during the prenatal ultrasonography; however, they can also present symptoms such as severe pain due to a large and invasive tumor, normally in the sympathetic chain. In cases where the tumor has metastasized, patients commonly present symptoms at the time of diagnosis; they usually have general symptoms such as weakness, fever, easy bruising or anorexia, and more specific symptoms of the disease such as bone pain and bone marrow failure[23].

The diagnosis of NB can be made, according to the criteria established by the International Neuroblastoma Risk Group Task force, if there are high levels of the catecholamine metabolites homovanillic acid (HVA) and vanillylmandelic acid (VMA) present in the urine or serum, and if there are NB cells detected in the bone marrow or in the tumor biopsy[57,58]. More than 85% of NB patients show elevated urine levels of HVA and VMA, which is derived from adrenaline (epinephrine) and noradrenaline (norepinephrine) respectively; hormones secreted by the adrenal medulla of the adrenal glands.

To determine the diagnosis, the following test can be used: (i) Blood and urine test to analyze the presence of anemia, evaluate liver and kidney functions and detect catecholamine metabolites; (ii) Tumor excision and histopathological assessment of tumor biopsy; (iii) Bone marrow aspiration and biopsy to determine presence of NB cells in this location; (iv) Computed tomography scan (CT), magnetic resonance imaging (MRI), MIBG scan and Positron emission tomography (PET) in order to detect possible abnormalities or tumoral formations in the body and to localize and measure the tumor[59,60].

Genetic studies are also performed in order to better determine the diagnosis, the risk-group of the patient and the prognosis of the tumor, and thereby define possible therapy targets. The methods used for the genetic analysis include: (i) Fluorescent *in-situ* hybridization (FISH) for the detection of specific copy number alterations (CNAs), (ii) Comparative genomic hybridization (aCGH) to screen genome-wide CNVs, (iii) Single nucleotide polymorphism array (SNP-array) to detect CNVs, polyploidies and loss of heterozygosity, (iv) Sanger sequencing and next generation sequencing[61-63].

Prognostic factors

NB is a disease characterized by its clinical heterogeneity. The course of the tumor can greatly vary; some of the tumors may regress spontaneously while others may become highly aggressive, capable of progressing in spite of intensive and multimodal treatment. Due to the complexity of this disease, it is difficult to stratify and assess the prognosis and treatment strategy. The parameters utilized in the stratification of the patients are the age of the patient at the time of diagnosis, tumor histology, tumor stage, DNA ploidy, chromosomal alteration, *MYCN* oncogene status and TRKA (Neutrofic Tyrosine kinase receptor) expression[44,64].

Tumor histology

It is relevant to know that neuroblastic tumors can be histologically divided in three types: ganglioneuroma (GN), ganglioneuroblastoma (GNB) and NB, according to the degree of neuroblast differentiation, maturation of schwannian stroma cells and mitosis-karyorrhexis index (MKI)[65]. (i) GN is the most benign tumor of the three and is the most differentiated being composed of gangliocytes and mature schwannian stroma cells. (ii) GNB has intermediate malignant potential; it is a poorly differentiated tumor composed of mature gangliocytes and immature neuroblast. It is considered a heterogeneous tumor since it has characteristics of the other two types. Moreover, GNB can be divided in two subgroups, intermixed and nodular, depending on the degree of differentiation. (iii) NB is the most malignant, since it is a highly undifferentiated, stroma poor and the most immature tumor of the three[66].

The International Neuroblastoma Pathology Classification (INPC) considers that the tumors with favorable histology are: GN (stroma-dominant), GNB intermixed (stroma-rich), NB (stroma-poor) that is differentiating or poorly differentiated with low/intermediate MKI in children younger than 1,5 years of age at diagnosis and NB (stroma-poor) that is differentiating with low MKI in children between 1,5-5 years at the time of diagnosis.

Tumors considered with unfavorable histology are: GNB nodular (composite by stroma-rich/dominant and stroma-poor) and NB (stroma-poor) in all cases that are not contemplated in the favorable histology group[65].

Risk-stratification

In 1986, the International Neuroblastoma Staging System (INSS) was proposed in order to categorize the stages of NB and facilitate the comparison of clinical trials. INSS stages (revised in 1993) are based on the age of diagnosis, the spread of the disease and the excision possibilities of the tumor[67] (Table 1). The tumors are graded from 1 to 4, stage 4 being the most aggressive. To summarize the groups, stage 1 and 2 have a localized, non-metastatic tumor that usually responds favorably to radiation and chemotherapy while stages 3 and 4 are metastatic tumors which can present resistance to radiation and chemotherapy or tumor relapse in a high number of cases. Stage 4S constitutes an intriguing exception of metastatic disease. In the case of stage 4S, the patients have a very

good prognosis despite the metastatic appearance and the tumor may regress spontaneously even without the necessity of treatment[17,23,57,68,69].

However, since INSS conducts the classification of the tumor after the excisions of the primary tumor (INRG), it is not suitable as a pre-treatment staging system and risk assessment. As a consequence, in 2009 the International Neuroblastoma Risk Group Staging System (INRGSS) was established. This classification is based on tumor imaging instead of directly looking at the extent of the surgical resection (Table 2). Furthermore, in order to stratify the risk of the patients, the INRGSS classification also includes INRG classification, the age of diagnosis, histology of the tumor, grade of tumor differentiation, *MYCN* status (amplified or non-amplified), 11q status and tumor cell ploidy[57] (Table 3).

Table 1. International Neuroblastoma Staging System (INSS)

INSS Stage	Description
1	Localized tumor with complete gross excision; ipsilateral lymph nodes negative for tumor (Lymph nodes attached to and removed with the primary tumor may be positive).
2A	Localized tumor with incomplete gross excision; ipsilateral lymph nodes negative for tumor.
2B	Localized tumor with complete or incomplete gross excision; ipsilateral lymph nodes positive for tumor; Contralateral lymph nodes must be negative for tumor.
3	Tumor infiltrating across the midline (vertebral column) with or without regional lymph node involvement; or localized unilateral tumor with contralateral regional lymph node involvement; or midline tumor with bilateral extension by infiltration or by lymph node involvement.
4	Dissemination of tumor to distant lymph nodes, bone, bone marrow, liver, skin and/or other organs (except as defined in stage 4S).
4S	Localized primary tumor as defined for stage 1 or 2 with dissemination limited to skin, liver and/or bone marrow (<10% tumor of total nucleated cells) in infants <1 year of age.

Adapted from Brodeur *et al.* 1993[67]

Table 2. International Neuroblastoma Risk Group Staging System Image-defined risk factors

INRG Stage	Description
L1	Localized tumor not involving vital structures as defined by the list of image-defined risk factors and confined to one body compartment.
L2	Locoregional tumor with presence of one or more image-defined risk factors
M	Distant metastatic disease (except stage MS)
MS	Metastatic disease in children younger than 18 months with metastases confined to skin, liver and/or bone marrow

Adapted from Monclair *et al.* 2009[57]

Table 3. International Neuroblastoma Risk Group Staging System (INRG)

INRG stage	Age (mos)	Histologic category	Grade of differentiation	MYCN status	11q-del	Ploidy	Pretreatment Risk Group
L1/L2		GN maturing, GNB intermixed					A Very low
L1		Any, except GN maturing or GNB intermixed		NA			B Very low
				AMP			K High
L2	< 18	Any, except GN maturing or GNB intermixed		NA	No		D Low
					Yes		G Intermediate
	≥ 18	GNB nodular, neuroblastoma	Differentiating	NA	No		E Low
					Yes		H Intermediate
			Poorly differented or undifferented	NA			
				Amp			N High
M	< 18			NA		Hyper-diploid	F Low
	< 12			NA		Diploid	I Intermediate
	12 to < 18			NA		Diploid	J Intermediate
	< 18			Amp			O High
	≥ 18						P High
MS	< 18			NA	No		C Very low
					Yes		Q High
				Amp			R High

(GN = ganglioneuroma, GNB = ganglioneuroblastoma, NA = no amplified, Amp = amplified).
Adapted from Cohn *et al.* 2009[44]

Treatment

The treatment of NB is composed of induction chemotherapy to achieve remission followed by surgery and local radiotherapy. Autologous stem cell transplant and retinoic acid differentiation therapy are then conducted in order to consolidate the remission. Anti-GD2 immunotherapy may or may not be supplied to maintain the remission[70,71]. The therapy can also include novel treatment strategies based on targeted therapy, such as in patients with ALK mutated tumors, which can receive ALK inhibitors[72].

Neuroblastoma alterations

Epigenetics

Epigenetic processes modify gene expression by DNA alterations that do not change the genetic code *per se*, such as DNA methylation or histone modifications. This process is essential in normal development[73,74], but alterations in epigenetic regulation can promote cancer. DNA hypermethylation frequently inactivates tumor suppressor genes, while hypomethylations can activate oncogenes and initiate chromosome instability[75]**.** Accumulation of epigenetic errors can aid in the transformation of a normal cell into a metastatic tumor cell. The number of methylated genes is associated with the stage of the disease in NB. Besides, inactivated genes via promoter methylations have been found in NB, such as *RASSF1A,* a tumor suppressor gene involved in cell cycle arrest, and *CASP8,* which is involved in cell apoptosis execution[76].

Expression profiling of neurotrophic receptors

The expression pattern of different neutrophin receptors of the tyrosine kinase family (TrkA, TrkB and TrkC) and their ligands are involved in the regulation of the differentiation, tumorigenesis and metastasis of NB; therefore having an important role in the biology and clinical outcome of the disease[77,78].

TrkA expression is associated with good prognosis of NB, while low or absent expression of the receptor is related with poor prognosis. TrkA is activated by its ligand NGF (nerve growth factor), promoting neuronal differentiation[78-80]

TrkC expression is also related with a favorable tumor prognosis in the absence of *MYCN*-amplification[81,82].

TrkB expression is mainly found in *MYCN*-amplified poor-prognosis tumors. TrkB is activated by its ligand BDNF (brain derived neurotrophic factor), helping in the NB cell proliferation and survival[77,78,83]. Tumors lacking amplification of *MYCN* generally do not express TrkB[83]

Tumor ploidy

Recurrent genomic alterations with relevance in the diagnostic and prognostic evaluation of NB are related to tumor ploidy or CNVs[44]. Tumors with a "numerical only" genomic profile (gain/loss of the whole chromosomes) and near-triploid karyotypes are associated with good prognosis. Whole chromosome 7 gain is the most common numerical copy alteration in this cancer type[84,85], followed by gain of chromosomes 18, 17, 12 and 13[85].

Genetic segmental abnormalities

Tumors with segmental aberrations and near di- or tetraploid karyotypes are generally associated with poor prognosis[86,87]. The most common alterations found in HR-NB are 17q-gain, 1p-deletion *MYCN*-amplification and 11q-deletion (Figure 3).

HR-NB with segmental aberrations can be divided into two main groups: *MYCN*-amplified tumors and 11q-deleted tumors, since they are almost mutually exclusive events. *MYCN*-amplification has been detected in around 20-30% of all HR-NB cases[88,89], while 11q-deletion has been found in around 35-45% of the tumors [90-92]. In those cases with segmental aberrations without *MYCN* being amplified or 11q being deleted, 17q-gain is an indicator of worse prognosis compared to the cases lacking this alteration, which have a more favorable outcome[92].

Even though *MYCN*-amplified tumors and 11q-deleted tumors present a similar overall survival, they have a clearly different genetical pattern[92]. *MYCN*-amplified tumors frequently present 1p-deletion and 17q-gain but very few other segmental aberrations. Nevertheless, 11q-deleted tumors often show 17q-gain but a major chromosomal instability with a high chromosomal break, including several segmental losses of other chromosomes (Figure 4).

Rearrangements in *TERT* or *ATRX*, two genes related to the telomere length, are commonly detected in 11q-deleted tumors, but not in *MYCN*-amplified tumors. Furthermore, *MYCN*, *TERT* and *ATRX* have been noted to be almost mutually exclusive, leading to alterations in the maintenance of the telomers, and they are related with HR-NB[93,94].

These and other recurrent chromosomal segmental alterations are explained below in more detail.

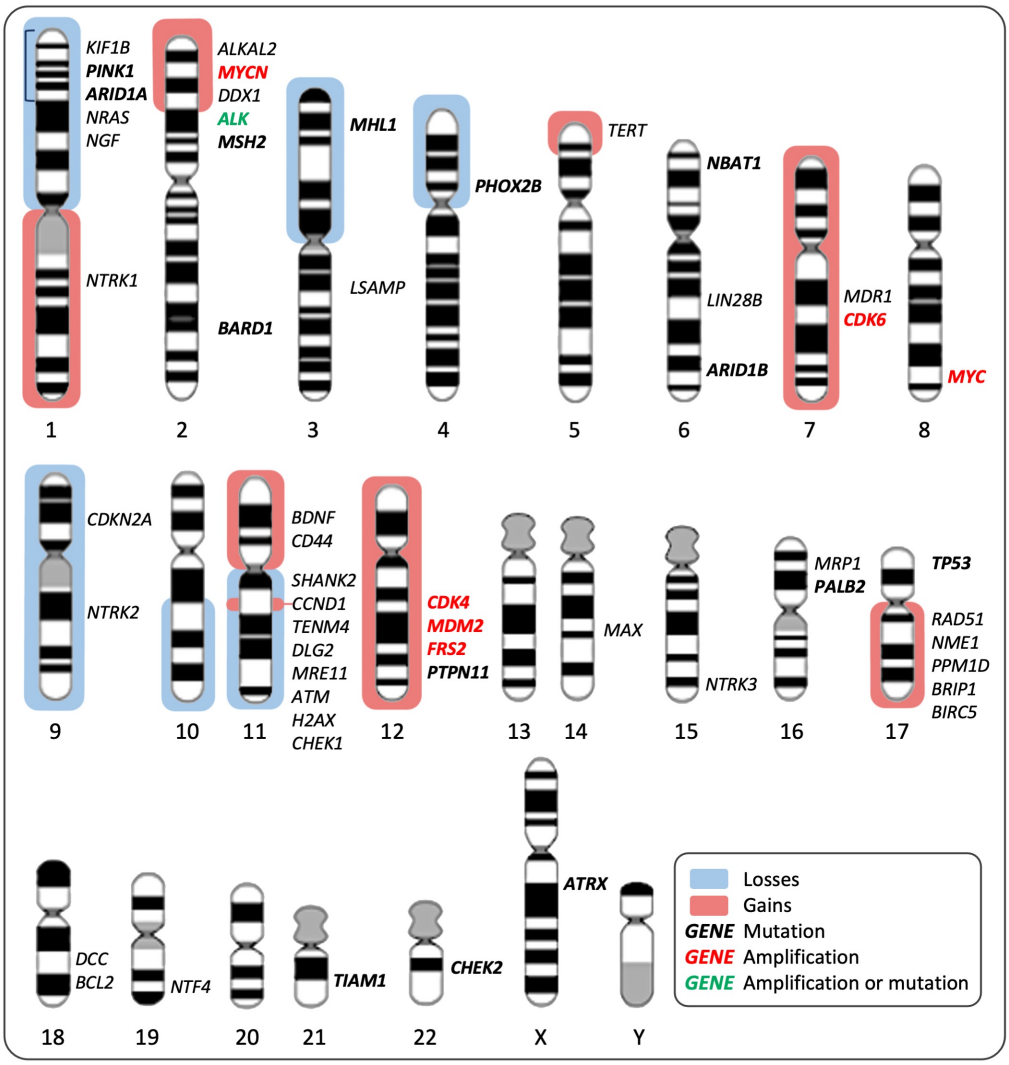

Figure 3. Representation of recurrent genetic alterations in HR-NB. Chromosomal segmental variants are marked in blue (losses) and red (gains); a line next to the chromosome indicates the most common area altered. Mutated genes are marked in bold, amplified genes are marked in bold red and genes that can be amplified or mutated are marked in bold green. Information based on our own data, Maris *et al*. 1999[88] and Depuydt *et al.* 2018[95].

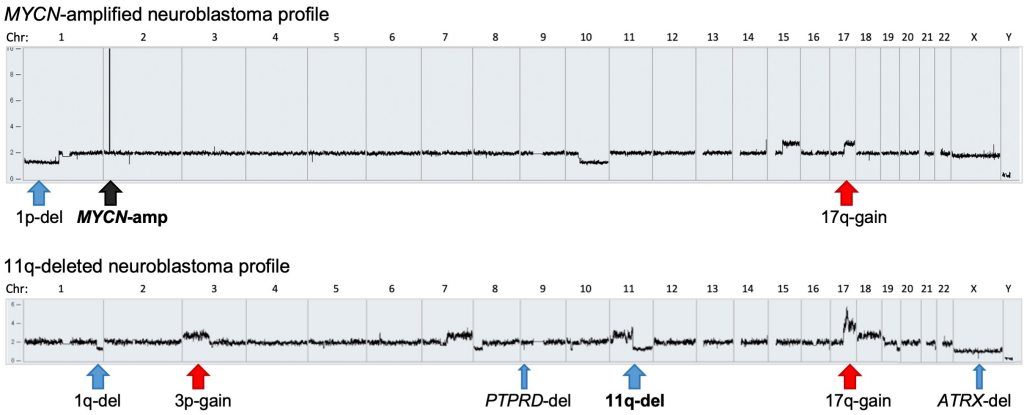

Figure 4. SNP-array genomic profile of two HR-NB tumors. Upper panel: a *MYCN*-amplified tumor showing few other segmental alterations. Lower panel: a 11q-deleted tumor, presenting a high degree of chromosomal instability and segmental changes, including an *ATRX*-deletion.

1p-deletions

Around 25-35% of NB tumors present a hemizygous deletion of part of the short arm of chromosome 1 (1p36)[44,96,97]. This aberration has a higher frequency in HR-NB: it has been detected in approximately 70% of the cases and it is correlated with *MYCN*-amplification[98-100]. Studies carried out to investigate the function of the 1p in NB showed that the transfer of the 1p arm into 1p-deleted NB cell lines induces neuronal differentiation, reduces proliferation and activates apoptosis[101]. Moreover, a recent study using genome editing showed that loss of the syntenic 1p36 locus caused impaired neuronal differentiation and led to increased proliferation and migration in mouse neural crest cells[102].

The shortest region of overlap (SRO) is usually located in 1p36.2-3 but there is not a final consensus about the specific SRO location[43,100,103,104]. Looking in more detail into 1p36 region, several tumor suppressor genes associated with the reduction of cell proliferation and the activation of apoptosis are identified, such as: *CAMTA1*, *CHD5*, *KIF1B*, *CASZ1* and *ARID1A*[105-112].

2p arm: MYCN, ALK and ALKAL2

MYCN, *ALK* and the ALK ligand encoding gene *ALKAL2* (FAM150B) are all located on the short arm of chromosome 2.

MYCN-amplification was identified in NB in 1983[39,113] and it still continues to be a relevant genetic marker to stratify risk in NB. *MYCN* is located at 2p24 and encodes a transcription factor that is normally expressed during the embryonic

development of the nervous system, even if it plays a key role in the NB development when amplified[114]. *MYCN*-amplification leads to high expression levels of MYCN, which has been associated with a poor prognosis and unfavorable outcome of the disease[88,89,115]. Additionally, high expression levels of *MYC* oncogene, another MYC family member located at 8q24, have been related with an independent subset of HR-NB cases[89,116,117].

ALK is located at 2p23.2 and encodes an anaplastic lymphoma kinase which is involved in differentiation, proliferation and apoptosis through PI3K/AKT, RAS/MAPK and STAT3 pathways[118,119]. Apart from being the most common mutated gene in familial NB, somatic *ALK* mutations has been detected in around 10% and amplifications in 3-4% of the sporadic NB cases[120,121]. Interestingly, *ALK* amplification is almost only detected in conjunction with *MYCN*-amplification[31].

2p-gain has been detected as a recurrent chromosomal alteration in association with poor prognosis in NB, probably because of the alteration combination of the genes mentioned above (*MYCN*, *ALK*, *ALKAL2*), which affects ALK signaling that has a role in the development of the nervous system providing advantages to NB progression[122,123].

11q-deletion

Hemizygous deletion of the long arm of chromosome 11 is mainly exclusive of non-*MYCN*-amplified tumors, as mentioned before. This alteration is seen as a biomarker for poor prognosis in tumors that do not have a *MYCN*-amplification[90-92]. Given the frequent deletion of 11q, it is believed that this may cause loss of one or several tumor suppressor genes in this area with relevance in NB tumorigenesis. For this reason, it is important to investigate in more detail the genes located in this specific area in order to find specific genetic markers of this NB subtype. Several candidate genes involved in DNA-damage response have been observed in this region, such as *ATM*, *CHEK1*, *MRE11* and *H2AFX*[17,92,124]. Recent studies have pointed at additional candidate genes located in this region, such as *TENM4, DLG2* and *SHANK2*, to play a relevant role in neurodevelopment processes[125-128]. *TENM4* regulates neurite outgrowth through the FAK signaling pathway[129], while *DLG2* and *SHANK2* have been directly related to differentiation processes[126,127]. Interestingly, *DLG2* has been described as downregulated target of oncogenic ALK signaling[126].

17q-gain

The gain of the distal part of chromosomal arm 17q is the most common genetic alteration in NB, which occurs in approximately 50% of the cases, and it has mainly been detected in the aggressive disease[44,130,131]. This alteration has been correlated with unbalanced translocations in other chromosomes, especially with the short arm of chromosome 1, where the translocation leads to chromosome 1p-loss with simultaneous chromosome 17q-gain, but not at a specific breakpoint[132,133]. The alternated 17q area contains several genes connected to cancer, such as *NME1*, *BIRC5* (Survivin), *BRIP1, RAD51* and *PPM1D*; the high expression of those genes contributes to NB growth[134-136]. Recent studies in mice indicate that WIP1-phosphatase, encoded by *PPM1D*, is activated by frequent segmental 17q-gain, but the tumor growth can be suppressed by pharmacological inhibition of WIP1[137].

9p-deletion

Loss of chromosome 9p has been found in several NB cases, commonly producing homozygous or heterozygous deletions in *CDKN2A*, mainly on the 11q-deleted tumor group. This gene is a well-known tumor suppressor gene which generates several transcripts, related to the inhibition of CDK4 kinase function, and also blocks the induction of p53 degradation by MDM2. In this way *CDKN2A* is involved in the cell cycle regulation, capable of inducing cell cycle arrest[131].

12q-amplification

High-grade amplification of 12q13.3-14.1 and 12q15 has been reported by us and others in a subset of NB tumors where the two regions are usually co-amplified[138,139]. Amplifications in chromosome 12 have also been detected in other types of cancer as sarcoma, glioma and melanoma among others[140,141]. The genes located in the amplified areas, 12q13.3-14.1 and 12q15, have an important role in cancer growth and progression, affecting mainly the *CDK4* gene, which is involved in the cell cycle progression, and the *MDM2* proto-oncogene. It is known that these two genes have an effect in NB tumor progression and both genes are therapeutic targets[142,143]. It should be noted that the *FRS2* gene, located distally of *MDM2*, is frequently amplified together with *MDM2*. This gene has been related to the activation of MAKP pathway and tumorigenesis[138,144,145].

Alterations related to telomeric maintenance

Rearrangements in and around the *TERT* locus occur in around 23% of HR-NB cases, according to Versteeg *et al*. 2015 dataset. These cases are associated with very poor prognosis. *TERT* is located in the distal part of chromosomal arm 5p and its rearrangements promote *TERT* overexpression, leading to a maintenance of the chromosome telomere length, which increases the lifetime of the cell[93].

Rearrangements in *TERT* have been exclusively found in non-*MYCN*-amplified NB tumors. However, *MYCN* is a transcriptional activator of *TERT,* leading to overexpression of *TERT* in *MYCN*-amplified tumors[93,94]. Another mechanism for telomere length retention is achieved through *ATRX* loss-of-function alterations/mutations, that produce a telomerase independent alternative lengthening of telomeres (ALT) through an homologous recombination-associated process[146,147]. *ATRX* is located in Xq21.1: mutations of this gene have been detected in around 11% of the patients, thereby becoming one of the few genes with recurrent mutations in NB.

Interestingly, alterations in connection with *TERT* or *ATRX* have almost been exclusively found in non-*MYCN*-amplified NB tumors. However, MYCN is a transcriptional activator of *TERT,* leading to overexpression of *TERT* in *MYCN*-amplified tumors[93,94].

Aims

The general aim of our studies is to find genomic profiles that predispose to develop a HR-NB or contribute to the relapse, metastatic or non-responsive status of the tumor in order to improve the biological understanding and the clinical stratification. To accomplish this goal, we performed comprehensive molecular characterization by NGS in combination with functional exploration of novel recurrent somatic aberrations.

The specific aims of the investigation included in this thesis are:

Paper I

- To study the genetic stability of NB patient-derived orthotopic xenograft (PDOXs) models over time after *in vivo* passaging.
- To explore NB ITH by multi-sample implantation from a single tumor into PDOXs.

Paper II

- To investigate the genetic differences between tumor at diagnosis and tumor at relapse by whole genome sequencing in order to identify alterations that can be used for prognostic purposes or for novel combination therapies.

Paper III

- To characterize and study the possible role of *LSAMP*, a recurrent genetic alteration in HR-NB patients, in the progression of NB tumors.

Paper IV

- To perform comprehensive genetic and clinical description of a subgroup of NB tumors that show high level of 12q-amplification.
- To investigate *in vitro* if MDM2 and/or CDK4 targeted therapy can be beneficial for this patient subgroup.

Material and methods

Samples and models

Neuroblastoma patient cohort and ethics

NB tumors were collected from Swedish patients after either written or verbal informed consent was obtained from parents/guardians according to ethical permits approved by the local ethics committee at: Karolinska Institutet and Karolinska University Hospital (03-736 and 2009/1369-31/1) in accordance with the Declaration of Helsinki, Regional Ethics Board of Southern Sweden (289-2011), Valencia/INCLIVA University (for Spanish patients), Vienna University (for Austrian patients), Wroclaw medical University (for Polish patients) and Oslo University hospital (for Norwegian patients). Clinical data on all patients was obtained from hospital records and/or the Swedish Childhood Cancer Registry.

Neuroblastoma cell lines

NB1, NB69, SK-N-AS, SK-N-BE(2), SK-N-F1, SK-N-DZ, SK-N-SH, SH-SY5Y, KELLY, IMR-32, LS, NGP, CLB-GAR, CLB-BAR, CLB-PE and CLB-GE human cell lines were used. All cell lines were cultured in high glucose DMEM supplemented with glutaMAX and 10% FBS and without antibiotics, at 37°C in 5% CO_2 environment. All cell lines were verified by SNP-Array analyses and routinely checked to be negative for mycoplasma, bacterial and fungal infection.

Patient-derived orthotopic xenografts

In *paper I*, patient-derived orthotopic xenografts (PDOXs) were established. For this purpose, undissociated patient tumor material was implanted into the tumor origin organ in immunodeficient NGS mice, in order to mimic the pathophysiology and biological characteristics of the patient tumor including the integration of the tumor with the host stroma and the development of tumor microenvironment. In this case, the fragment of the tumor was implanted into the para-adrenal space[148,149].

Serial orthotopic transplantation of tumor material from 5 patients was performed establishing up to eight *in vivo* generations to study molecular stability. Additionally, ten PDOXs were established from a unique patient to study the ITH. Tissue sections in paraffin were analyzed by immunohistochemistry and microscopy. All animal experiments (M146-13) were approved by Malmö–Lund Ethical Committee for the use of laboratory animals.

Molecular analyses

DNA extraction

DNA was extracted from tumor material, blood and cell line pellets using the DNeasy blood and tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNA preparations used in WES and WGS were evaluated using absorbance measurements, fluorometric quantitation and DNA integrity assessment on an Agilent TapeStation (Agilent, Santa Clara, CA) before sequencing.

SNP-array analysis

SNP-arrays analysis of tumor material of NB patients (*paper I*, *III* and *IV*), PDOXs (*paper I*) and NB cell lines (*paper III* and *IV)* were performed in order to investigate DNA segmental variants such as chromosome insertions, deletions, duplications, amplifications and LOH. The Affymetrix CytoScan HD platform was used to conduct SNP-array analysis for a substantial part of the patient samples as well as all the NB cell line samples. This type of array is currently able to detect more than 2.6 million markers, including 750.000 SNP positions, within the same chip. This provides a dense genome coverage enabling detection of multiple chromosomal aberrations from a single sample simultaneously. This technique is able to detect CNVs and LOH down to 25-50kb in size[150-152].

Sanger sequencing analysis

In order to analyze and verify *LSAMP* breakpoints (*paper III*) and *TP53* mutations (*paper IV*), PCRs were conducted at our laboratory using PCR primers designed by us, followed by sanger sequencing of the amplicons performed by GATC (GATC, Konstanz, Germany) following standard conditions.

Exome sequencing

Exome sequencing was performed, for *paper I,* in order to find and compare possible changes in gene coding regions. The aim was to study the genetic stability in serial passaging PDOXs model and ITH. Tumor DNA from five NB patients and all consecutive developed PDOXs as well as constitutional DNA (from blood) from three NB patients were subjected for exome sequencing.

The study was conducted by paired-end sequencing (2x125bp) on Illumina platforms after exome capture using SureSelect Human All Exon V6 (Agilent, Santa Clara, California) performed by GATC Biotech (Constance, Germany), finally generating about 60-70 million paired reads per sample. CLC Genomics Workbench software (https://www.qiagenbioinformatics.com) was used in order to perform the read trimming and the variant calling, mapping the human reference genome hg19. In the patients, only variants with a minimum of 10% allele frequency and a total read coverage of 10 were considered. In the case of the PDOXs, variants with allele frequency below 15 % were discarded to avoid possible variants called due to presence of DNA from mouse in the PDOX tumors.

In parallel, with the aim of removing all mouse content and keep PDOX unique variant calls, reads uniquely mapping to mouse mm9 reference genome were removed before trimming and remapping to the human hg19 genome. The variant lists from both approaches were merged and reviewed by manual inspection using IGV.

Furthermore, to avoid filtering out possible low allelic fraction variants, all variants detected in a PDOX linage were further inspected in all sequenced samples of that specific PDOX linage in order to confirm their presence.

Whole genome sequencing

Whole genome sequencing (WGS) is a highly sensitive technique that provides information about single nucleotide variants as well as structural variants and CNAs along the genome.

WGS was used to investigate possible genetical changes that potentially drive tumor relapse (*paper II*) and to look for recurrent alterations between different NB patients (*paper II*, *III* and *IV*). For this purpose, DNA from tumor material (at diagnosis and at relapse) and corresponding constitutional DNA from the blood of NB patients was analyzed.

WGS was conducted with Illumina xTen or NovaSeq instrumentation (Illumina, San Diego, CA, USA) located at Clinical Genomics, SciLife Laboratories, Stockholm/Gothenburg, Sweden. Mapping to human reference genome hg19 and variant calling was done using Sentieon analysis pipelines and tools (Sentieon Inc, San Jose, CA, USA). Only high-quality somatic called variants with a minimum 10% variant allele frequency and a total read coverage of ten were considered for further analysis. The germline variants were analyzed using a more stringent filtering approach excluding all variants with a population allele frequency above 1.0%; unless being an already established pathogenic variant or gene variants with connections to hereditary and/or sporadic cancer genes. All synonymous variants or variants in non-coding regions were excluded, except those affecting canonical splice sites. Remaining variants were assessed manually using the Integrative Genomics Viewer (IGV) to remove calls due to mapping artifacts and paralogs. The filtering of somatic and germline variants was done using the ingenuity variant analysis software v5.6 (Qiagen, Hilden, Germany). The Canvas tool (version 1.38.0.1554) was used to call CNAs, with gains and losses of genomic regions as well as LOH regions.

Gene expression analyses

RNA was extracted and reverse transcribed from NB tumor material and cell lines using commercially available kits, under manufacturer's recommendations, and stored at -80 °C.

In *paper III*, qPCR was conducted, in order to investigate *LSAMP* gene expression in 14 NB cell lines and also in transfected cells. *LSAMP* TaqMan pre-designed probes cover three different exon boundaries (exon 1-2, exon 2-3 and exon 4-5), with the aim of studying possible effects of the structural alterations on the gene expression.

In *paper IV*, cDNA of 36 NB tumors and 2 NB cell lines (LS and NGP) was loaded into TLDA cards to determine the expression levels of 31 genes located within the two common 12q-amplified regions. TLDA cards were conducted according to Applied Biosystem protocol.

In both studies, the analysis of gene expression data was conducted following the "Delta Delta C(t) method", prior to normalization against multiple housekeeping genes.

Western blot

In *paper IV*, protein expression analysis of CDK4/6 and MDM2 and their pathway partners has been conducted using the western blot technique, in order to investigate the downstream effects of CDK4/6 and MDM2 inhibition. Proteins from 72h treated cells, at their corresponding IC50 concentrations, were extracted using RIPA lysis buffer and a cocktail of proteinase and phosphatase inhibitors.

Protein levels were analyzed and quantified, prior to normalization against KU80, using Image J.

Transcriptome, proteomic and phosphoproteomic analyses

In *paper I*, RNA sequencing (RNA-seq) was conducted to analyze the whole transcriptome pattern in order to study the transcriptional stability of serial passaging in PDOX models as well as in the possible ITH. RNA-seq of all primary tumors and all PDOX tumors was performed following Ion AmpliSeq Transcriptome Human Gene Expression indications from Thermo Fisher Scientific. In addition, proteomic and phosphoproteomic analyses were performed on ten different tumors of PDOX #5 (G1) for further investigations of ITH.

Cell culture

Cell transfection, proliferation and viability

In order to evaluate the role of *LSAMP* in NB tumor progression, *in vitro* experiments were conducted in four NB cell lines in *paper III*.

Silencing of LSAMP was performed in two expressing *LSAMP* NB cell lines using shRNA lentivirus particles from Santa Cruz Biotechnology (Santa Cruz, CA). The transformation was performed using Polybrene following the standard protocol.

Re-/over-expression of *LSAMP* was performed in two *LSAMP* deleted NB cell lines (one homozygous and one heterozygous) using pCMV6-AC plasmids (GeneArt, Thermo Fisher). The transformation was conducted using Lipofectamine 3000.

The *LSAMP* silenced cells were selected with puromycine while the *LSAMP* re-/over-expressed cells were selected with neomycin to obtain stable transfects during time.

Proliferation of the transfects was monitored in real-time during 96h using the xCELLigence RTCA DP instrument; the experiment was repeated in triplicate. 2-way Anova was used for the statistical analysis of the proliferation results during time.

Viability of *LSAMP* transfects was measured after 72h of seeding using a Presto Blue HS cell viability reagent (Thermo Fisher). T-test was performed to statistically analyze the results of viability. Moreover, the expression levels of *LSAMP* were confirmed by qPCR.

Single drug and combination drug treatment

In *paper IV*, ten NB cell lines with different genetic backgrounds were used to study the cell viability effects after CDK4/6 and/or MDM2 inhibition. The cells were seed in 96-well plates 24h prior treatment. For a single drug assay, the cells were treated with CDK4/6i (Abemaciclib or Ribociclib) or with MDM2i (Nutlin-3a) during a total of 72h. Cell viability was measured at 24, 48 and 72h using a Presto Blue HS.

IC50 values, for each drug and cell line, were calculated with Graph Pad Prism software version 8.4.3 using the dose response sigmoidal curve with variable slope analysis (the log [inhibitor] vs. normalized response – Variable slope). In order to analyze the statistical significance of the IC50 values of $p53^{wt}$ vs. $p53^{mut}$ cell lines, t-test was conducted.

With the aim of investigating a possible synergy effect of dual MDM2 and CDK4/6 inhibition, the cells were treated with single drugs and with different drug combinations following the dose-response matrix suggested by Synergy Finder[153]. The viability was measure using Presto Blue SH after 72h of treatment. The synergy score of the different drug combination responses was calculated based on HAS reference model using Synergyfinder.

Results and discussion

Paper I

This paper was a collaborative project with Daniel Bexell's laboratory at Lund University in which our lab contributed with the genetic studies.

In order to study NB tumor stability over time (a lapse of 2 years) in PDOXs after serial in vivo passaging (up to eight generations), PDOXs were established from tumor material of five different NB patients. Besides, ten PDOXs from multi-sample implantation of the same patient tumor (linage #5, generation 1) were established to investigate NB ITH (Figure 5).

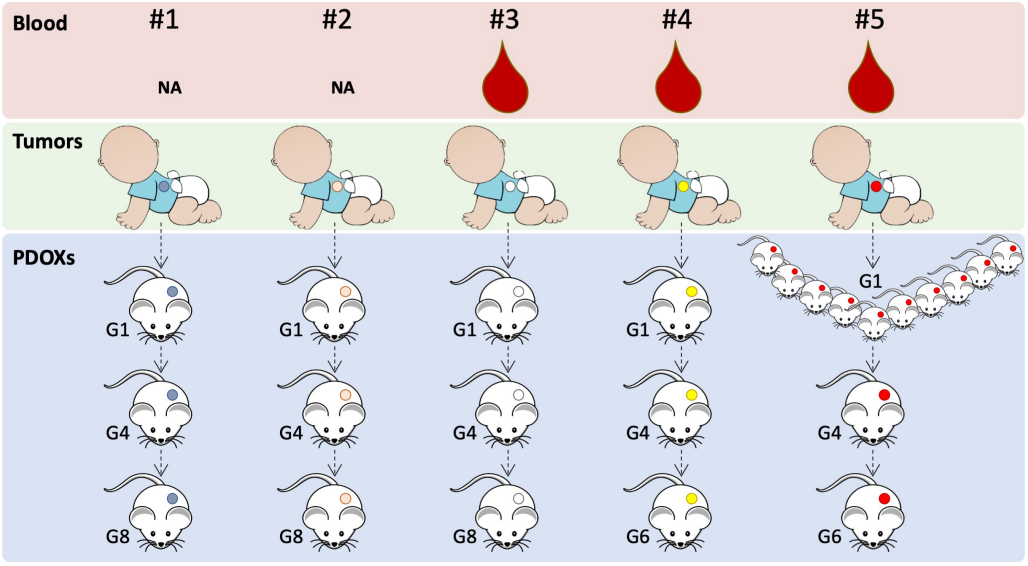

Figure 5. Schematization of the PDOXs established from five different neuroblastoma patients in order to study the tumor stability (#1 to #5) as well as the multi-sample implantation to study intra-tumoral heterogeneity (#5 – G1). Tumor material from the generations (G) represented in the figure was used in the molecular analyses.

PDOXs were cataloged according to the generation as LowG-PDOXs (G1-2) and HighG-PDOXs (G5-8) in reference to the serial passage. Previous studies of Braekeveldt *et al*. showed that LowG-PDOXs maintained cellular morphology, protein marker expression and a similar behavior to the tumors of the patients[154,155]. These features are also retained in HighG-PDOXs, including poor differentiation, expression of NB markers as well as spontaneous metastatic

capacity to lungs, bone, bone marrow and liver, as it was observed in this study. Parallelly, whole-transcriptome analysis (RNA-seq) was performed in patient tumor material and corresponding PDOXs. According to the gene expression results, each linage was located in a different cluster, meaning that different linages had patient specific patterns of gene expression that was maintained over time within each linage.

During WES analysis, mutations in genes previously associated with NB progression and/or relapse were detected, such as *NRAS* (p.Q61K), *ALK* (p.F1174L; p.L1240V), *ARID1B* (splice site mutation) and *MYCN* (p.44L). The variants in *MYCN* and *ALK* (L1240V) in linage #5 and *NRAS* in linage #3 were all present in the tumor material of the patient and remained at least until G6-8. Whereas in linage #4, *ALK* (F1174L) and *ARID1B* were detected in G1 and G4 respectively but not in the initial patient sample, highlighting the possibility of ITH, as the mutations may have existed in another area of the primary tumor. Even though all PDOX samples had unique variants, PDOXs from each linage remained genetically stable, as the majority of alterations found in the patient tumor material were retained in the PDOXs along the time, including NB markers and gene expression signatures. Besides, CNV characterization showed that large chromosomal changes typically associated with NB, such as 1p36 deletion, *MYCN*-amplification and 17q-gain, were also maintained in the PDOXs.

For the analysis of spatial heterogeneity of NB tumors, PDOXs from ten different regions of a tumor from a single patient were established. Interestingly, a striking difference in time-to-tumor burden was noted between PDOXs, thus classifying them in three groups based on this feature. In spite of the remarkable differences based on the tumor *in vivo* growth time in the PDOXs, SNP-microarray and WES analysis did not detect any significant genetic changes between the samples. Gene ontology (GO) analysis from RNA-seq as well as from proteomic and phosphoproteomic data reveals molecular changes in pathways associated with neuronal differentiation, neurogenesis, mesenchymal transition, cell cycle regulation, stromal composition and angiogenesis. This demonstrates a phenotypic heterogeneity between the different samples, although the changes were not significantly matched with the growth group classification previously mentioned. Differences in growth dynamics as well as gene expression and functional diversity in genetically identical tumor samples have also been detected in *in vivo* colorectal cancer models[156].

The differences in the tumor *in vivo* growth time in the distinct PDOXs can derive from the tumor microenvironment prior to tumor implantation or once implanted in the mouse. The tumor material implanted in group 3 could for instance derive from a hypoxic area of the tumor, inducing a slower *in vivo* tumoral growth, or could instead derive from a hypoxic microenvironment after the implantation in the mouse[157].

Despite genetic analysis not detecting any significant genetic changes between the samples, variations in their gene expression and protein/phosphoprotein expression revealed molecular spatial ITH in NB.

We showed that NB PDOXs can have phenotypic, genetic and transcriptional characteristics that remain stable for more than two years of growth in immunodeficient NSG mice. The relationship between ITH, tumor evolution and treatment response has been identified in different cancer types as well as in NB. The PDOX model for the study of ITH can be used to find new biomarkers and to predict the possible response to the diverse treatments. Only one patient was included in the ITH study in our case, hence limiting the investigation. In the future, it would be interesting to add more patients in this analysis, in order to continue studying the difference in the clonal development of a tumor as well as the ITH over time.

Paper II

Triplicate samples of tumor at diagnosis together with their corresponding relapsed/refractory tumors and constitutional DNA from seven HR-NB patients were genetically characterized in order to explore possible molecular features related to NB disease progression despite multimodal treatment. All patients presented a metastatic disease at time of diagnosis and all of them were under similar treatment conditions: including chemotherapy, surgery, irradiation, stem cell rescue and retinoids maintenance therapy. Six of the patients responded to the treatment showing a complete remission of the tumor prior to tumor recurrence, while one of them experienced a refractory disease in which the tumor progressed during treatment.

During genetical analysis, constitutional alterations in genes involved in cancer predisposition were found in 3 out of 7 patients. Relevant recurrent mutations associated with constitutional and sporadic NB are *PHOX2B* and *ALK*[31,158,159].

Germline mutations in *PHOX2B* have not been detected in the studied cohort, but one of the patients (NB67R5) presented a somatic frameshift mutation, p.G230fs, in both primary and relapsed tumors. *PHOX2B* (p.G230fs) is expected to produce a truncated protein with dominant-negative effect in the differentiation of the sympathetic nervous system leading to susceptibility to other transforming events[160]. Furthermore, a novel constitutional frameshift mutation in *ALK* (p.V1471fs*6) has been found in one of the patients (NB63R4), which is predicted to produce a truncated protein. Despite the relevance of *ALK* alterations in NB, it is difficult to predict the possible oncogenic role of this novel variation, as it is expected to lose essential ALK activation phosphorylation sites. Moreover, rare variants in cancer predisposition genes in the germline DNA were detected: *BARD1* nonsense variant in patient NB63R4, *WRN* missense mutation in NB59R9 and missense variants in *CHEK2* and *BRCA2* in NB67R9. Constitutional mutations in *BARD1* and *CHEK2* have been described as enriched in NB patients[51,161], while *BRCA2* mutations have been described in pediatric cancer patients[162]. *WRN* is relevant in the genome instability maintenance, since the constitutional missense variant detected (p.S1141L) affects an essential ATM phosphorylation site promoting high genomic instability[163].

With regards to somatic mutations, 529 nonsynonymous variants were detected in total in the analyzed tumors. Comparing the overall somatic variants between time of diagnosis and time of recurrence, the mutational ratio of relapsed/progression tumors was significantly higher than the tumors at the time of diagnosis. Additionally, the increase of non-synonymous single nucleotide variants (SNVs) were also correlated with the age of the patients at the time of diagnosis or at the time of relapse, in line with previous studies of relapsed and refractory NB[51,52]. Approximately 50% of all the mutations detected in the tumors at the time of diagnosis were maintained in the relapsed/refractory tumor, constituting around 23% of the mutations for the latter tumor. This excludes patient NB60R6: no shared somatic nonsynonymous SNVs could be detected in this case (but the tumor set shares an identical translocation between chromosomes 11 and 17) (Figure 6).

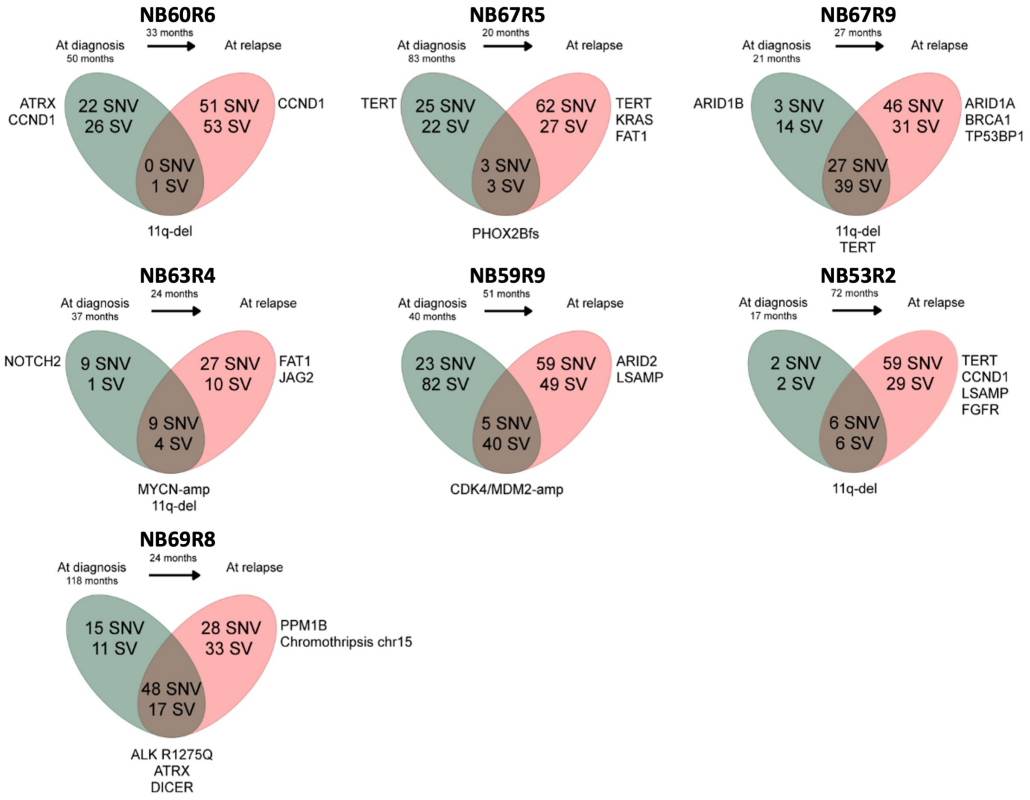

Figure 6. Case-by-case overview of biologically relevant somatic SVs and SNVs in primary and relapsed/progressive tumors[53].

Tumors from 4 out of 7 patients presented somatic alterations related to ALK-MAPK-RAS signaling pathway, which has previously been associated with relapsed NB[50]. The mutations found were *ALK*, *MAP3K14*, *RAS*, *FGR1* and indirectly *FAT1,* which is a tumor-suppressor gene that regulates the activity of YAP, a critical effector of RAS.

CNVs and SVs were also increased at the time of relapse/progression compared to the primary tumors. Relapsed tumors presented CNVs that are related to poor prognosis and were already present in the primary tumor such as 17q-gain (present in 6 out of 7 patients), whole chromosome 17 gain (in 1 patient), 11q-deletion (in 4 patients), *MYCN*-amplification (in 1 patient), *CDK4*/*MDM2*/*FRS2* amplification (in 1 patient) and 1p-deletion (in 3 patients). Besides, 1p-deletion was also found in NB60R6 only in the primary tumor, in NB53R2 only in the relapsed tumor and in both primary and relapsed tumors of NB67R5, but at different breakpoints.

Recurrent SVs in association with *TERT* (3 cases, 1 relapsed specific) were detected, which is a gene involved in telomere maintenance. Interestingly, two of the patients had a tandem duplication that causes *ATRX* disruption, a gene involved in chromatin remodeling. *CCND1* SVs (3 cases, 1 relapsed specific), a gene involved in the cell cycle progression, were also found. Surprisingly, *TERT* SVs in NB67R5 and *CCND1* SVs in NB60R6 are present in both the primary and relapsed tumors, but they have different breakpoints and different translocation partners, which indicates that those alterations may have emerged as parallel events. It should be noted that in addition to two different *TERT* SVs in NB67R5, this patient also presented two different mutations in the chromatin-associated genes, *ARID1A* and *ARID1B*, at time of diagnosis and time of relapse, respectively.

From all determined genetic variants, recurrent genetic alterations in 32 genes have been detected in two patients of which 8 were relapse specific: *MUC16*, *LSAMP*, *CDK5RAP1*, *CFAP44*, *NUBPL*, *POLR2A*, *PRR12* and *SC6A18*.

To sum up, we detected recurrent somatic mutations in genes related with RAS-MAPK pathway including: *ALK* (in primary and relapse), *KRAS* (relapse specific), *FGFR1* (relapse specific), *MAP3K14* (in primary and relapse) and *FAT1* (in primary and relapse). Apart from the *MYCN*-amplified case, in which *TERT* expression may be elevated driven by MYCN producing sustained telomer length, all six non-*MYCN*-amplified NB cases contain alterations in genes related to telomere maintenance or chromatin remodeling. Three of the cases presented rearrangements in *TERT* (one of these cases also has mutations in *ARID1A* at diagnosis, and *ARID1B* at relapse), two of the cases had alterations in *ATRX*, and one presented a mutation in *ARID2*.

This study remarks the complexity of NB tumors and the high intra-tumoral and inter-patient heterogeneity, hindering the prognosis and treatment of the disease. The findings of this project highlight the relevance of developing a possible combination therapy inhibiting MAPK signaling, cell cycle progression and telomere activity.

Paper III

The analysis of the SVs, performed in our lab, detected a recurrent focal chromosomal rearrangement at chromosomal region 3q13.31 using WGS, which was confirmed by SNP-array. This rearrangement is affecting directly inside of the limbic system-associated membrane protein gene (*LSAMP*) (Figure 7). LSAMP has been reported to be present in chick neural crest cells, at level of the trunk and also in the notochord, suggesting that it could be involved in the interaction and segregation of neural crest cells[164]. Furthermore, this gene has been found to play a selective role during neuronal growth and axon guidance in the developing limbic system, affecting neurite outgrowth[165]. Apart from that, *LSAMP* has also been implicated as a tumor suppressor gene in myeloid leukemia, ovarian cancer, osteosarcoma, prostatic cancer, epithelioid glioblastoma[166-173] and with mental disorders[174,175] .

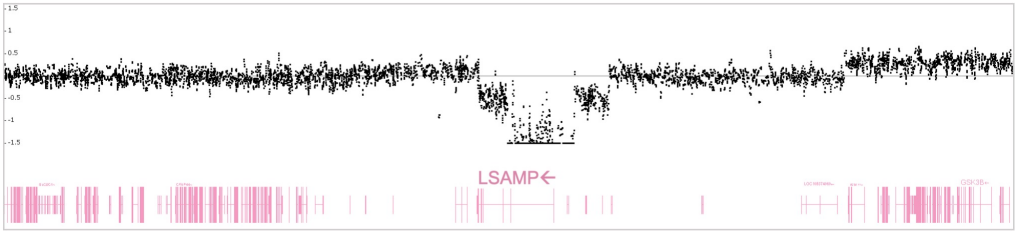

Figure 7. SNP-array profile of a NB cell line, showing a focal chromosomal deletion in *LSAMP*.

Due to the relationship of *LSAMP* in cell adhesion, neurite growth and its demonstrated implication as a tumor suppressor gene in other cancer types, we wanted to further study the implication of *LSAMP* in NB.

LSAMP SVs were detected by WGS analyses of tumor DNA in 6 out of a total of 35 patients, which was confirmed by SNP-microarray analysis. Two of the patients with *LSAMP* SVs presented the alteration only in the relapsed tumor, as we described in our primary/relapse investigation[53]. SNP-microarray performed on NB cell lines showed that 7 out of 16 cell lines were positive for rearrangements in *LSAMP*. These rearrangements are targeting the gene and are expected to produce an expressional or structural dysregulation. The higher occurrence in cell lines relative to patient tumor samples indicates that this might be a later event contributing to tumor progression rather than initiation.

Functional evaluations using *in vitro* experiments indicate that *LSAMP* has a tumor suppressor role in NB, as silencing of this gene causes an increase of cell proliferation and viability while re-/over-expression of it produces the opposite.

Moreover, a study of 498 NB patients in the R2 genomic analysis and visualization platform shows that patients with low *LSAMP* expression have worse overall and eventfree survival probability than those with high expression.

Further investigations regarding LSAMP should be performed in order to completely understand the mechanisms by which this gene may be involved in the progression of NB.

Paper IV

Although amplifications in chromosome 12 are not common in primary NB tumors, high-grade amplification of one or both chromosomal regions 12q13.3-14.1 and 12q15 were detected in a subset of NB tumors, commonly involving the *CDK4* (12q14.1) and *MDM2* (12q15) genes (Figure 8), which are potential oncogenic targets. Interestingly, amplifications between these amplicons, such as an amplification block, have not been detected. The two loci were co-amplified in 13 out of 17 tumors from this subset. Three other tumors presented *CDK4* amplification in the absence of *MDM2* amplification, while one showed *MDM2* amplification without *CDK4* amplification. Interestingly, some of the tumors with 12q-ampification had unique clinical features, including initial suspicion of Wilms' tumor and an atypical metastatic pattern. Besides, specific 12q-amplifications have also been detected in other cancer types such as sarcoma, glioma and melanoma[140,141].

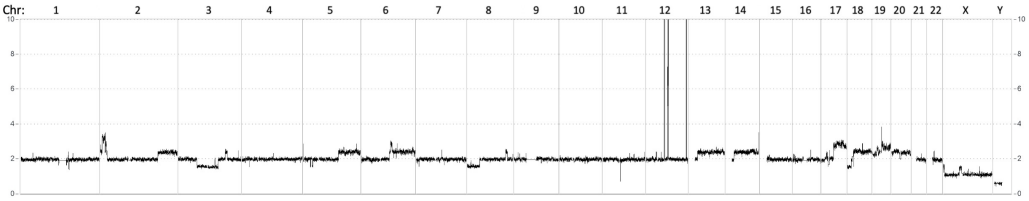

Figure 8. SNP-array profile of a NB cell line, which presents 12q13.3-14.1 and 12q15 amplifications.

The genes *CDK4* and *MDM2*, commonly included in the amplicons, play an important role in cancer growth and progression. *CDK4* is involved in the cell division cycle, helping to drive the progression of the cells into DNA synthetic (S) phase[176], whereas *MDM2* is a negative regulator of the tumor suppressor p53, having a relevant role in its transcriptional activity and protein stability[177].

Gene expression status of 31 genes located in the 12q-amplified regions were investigated for better understanding of the possible implications of these alterations, showing that 23 of these genes responded to the amplification with a significant increase in the expression levels.

Taking into consideration that *CDK4* and *MDM2* are pharmacological targets already used in cancer therapy investigations, we were interested in the study of the effects of their inhibitors, either as single drug or in combination (MDM2i and CDK4/6i), in the progression of NB, and if a combinational therapy would be beneficial for this subset of patients. Ten NB cell lines with different genetic background were used for this purpose. Viability experiments show that almost all studied cell lines, except one, were sensitive to CDK4/6 inhibitors, Ribociclib and Abemaciclib. Interestingly, the resistant cell line, SK-N-FI, has high expression levels of *MDR1* (Multidrug Resistance Protein 1), which could possibly lead to the mentioned resistance. Regarding MDM2i (Nutlin-3a), the response of the cell lines to the treatment was related to the p53 mutational status. Cell lines with wild type p53 function were sensitive to Nutlin-3a, including the two cell lines (LS and NGP) with *CDK4* and *MDM2* co-amplification that showed a level of sensitivity similar to the other $p53^{wt}$ cell lines, while the ones with $p53^{mut}$ showed resistance to the treatment.

In connection with combined MDM2 and CDK4/6i therapy, even though non-significant synergetic effects were observed in $p53^{wt}$ cell lines, the viability results showed an additive effect between the drugs; the same result has been obtained in *CDK4* and *MDM2* co-amplified cell lines. In the case of $p53^{mut}$ cell lines, no positive response (even lightly antagonistic effect) of the drug combination was detected, probably due to lack of response to Nutlin-3a in these cells.

Considering that less than 2% of NB patients present p53 mutations at the time of diagnosis and about 15% at relapse time[178,179], a large group of NB patients may benefit from MDM2i and from a possible combination with CDK4/6i. Further investigations are required in order to analyze, *in vivo*, the efficacy of

the drug combination as well as the toxicity and the possibility of long-term treatment resistance.

Conclusions

Paper I

- Molecular analyses of NB PDOXs show that this model maintains strong intrinsic genetic, transcriptional and phenotypic stability after serial passaging for more than 2 years, preserving NB associated gene signatures.
- Multiple PDOXs established from a single patient tumor reveal functional and molecular spatial ITH presenting diverse growth rates as well as transcriptional, proteomic and phosphoproteomic profiles.

Paper II

- Relapsed tumors generally present higher mutational load and additional segmental variants compared to tumors at the time of diagnosis.
- WGS of recurrent NB tumors reveals somatic alterations in the relapsed tumors which affects crucial promotors of tumor progression, including genes involved in RAS-MAPK signaling, cell cycle progression and telomere maintenance.
- Rare genetic germline variants in DNA repair genes were detected which may be cooperating with the acquisition of somatic variants, contributing to the development of highly aggressive recurrent NB.

Paper III

- Focal rearrangement in the chromosomal region 3q13.31 within the *LSAMP* gene was detected.
- *LSAMP* presented a tumor suppressing function in NB.

Paper IV

- High grade amplification on 12q was detected in a subgroup of NB patients involving the oncogenes *CDK4*, located at 12q13-14 and/or *MDM2*, located at 12q15.
- The majority of 12q-amplified tumors arose in the abdominal area, some with an atypical metastatic pattern.

- MDM2 inhibition by Nutlin-3a is highly dependent on *TP53* status.
- Combined inhibition of CDK4/6 and MDM2 presented an additive effect in p53 wild-type cell lines.

Future perspectives

In spite of the neuroblastoma multimodal treatment advances, scientific efforts and substantial prognosis during the last decade, the long-term survival of HR-NB children remains below 50% at the time of diagnosis and below 10% in case of relapsed and refractory metastatic disease. Furthermore, the therapy in many cases produces long-term side effects due to the toxicity of the treatment, which commonly targets fast growing cells, being especially problematic for children whose bodies are growing.

We believe that the incorporation of next generation sequencing analysis, such as WGS, into clinical practice could increase the survival rate of the patients. WGS is a versatile tool which provides reliable patient specific information of somatic mutations in tumors including SNV, CNVs and SVs as well as constitutional alterations in germline. WGS can provide a more comprehensive approach for detection of genomic abnormalities with rapid determination of biomarkers for diagnosis/prognosis, and most importantly, it is a suitable starting point for personalized treatment, based on specific tumor targets for a specific patient. The new findings can lead not only to a better stratification of the patients, but also to avoid unnecessary treatment.

During these studies we have, by using SNP-microarrays and next generation sequencing, characterized a neuroblastoma subset (12q13.3-14.1 and 12q15 co-amplified) that shares distinct clinical presentation as well as genetical alterations, allowing us to analyze possible therapeutic targets. We also detected recurrent breakpoints affecting *LSAMP,* a gene which seems to have an anti-proliferative effect in NB cell lines. Moreover, we could explore the ITH in PDOXs and genetical differences between primary/relapsed tumor sets which highlighted the importance of MAPK signaling, cell cycle progression and telomere activity in this specific group.

Tumors with high degree of ITH are often more aggressive due to subclones that can escape to the therapy leading to chemoresistance and relapse. Capturing ITH and clonal evolution during time can guide us to relevant information in order to establish appropriate specific therapeutic strategies. Consequently, we believe in the need to build routine protocols in the clinic for cell-free DNA extraction (liquid biopsies), a technique based on circulating cell-free DNA from

the tumors that will allow us to study the genetics of the tumor over time in a non-invasive way.

Our ultimate goal is to better understand the genetic alterations that leads to HR-NB to facilitate the prognosis, helping also to approach to a truly personalized therapy for NB patients, although this is a complex task due to the complications of ITH and sub-clonal evolution.

Acknowledgments

This journey has come to an end, thereby I would like to thank all of you who have made it possible:

My supervisor, **Susanne Fransson**, for giving me the opportunity to work on this interesting and challenging project, allowing me to be independent but still being there when I needed it. Thanks for the interesting discussions, for your always optimistic attitude and for helping me to successfully reach the end. I really appreciate all your funny “bad” jokes and chocolate candies.

My co-supervisor, **Tommy Martinsson**, for sharing your great knowledge. I could always knock on your door when I was in doubt. Thanks for all the scientific/life fun facts that you share with us, such as “Have you heard that if you put your finger in DMSO, you get a garlic taste in your mouth?”. I truly enjoyed them.

All members in Tommy Martinsson’s group. It has been a pleasure to work with you. **Anna Djos**, for our scientific and regular day life talks, I love them. Many thanks for always caring about how I am doing. **Niley**, for all our fun talks, late at the lab, that made me laugh and de-stress. With you I have learned adorable cultural details that we, Iranians and Spaniards, share. **Jennie**, I am grateful for having you as a lab-partner these last years. It has been very inspiring to see your enthusiasm to learn and to work hard. I love your great sense of humor that light up my mood when I need it, you have been an awesome colleague. And to **Rose-Marie**, for our nice talks and your advice, for taking care of me since my beginning in the lab and for believing in me. You have been an important support for me, and I am sure that for the rest of the group as well.

The people at Clinical Genetics for creating a nice working environment, especially to **Maria Yhr, Catarina Darnfors** and recently retired **Ylva Sirenius**, for the nice chats in the lunchroom, for your kindness and for always willing to help.

SciLifeLab Clinical Genomics core facility and Bioinformatics core facility in Gothenburg, for your assistance. You have always been so friendly.

My previous colleagues: **Camilla Ingeson-Carlsson**, **Tajana Tesan Tomic** and **Susanne Reingsbach**, for your guidance.

My lovely friends from Cancer Center: **Maryam** and **Soheila** for your kindness and fun, and for introducing me to the fascinating world of Iranian food! **Elena**, **Paloma** and **Clo**, thanks for the nice talks and laughs. It is always nice to see you!

My **biologuillos**, we started in the science world together and here we are, still sharing a passion. Specially to **Moises, Cosme**, **Jose** and **Pili**, you made my time in university the best. My Mari Pili, thanks for your help and for inspiring me, I wouldn't have gotten this far without you. You are amazing friends!

Thank you to all my dear friends who give me the strength to go through the difficulties and made my life more gorgeous and fun. **Kristell** and **Esther**, my unconditional friends who have always been there, no matter what, for both good and bad, I am eternally grateful to have you in my life. **Emil** and **Jonas**, my favorite Swedish people, you are so much fun guys. Also, to **Eva**, **Mima**, **Alfredo**, **Bea D., Raquel, Xisco, Nuria, Bea, Glo, Julio, Vero, Patricia** and **Sus,** you are gold!

My family "in-law", for all the love and care. Thanks for your wonderful musical contributions during my writing, it helped me a lot! I really appreciate you.

My beautiful family. Gracias a mi familia por todo el apoyo y el cariño que me habéis dado. Especialmente a mis padres, **Ángela** y **Marcelino**, sois mis mejores referentes en la vida, no tengo palabras para agradeceros todo lo que habéis hecho siempre por mi; no estaría donde estoy si no fuese por vosotros. Gracias por creer en mi y darme la motivación para seguir avanzando, por estar a mi lado y por mimarme tanto. A mi hermano, **Jorge**, gracias por tu apoyo incondicional, siempre sabes como animarme y sacarme una sonrisa, ¡eres lo más! A mi abuela, **Catalina**, tenerte es un regalo, gracias por además de mi abuela, ser mi amiga. ¡Os quiero!

My dear **Iñigo**, you have been my great support from the day one. Without you, none of this would have been possible. Thanks for giving me so much. Zuri, nire alboan egon zarenari, behar nuen makulua izan zarenari, inoiz ezingo dizut emandako laguntza nahikoa eskertu. Zu nire bizitzan izateak zortedun sentiarazten nau.

Finally, I would like to extend my sincere gratitude to all the families of the children with neuroblastoma for willing to contribute to cancer research.

To start a great project, it takes courage. To finish a great project, it takes perseverance. – Anonymous

References

1. ECIS. European Cancer Information System. 2020 Available from: https://ecis.jrc.ec.europa.eu; accessed on 30/December/2020
2. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011; 144: 646–74.
3. Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Diaz LA, Kinzler KW. Cancer genome landscapes. Science 2013; 339: 1546–58.
4. Lee EYHP, Muller WJ. Oncogenes and Tumor Suppressor Genes. Cold Spring Harbor Perspectives in Biology 2010; 2: a003236–6.
5. White MC, Holman DM, Boehm JE, Peipins LA, Grossman M, Henley SJ. Age and cancer risk: a potentially modifiable relationship. Am J Prev Med 2014; 46: S7–15.
6. CDC. Centers for Disease Control and Prevention. Available from: https://www.cdc.gov/nchs/fastats/child-health.htm, accessed on 15/December/2020
7. Saletta F, Seng MS, Lau LMS. Advances in paediatric cancer treatment. Transl Pediatr 2014; 3: 156–82.
8. Tomasetti C, Vogelstein B. Cancer etiology. Variation in cancer risk among tissues can be explained by the number of stem cell divisions. Science 2015; 347: 78–81.
9. Miller RW, Young JL, Novakovic B. Childhood cancer. Cancer 1995; 75: 395–405.
10. Linet MS, Wacholder S, Zahm SH. Interpreting epidemiologic research: lessons from studies of childhood cancer. Pediatrics 2003; 112: 218–32.
11. Ward E, DeSantis C, Robbins A, Kohler B, Jemal A. Childhood and adolescent cancer statistics, 2014. CA A Cancer Journal for Clinicians 2014; 64: 83–103.
12. Scotting PJ, Walker DA, Perilongo G. Childhood solid tumours: a developmental disorder. Nat Rev Cancer 2005; 5: 481–8.
13. Rahal Z, Abdulhai F, Kadara H, Saab R. Genomics of adult and pediatric solid tumors. Am J Cancer Res 2018; 8: 1356–86.
14. Tomasetti C, Vogelstein B, Parmigiani G. Half or more of the somatic mutations in cancers of self-renewing tissues originate prior to tumor initiation. Proceedings of the National Academy of Sciences 2013; 110: 1999–2004.
15. Matthay KK, Maris JM, Schleiermacher G, Nakagawara A, Mackall CL, Diller L, Weiss WA. Neuroblastoma. Nature Publishing Group 2016; 2: 16078–22.
16. Park JR, Bagatell R, London WB, Maris JM, Cohn SL, Mattay KK, Hogarty M. Children“s Oncology Group”s 2013 blueprint for research: neuroblastoma. Pediatr Blood Cancer 2013; 60: 985–93.
17. Brodeur GM. Neuroblastoma: biological insights into a clinical enigma. Nat Rev Cancer 2003; 3: 203–16.
18. Maris JM. Recent advances in neuroblastoma. N Engl J Med 2010; 362: 2202–11.
19. Park JR, Eggert A, Caron H. Neuroblastoma: biology, prognosis, and treatment. Hematol Oncol Clin North Am 2010; 24: 65–86.

20. Gatta G, Ferrari A, Stiller CA, Pastore G, Bisogno G, Trama A, Capocaccia R, RARECARE Working Group. Embryonal cancers in Europe. Eur J Cancer 2012; 48: 1425–33.

21. Vo KT, Matthay KK, Neuhaus J, London WB, Hero B, Ambros PF, Nakagawara A, Miniati D, Wheeler K, Pearson ADJ, Cohn SL, DuBois SG. Clinical, biologic, and prognostic differences on the basis of primary tumor site in neuroblastoma: a report from the international neuroblastoma risk group project. JCO 2014; 32: 3169–76.

22. Tolbert VP, Matthay KK. Neuroblastoma: clinical and biological approach to risk stratification and treatment. 2018; 372: 1–15.

23. Maris JM, Hogarty MD, Bagatell R, Cohn SL. Neuroblastoma. Lancet 2007; 369: 2106–20.

24. Oberthuer A, Juraeva D, Hero B, Volland R, Sterz C, Schmidt R, Faldum A, Kahlert Y, Engesser A, Asgharzadeh S, Seeger R, Ohira M, Nakagawara A, Scaruffi P, Tonini GP, Janoueix-Lerosey I, Delattre O, Schleiermacher G, Vandesompele J, Speleman F, Noguera R, Piqueras M, Bénard J, Valent A, Avigad S, Yaniv I, Grundy RG, Ortmann M, Shao C, Schwab M, Eils R, Simon T, Theissen J, Berthold F, Westermann F, Brors B, Fischer M. Revised risk estimation and treatment stratification of low- and intermediate-risk neuroblastoma patients by integrating clinical and molecular prognostic markers. Clinical Cancer Research 2015; 21: 1904–15.

25. Cheung N-KV, Dyer MA. Neuroblastoma: developmental biology, cancer genomics and immunotherapy. Nat Rev Cancer 2013; 13: 397–411.

26. Tsubota S, Kadomatsu K. Origin and initiation mechanisms of neuroblastoma. Cell Tissue Res 2018; 372: 211–21.

27. Furlan A, Dyachuk V, Kastriti ME, Calvo-Enrique L, Abdo H, Hadjab S, Chontorotzea T, Akkuratova N, Usoskin D, Kamenev D, Petersen J, Sunadome K, Memic F, Marklund U, Fried K, Topilko P, Lallemend F, Kharchenko PV, Ernfors P, Adameyko I. Multipotent peripheral glial cells generate neuroendocrine cells of the adrenal medulla. Science 2017; 357: eaal3753–12.

28. Tsubota S, Kadomatsu K. Origin and mechanism of neuroblastoma. Oncoscience 2017; 4: 70–2.

29. Mohlin SA, Wigerup C, Påhlman S. Neuroblastoma aggressiveness in relation to sympathetic neuronal differentiation stage. Seminars in Cancer Biology 2011; 21: 276–82.

30. Cheung N-KV, Dyer MA. Neuroblastoma: developmental biology, cancer genomics and immunotherapy. Nat Rev Cancer 2013; 13: 397–411.

31. Mossé YP, Laudenslager M, Longo L, Cole KA, Wood A, Attiyeh EF, Laquaglia MJ, Sennett R, Lynch JE, Perri P, Laureys G, Speleman F, Kim C, Hou C, Hakonarson H, Torkamani A, Schork NJ, Brodeur GM, Tonini GP, Rappaport E, Devoto M, Maris JM. Identification of ALK as a major familial neuroblastoma predisposition gene. Nature 2008; 455: 930–5.

32. Knudson AGJ, Strong LC. Mutation and cancer: neuroblastoma and pheochromocytoma. Am J Hum Genet 1972; 24: 514–32.

33. Kushner BH, Gilbert F, Helson L. Familial neuroblastoma. Case reports, literature review, and etiologic considerations. Cancer 1986; 57: 1887–93.

34. Liu Z, Thiele CJ. ALK and MYCN: when two oncogenes are better than one. Cancer Cell 2012; 21: 325–6.

35. Tolbert VP, Coggins GE, Maris JM. Genetic susceptibility to neuroblastoma. Current Opinion in Genetics & Development 2017; 42: 81–90.

36. Trochet D, Bourdeaut F, Janoueix-Lerosey I, Deville A, de Pontual L, Schleiermacher G, Coze C, Philip N, Frebourg T, Munnich A, Lyonnet S, Delattre

O, Amiel J. Germline mutations of the paired-like homeobox 2B (PHOX2B) gene in neuroblastoma. Am J Hum Genet 2004; 74: 761–4.

37. Raabe EH, Laudenslager M, Winter C, Wasserman N, Cole K, LaQuaglia M, Maris DJ, Mosse YP, Maris JM. Prevalence and functional consequence of PHOX2B mutations in neuroblastoma. Oncogene 2008; 27: 469–76.

38. Serra A, Häberle B, König IR, Kappler R, Suttorp M, Schackert HK, Roesner D, Fitze G. Rare occurrence of PHOX2b mutations in sporadic neuroblastomas. J Pediatr Hematol Oncol 2008; 30: 728–32.

39. Kohl NE, Kanda N, Schreck RR, Bruns G, Latt SA, Gilbert F, Alt FW. Transposition and amplification of oncogene-related sequences in human neuroblastomas. Cell 1983; 35: 359–67.

40. Chen Y, Takita J, Choi YL, Kato M, Ohira M, Sanada M, Wang L, Soda M, Kikuchi A, Igarashi T, Nakagawara A, Hayashi Y, Mano H, Ogawa S. Oncogenic mutations of ALK kinase in neuroblastoma. Nature 2008; 455: 971–4.

41. Bosse KR, Maris JM. Advances in the translational genomics of neuroblastoma: From improving risk stratification and revealing novel biology to identifying actionable genomic alterations. Cancer 2015; 122: 20–33.

42. Tonini GP. Growth, progression and chromosome instability of Neuroblastoma: a new scenario of tumorigenesis? BMC Cancer 2017; 17: 1–6.

43. Multicentre analysis of patterns of DNA gains and losses in 204 neuroblastoma tumors: how many genetic subgroups are there? 2001; 36: 5–10.

44. Cohn SL, Pearson ADJ, London WB, Monclair T, Ambros PF, Brodeur GM, Faldum A, Hero B, Iehara T, Machin D, Mosseri V, Simon T, Garaventa A, Castel V, Matthay KK. The International Neuroblastoma Risk Group (INRG) classification system: an INRG Task Force report. JCO 2009; 27: 289–97.

45. Chen Q-R, Bilke S, Wei JS, Whiteford CC, Cenacchi N, Krasnoselsky AL, Greer BT, Son C-G, Westermann F, Berthold F, Schwab M, Catchpoole D, Khan J. BMC Genomics. BMC Genomics 2004; 5: 70–13.

46. Molenaar JJ, Koster J, Zwijnenburg DA, van Sluis P, Valentijn LJ, van der Ploeg I, Hamdi M, van Nes J, Westerman BA, van Arkel J, Ebus ME, Haneveld F, Lakeman A, Schild L, Molenaar P, Stroeken P, van Noesel MM, Øra I, Santo EE, Caron HN, Westerhout EM, Versteeg R. Sequencing of neuroblastoma identifies chromothripsis and defects in neuritogenesis genes. Nature 2012; 483: 589–93.

47. Pandey GK, Mitra S, Subhash S, Hertwig F, Kanduri M, Mishra K, Fransson S, Ganeshram A, Mondal T, Bandaru S, Östensson M, Akyürek LM, Abrahamsson J, Pfeifer S, Larsson E, Shi L, Peng Z, Fischer M, Martinsson T, Hedborg F, Kogner P, Kanduri C. The Risk-Associated Long Noncoding RNA NBAT-1 Controls Neuroblastoma Progression by Regulating Cell Proliferation and Neuronal Differentiation. Cancer Cell 2014; 26: 722–37.

48. Estimation of copy number aberrations: Comparison of exome sequencing data with SNP microarrays identifies homozygous deletions of 19q13.2 and CIC in neuroblastoma. 2016; 48: 1103–16.

49. Whittle SB, Smith V, Doherty E, Zhao S, McCarty S, Zage PE. Overview and recent advances in the treatment of neuroblastoma. Expert Review of Anticancer Therapy 2017; 17: 369–86.

50. Schleiermacher G, Javanmardi N, Bernard V, Leroy Q, Cappo J, Rio Frio T, Pierron G, Lapouble E, Combaret V, Speleman F, de Wilde B, Djos A, Øra I, Hedborg F, Träger C, Holmqvist B-M, Abrahamsson J, Peuchmaur M, Michon J, Janoueix-Lerosey I, Kogner P, Delattre O, Martinsson T. Emergence of New ALKMutations at Relapse of Neuroblastoma. JCO 2014; 32: 2727–34.

51. Eleveld TF, Oldridge DA, Bernard V, Koster J, Colmet Daage L, Diskin SJ, Schild L, Bentahar NB, Bellini A, Chicard M, Lapouble E, Combaret V, Legoix-Né P, Michon J, Pugh TJ, Hart LS, Rader J, Attiyeh EF, Wei JS, Zhang S, Naranjo A,

Gastier-Foster JM, Hogarty MD, Asgharzadeh S, Smith MA, Guidry Auvil JM, Watkins TBK, Zwijnenburg DA, Ebus ME, van Sluis P, Hakkert A, van Wezel E, van der Schoot CE, Westerhout EM, Schulte JH, Tytgat GA, Dolman MEM, Janoueix-Lerosey I, Gerhard DS, Caron HN, Delattre O, Khan J, Versteeg R, Schleiermacher G, Molenaar JJ, Maris JM. Relapsed neuroblastomas show frequent RAS-MAPK pathway mutations. Nat Genet 2015; 47: 864–71.

52. Schramm A, Köster J, Assenov Y, Althoff K, Peifer M, Mahlow E, Odersky A, Beisser D, Ernst C, Henssen AG, Stephan H, Schröder C, Heukamp L, Engesser A, Kahlert Y, Theissen J, Hero B, Roels F, Altmüller J, Nürnberg P, Astrahantseff K, Gloeckner C, De Preter K, Plass C, Lee S, Lode HN, Henrich K-O, Gartlgruber M, Speleman F, Schmezer P, Westermann F, Rahmann S, Fischer M, Eggert A, Schulte JH. Mutational dynamics between primary and relapse neuroblastomas. Nat Genet 2015; 47: 872–7.

53. Fransson S, Martinez-Monleon A, Johansson M, Sjöberg R-M, Björklund C, Ljungman G, Ek T, Kogner P, Martinsson T. Whole-genome sequencing of recurrent neuroblastoma reveals somatic mutations that affect key players in cancer progression and telomere maintenance. Sci Rep 2020; 10: 22432.

54. Braekeveldt N, Stedingk von K, Fransson S, Martinez-Monleon A, Lindgren D, Axelson H, Levander F, Willforss J, Hansson K, Øra I, Backman T, Börjesson A, Beckman S, Esfandyari J, Berbegall AP, Noguera R, Karlsson J, Koster J, Martinsson T, Gisselsson D, Påhlman S, Bexell D. Patient-Derived Xenograft Models Reveal Intratumor Heterogeneity and Temporal Stability in Neuroblastoma. Cancer Res 2018; 78: 5958–69.

55. Javanmardi N, Fransson S, Djos A, berg R-MSX, Nilsson S, x000E9 KT, Kogner P, Martinsson T. Low Frequency ALK Hotspots Mutations In Neuroblastoma Tumours Detected By Ultra-deep Sequencing: Implications For ALK Inhibitor Treatment. Sci Rep 2019 Feb 6. doi: 10.1038/s41598-018-37240-z: 1–12.

56. Stedingk von K, Gisselsson D, Bexell D. Multidimensional intratumour heterogeneity in neuroblastoma. Oncotarget 2019; 10: 3–5.

57. Monclair T, Brodeur GM, Ambros PF, Brisse HJ, Cecchetto G, Holmes K, Kaneko M, London WB, Matthay KK, Nuchtern JG, Schweinitz von D, Simon T, Cohn SL, Pearson ADJ. The International Neuroblastoma Risk Group (INRG) staging system: an INRG Task Force report. JCO 2009; 27: 298–303.

58. Øra I, Eggert A. Progress in treatment and risk stratification of neuroblastoma: impact on future clinical and basic research. Seminars in Cancer Biology 2011; 21: 217–28.

59. Vik TA, Pfluger T, Kadota R, Castel V, Tulchinsky M, Farto JCA, Heiba S, Serafini A, Tumeh S, Khutoryansky N, Jacobson AF. (123)I-mIBG scintigraphy in patients with known or suspected neuroblastoma: Results from a prospective multicenter trial. Pediatr Blood Cancer 2009; 52: 784–90.

60. Yang J, Codreanu I, Servaes S, Zhuang H. I-131 MIBG post-therapy scan is more sensitive than I-123 MIBG pretherapy scan in the evaluation of metastatic neuroblastoma. Nucl Med Commun 2012; 33: 1134–7.

61. Moreno L, Barone G, DuBois SG, Molenaar J, Fischer M, Schulte J, Eggert A, Schleiermacher G, Speleman F, Chesler L, Geoerger B, Hogarty MD, Irwin MS, Bird N, Blanchard GB, Buckland S, Caron H, Davis S, de Wilde B, Deubzer HE, Dolman E, Eilers M, George RE, George S, Jaroslav Š, Maris JM, Marshall L, Merchant M, Mortimer P, Owens C, Philpott A, Poon E, Shay JW, Tonelli R, Valteau-Couanet D, Vassal G, Park JR, Pearson ADJ. Accelerating drug development for neuroblastoma: Summary of the Second Neuroblastoma Drug Development Strategy forum from Innovative Therapies for Children with Cancer and International Society of Paediatric Oncology Europe Neuroblastoma. Eur J Cancer 2020; 136: 52–68.

62. Savelyeva L, Schwab M. Amplification of oncogenes revisited: from expression profiling to clinical application. Cancer Lett 2001; 167: 115–23.

63. Zhao X, Li C, Paez JG, Chin K, Jänne PA, Chen T-H, Girard L, Minna J, Christiani D, Leo C, Gray JW, Sellers WR, Meyerson M. An integrated view of copy number and allelic alterations in the cancer genome using single nucleotide polymorphism arrays. Cancer Res 2004; 64: 3060–71.

64. Tanaka T, Hiyama E, Sugimoto T, Sawada T, Tanabe M, Ida N. trk A gene expression in neuroblastoma. The clinical significance of an immunohistochemical study. Cancer 1995; 76: 1086–95.

65. Shimada H, Ambros IM, Dehner LP, Hata J, Joshi VV, Roald B, Stram DO, Gerbing RB, Lukens JN, Matthay KK, Castleberry RP. The International Neuroblastoma Pathology Classification (the Shimada system). Cancer 1999; 86: 364–72.

66. Lonergan GJ, Schwab CM, Suarez ES, Carlson CL. Neuroblastoma, ganglioneuroblastoma, and ganglioneuroma: radiologic-pathologic correlation. Radiographics 2002; 22: 911–34.

67. Brodeur GM, Pritchard J, Berthold F, Carlsen NL, Castel V, Castelberry RP, De Bernardi B, Evans AE, Favrot M, Hedborg F. Revisions of the international criteria for neuroblastoma diagnosis, staging, and response to treatment. JCO 1993; 11: 1466–77.

68. Jiang M, Stanke J, Lahti JM. The Connections Between Neural Crest Development and Neuroblastoma. In: Cancer and Development. Elsevier, 2011. pages 77–127.

69. van Noesel MM, Versteeg R. Pediatric neuroblastomas: genetic and epigenetic 'danse macabre'. Gene 2004; 325: 1–15.

70. Modak S, Cheung N-KV. Neuroblastoma: Therapeutic strategies for a clinical enigma. Cancer Treatment Reviews 2010; 36: 307–17.

71. Swift CC, Eklund MJ, Kraveka JM, Alazraki AL. Updates in Diagnosis, Management, and Treatment of Neuroblastoma. Radiographics 2018; 38: 566–80.

72. Trigg RM, Turner SD. ALK in Neuroblastoma: Biological and Therapeutic Implications. Cancers 2018; 10: 1–16.

73. Grau E, Martinez F, Orellana C, Cañete A, Yañez Y, Oltra S, Noguera R, Hernandez M, Bermúdez JD, Castel V. Hypermethylation of apoptotic genes as independent prognostic factor in neuroblastoma disease. Mol Carcinog 2011; 50: 153–62.

74. Zhu J, Yao X. Use of DNA methylation for cancer detection and molecular classification. J Biochem Mol Biol 2007; 40: 135–41.

75. Sakai T, Toguchida J, Ohtani N, Yandell DW, Rapaport JM, Dryja TP. Allele-specific hypermethylation of the retinoblastoma tumor-suppressor gene. Am J Hum Genet 1991; 48: 880–8.

76. Michalowski MB, de Fraipont F, Plantaz D, Michelland S, Combaret V, Favrot M-C. Methylation of tumor-suppressor genes in neuroblastoma: The RASSF1A gene is almost always methylated in primary tumors. Pediatr Blood Cancer 2008; 50: 29–32.

77. Aygun N. Biological and Genetic Features of Neuroblastoma and Their Clinical Importance. Curr Pediatr Rev 2018; 14: 73–90.

78. Eggert A, Ikegaki N, Liu XG, Brodeur GM. Prognostic and biological role of neurotrophin-receptor TrkA and TrkB in neuroblastoma. Klin Padiatr 2000; 212: 200–5.

79. Kogner P, Barbany G, Dominici C, Castello MA, Raschellà G, Persson H. Coexpression of messenger RNA for TRK protooncogene and low affinity nerve growth factor receptor in neuroblastoma with favorable prognosis. Cancer Res 1993; 53: 2044–50.

80. Nakagawara A, Arima-Nakagawara M, Scavarda NJ, Azar CG, Cantor AB, Brodeur GM. Association between high levels of expression of the TRK gene and favorable outcome in human neuroblastoma. N Engl J Med 1993; 328: 847–54.

81. Rydén M, Sehgal R, Dominici C, Schilling FH, Ibáñez CF, Kogner P. Expression of mRNA for the neurotrophin receptor trkC in neuroblastomas with favourable tumour stage and good prognosis. British Journal of Cancer 1996; 74: 773–9.

82. Yamashiro DJ, Nakagawara A, Ikegaki N, Liu XG, Brodeur GM. Expression of TrkC in favorable human neuroblastomas. Oncogene 1996; 12: 37–41.

83. Nakagawara A, Azar CG, Scavarda NJ, Brodeur GM. Expression and function of TRK-B and BDNF in human neuroblastomas. Mol Cell Biol 1994; 14: 759–67.

84. Plantaz D, Mohapatra G, Matthay KK, Pellarin M, Seeger RC, Feuerstein BG. Gain of chromosome 17 is the most frequent abnormality detected in neuroblastoma by comparative genomic hybridization. The American Journal of Pathology 1997; 150: 81–9.

85. Juan Ribelles A, Gargallo P, Ferriol C, Segura V, Yáñez Y, Juan B, Cañada AJ, Font de Mora J, Cañete A, Castel V. Distribution of segmental chromosomal alterations in neuroblastoma. Clin Transl Oncol 2020 Sepdoi: 10.1007/s12094-020-02497-2

86. Ladenstein R, Ambros IM, Pötschger U, Amann G, Urban C, Fink FM, Schmitt K, Jones R, Slociak M, Schilling F, Ritter J, Berthold F, Gadner H, Ambros PF. Prognostic significance of DNA di-tetraploidy in neuroblastoma. Med Pediatr Oncol 2001; 36: 83–92.

87. Kaneko Y, Knudson AG. Mechanism and relevance of ploidy in neuroblastoma. Genes Chromosomes Cancer 2000; 29: 89–95.

88. Maris JM, Matthay KK. Molecular biology of neuroblastoma. JCO 1999; 17: 2264–79.

89. Huang M, Weiss WA. Neuroblastoma and MYCN. Cold Spring Harbor Perspectives in Medicine 2013; 3: a014415–5.

90. Guo C, White PS, Weiss MJ, Hogarty MD, Thompson PM, Stram DO, Gerbing R, Matthay KK, Seeger RC, Brodeur GM, Maris JM. Allelic deletion at 11q23 is common in MYCN single copy neuroblastomas. Oncogene 1999; 18: 4948–57.

91. Plantaz D, Vandesompele J, Van Roy N, Lastowska M, Bown N, Combaret V, Favrot MC, Delattre O, Michon J, Bénard J, Hartmann O, Nicholson JC, Ross FM, Brinkschmidt C, Laureys G, Caron H, Matthay KK, Feuerstein BG, Speleman F. Comparative genomic hybridization (CGH) analysis of stage 4 neuroblastoma reveals high frequency of 11q deletion in tumors lacking MYCN amplification. Int J Cancer 2001; 91: 680–6.

92. Carén H, Kryh H, Nethander M, Sjöberg R-M, Träger C, Nilsson S, Abrahamsson J, Kogner P, Martinsson T. High-risk neuroblastoma tumors with 11q-deletion display a poor prognostic, chromosome instability phenotype with later onset. Proceedings of the National Academy of Sciences 2010; 107: 4323–8.

93. Valentijn LJ, Koster J, Zwijnenburg DA, Hasselt NE, van Sluis P, Volckmann R, van Noesel MM, George RE, Tytgat GAM, Molenaar JJ, Versteeg R. TERT rearrangements are frequent in neuroblastoma and identify aggressive tumors. Nat Genet 2015; 47: 1411–4.

94. Peifer M, Hertwig F, Roels F, Dreidax D, Gartlgruber M, Menon R, Krämer A, Roncaioli JL, Sand F, Heuckmann JM, Ikram F, Schmidt R, Ackermann S, Engesser A, Kahlert Y, Vogel W, Altmüller J, Nürnberg P, Thierry-Mieg J, Thierry-Mieg D, Mariappan A, Heynck S, Mariotti E, Henrich K-O, Gloeckner C, Bosco G, Leuschner I, Schweiger MR, Savelyeva L, Watkins SC, Shao C, Bell E, Höfer T, Achter V, Lang U, Theissen J, Volland R, Saadati M, Eggert A, de Wilde B, Berthold F, Peng Z, Zhao C, Shi L, Ortmann M, Büttner R, Perner S, Hero B, Schramm A, Schulte JH, Herrmann C, O'Sullivan RJ, Westermann F, Thomas

RK, Fischer M. Telomerase activation by genomic rearrangements in high-risk neuroblastoma. Nature 2015; 526: 700–4.

95. Depuydt P, Koster J, Boeva V, Hocking TD, Speleman F, Schleiermacher G, De Preter K. Data Descriptor: Meta-mining of copy number profiles of high-risk neuroblastoma tumors. Sci Data 2018; 5: 1–9.

96. Michels E, Vandesompele J, De Preter K, Hoebeeck J, Vermeulen J, Schramm A, Molenaar JJ, Menten B, Marques B, Stallings RL, Combaret V, Devalck C, De Paepe A, Versteeg R, Eggert A, Laureys G, Van Roy N, Speleman F. ArrayCGH-based classification of neuroblastoma into genomic subgroups. Genes Chromosomes Cancer 2007; 46: 1098–108.

97. Tomioka N, Oba S, Ohira M, Misra A, Fridlyand J, Ishii S, Nakamura Y, Isogai E, Hirata T, Yoshida Y, Todo S, Kaneko Y, Albertson DG, Pinkel D, Feuerstein BG, Nakagawara A. Novel risk stratification of patients with neuroblastoma by genomic signature, which is independent of molecular signature. Oncogene 2008; 27: 441–9.

98. Maris JM, Weiss MJ, Guo C, Gerbing RB, Stram DO, White PS, Hogarty MD, Sulman EP, Thompson PM, Lukens JN, Matthay KK, Seeger RC, Brodeur GM. Loss of heterozygosity at 1p36 independently predicts for disease progression but not decreased overall survival probability in neuroblastoma patients: a Children's Cancer Group study. JCO 2000; 18: 1888–99.

99. Mora J, Cheung NK, Kushner BH, LaQuaglia MP, Kramer K, Fazzari M, Heller G, Chen L, Gerald WL. Clinical categories of neuroblastoma are associated with different patterns of loss of heterozygosity on chromosome arm 1p. J Mol Diagn 2000; 2: 37–46.

100. Ejeskär K, Sjoberg RM, Abel F, Kogner P, Ambros PF, Martinsson T. Fine mapping of a tumour suppressor candidate gene region in 1p36.2-3, commonly deleted in neuroblastomas and germ cell tumours. Med Pediatr Oncol 2001; 36: 61–6.

101. Bader SA, Fasching C, Brodeur GM, Stanbridge EJ. Dissociation of suppression of tumorigenicity and differentiation in vitro effected by transfer of single human chromosomes into human neuroblastoma cells. Cell Growth Differ 1991; 2: 245–55.

102. Xu C, Du Z, Ren S, Liang X, Li H. MiR-129-5p sensitization of lung cancer cells to etoposide-induced apoptosis by reducing YWHAB. J Cancer 2020; 11: 858–66.

103. Martinsson T, Sjoberg RM, Hallstensson K, Nordling M, Hedborg F, Kogner P. Delimitation of a critical tumour suppressor region at distal 1p in neuroblastoma tumours. Eur J Cancer 1997; 33: 1997–2001.

104. Bauer A, Savelyeva L, Claas A, Praml C, Berthold F, Schwab M. Smallest region of overlapping deletion in 1p36 in human neuroblastoma: a 1 Mbp cosmid and PAC contig. Genes Chromosomes Cancer 2001; 31: 228–39.

105. Bagchi A, Papazoglu C, Wu Y, Capurso D, Brodt M, Francis D, Bredel M, Vogel H, Mills AA. CHD5 is a tumor suppressor at human 1p36. Cell 2007; 128: 459–75.

106. Fransson S, Martinsson T, Ejeskär K. Neuroblastoma tumors with favorable and unfavorable outcomes: Significant differences in mRNA expression of genes mapped at 1p36.2. Genes Chromosomes Cancer 2007; 46: 45–52.

107. García-López J, Wallace K, Otero JH, Olsen R, Wang Y-D, Finkelstein D, Gudenas BL, Rehg JE, Northcott P, Davidoff AM, Freeman KW. Large 1p36 Deletions Affecting Arid1a Locus Facilitate Mycn-Driven Oncogenesis in Neuroblastoma. CellReports 2020; 30: 454–5.

108. Katoh M, Katoh M. Identification and characterization of FLJ10737 and CAMTA1 genes on the commonly deleted region of neuroblastoma at human chromosome 1p36.31-p36.23. Int J Oncol 2003; 23: 1219–24.

109. Henrich K-O, Schwab M, Westermann F. 1p36 tumor suppression--a matter of dosage? Cancer Res 2012; 72: 6079–88.

110. Liu Z, Yang X, Li Z, McMahon C, Sizer C, Barenboim-Stapleton L, Bliskovsky V, Mock B, Ried T, London WB, Maris J, Khan J, Thiele CJ. CASZ1, a candidate tumor-suppressor gene, suppresses neuroblastoma tumor growth through reprogramming gene expression. Cell Death and Differentiation 2011; 18: 1174–83.

111. Yang HW, Chen YZ, Takita J, Soeda E, Piao HY, Hayashi Y. Genomic structure and mutational analysis of the human KIF1B gene which is homozygously deleted in neuroblastoma at chromosome 1p36.2. Oncogene 2001; 20: 5075–83.

112. Shi H, Tao T, Abraham BJ, Durbin AD, Zimmerman MW, Kadoch C, Look AT. ARID1A loss in neuroblastoma promotes the adrenergic-to-mesenchymal transition by regulating enhancer-mediated gene expression. Sci Adv 2020; 6: eaaz3440.

113. Schwab M, Alitalo K, Klempnauer KH, Varmus HE, Bishop JM, Gilbert F, Brodeur G, Goldstein M, Trent J. Amplified DNA with limited homology to myc cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumour. Nature 1983; 305: 245–8.

114. Grimmer MR, Weiss WA. Childhood tumors of the nervous system as disorders of normal development. Curr Opin Pediatr 2006; 18: 634–8.

115. Zaizen Y, Taniguchi S, Noguchi S, Suita S. The effect of N-myc amplification and expression on invasiveness of neuroblastoma cells. J Pediatr Surg 1993; 28: 766–9.

116. Wang LL, Teshiba R, Ikegaki N, Tang XX, Naranjo A, London WB, Hogarty MD, Gastier-Foster JM, Look AT, Park JR, Maris JM, Cohn SL, Seeger RC, Asgharzadeh S, Shimada H. Augmented expression of MYC and/or MYCN protein defines highly aggressive MYC-driven neuroblastoma: a Children's Oncology Group study. British Journal of Cancer 2015; 113: 57–63.

117. Zimmerman MW, Liu Y, He S, Durbin AD, Abraham BJ, Easton J, Shao Y, Xu B, Zhu S, Zhang X, Li Z, Weichert-Leahey N, Young RA, Zhang J, Look AT. MYC Drives a Subset of High-Risk Pediatric Neuroblastomas and Is Activated through Mechanisms Including Enhancer Hijacking and Focal Enhancer Amplification. Cancer Discovery 2018; 8: 320–35.

118. Chiarle R, Voena C, Ambrogio C, Piva R, Inghirami G. The anaplastic lymphoma kinase in the pathogenesis of cancer. Nat Rev Cancer 2008; 8: 11–23.

119. Palmer RH, Vernersson E, Grabbe C, Hallberg B. Anaplastic lymphoma kinase: signalling in development and disease. Biochem J 2009; 420: 345–61.

120. George RE, Sanda T, Hanna M, Fröhling S, Luther W2, Zhang J, Ahn Y, Zhou W, London WB, McGrady P, Xue L, Zozulya S, Gregor VE, Webb TR, Gray NS, Gilliland DG, Diller L, Greulich H, Morris SW, Meyerson M, Look AT. Activating mutations in ALK provide a therapeutic target in neuroblastoma. Nature 2008; 455: 975–8.

121. Mossé YP. Anaplastic Lymphoma Kinase as a Cancer Target in Pediatric Malignancies. Clinical Cancer Research 2016; 22: 546–52.

122. Jeison M, Ash S, Halevy-Berko G, Mardoukh J, Luria D, Avigad S, Feinberg-Gorenshtein G, Goshen Y, Hertzel G, Kapelushnik J, Ben Barak A, Attias D, Steinberg R, Stein J, Stark B, Yaniv I. 2p24 Gain region harboring MYCN gene compared with MYCN amplified and nonamplified neuroblastoma: biological and clinical characteristics. The American Journal of Pathology 2010; 176: 2616–25.

123. Javanmardi N, Fransson S, Djos A, Umapathy G, Östensson M, Milosevic J, Borenäs M, Hallberg B, Kogner P, Martinsson T, Palmer RH. Analysis of ALK, MYCN, and the ALK ligand ALKAL2 (FAM150B/AUGα) in neuroblastoma patient samples with chromosome arm 2p rearrangements. Genes Chromosomes Cancer 2019 Juldoi: 10.1002/gcc.22790

124. Carén H, Abel F, Kogner P, Martinsson T. High incidence of DNA mutations and gene amplifications of the ALK gene in advanced sporadic neuroblastoma tumours. Biochem J 2008; 416: 153–9.

125. Boeva V, Jouannet S, Daveau R, Combaret V, Pierre-Eugène C, Cazes A, Louis-Brennetot C, Schleiermacher G, Ferrand S, Pierron G, Lermine A, Rio Frio T, Raynal V, Vassal G, Barillot E, Delattre O, Janoueix-Lerosey I. Breakpoint features of genomic rearrangements in neuroblastoma with unbalanced translocations and chromothripsis. PLoS ONE 2013; 8: e72182.

126. Siaw JT, Javanmardi N, Van den Eynden J, Lind DE, Fransson S, Martinez-Monleon A, Djos A, Sjöberg R-M, Östensson M, Carén H, Trøen G, Beiske K, Berbegall AP, Noguera R, Lai W-Y, Kogner P, Palmer RH, Hallberg B, Martinsson T. 11q Deletion or ALK Activity Curbs DLG2 Expression to Maintain an Undifferentiated State in Neuroblastoma. CellReports 2020; 32: 108171.

127. Lopez G, Conkrite KL, Doepner M, Rathi KS, Modi A, Vaksman Z, Farra LM, Hyson E, Noureddine M, Wei JS, Smith MA, Asgharzadeh S, Seeger RC, Khan J, Auvil JG, Gerhard DS, Maris JM, Diskin SJ. Somatic structural variation targets neurodevelopmental genes and identifies SHANK2 as a tumor suppressor in neuroblastoma. Genome Research 2020; 30: 1228–42.

128. Keane S, Améen S, Lindlöf A, Ejeskär K. Low DLG2 gene expression, a link between 11q-deleted and MYCN-amplified neuroblastoma, causes forced cell cycle progression, and predicts poor patient survival. Cell Commun Signal 2020; 18: 65.

129. Suzuki N, Numakawa T, Chou J, de Vega S, Mizuniwa C, Sekimoto K, Adachi N, Kunugi H, Arikawa-Hirasawa E, Yamada Y, Akazawa C. Teneurin-4 promotes cellular protrusion formation and neurite outgrowth through focal adhesion kinase signaling. The FASEB Journal 2014; 28: 1386–97.

130. Abel F, Ejeskär K, Kogner P, Martinsson T. Gain of chromosome arm 17q is associated with unfavourable prognosis in neuroblastoma but does not involve mutations in the somatostatin receptor 2(SSTR2) gene at 17q24. British Journal of Cancer 1999; 81: 1402–9.

131. Carén H, Erichsen J, Olsson L, Enerback C, Sjöberg R-M, Abrahamsson J, Kogner P, Martinsson T. High-resolution array copy number analyses for detection of deletion, gain, amplification and copy-neutral LOH in primary neuroblastoma tumors; Four cases of homozygous deletions of the CDKN2A gene. BMC Genomics 2008; 9: 353–13.

132. Savelyeva L, Corvi R, Schwab M. Translocation involving 1p and 17q is a recurrent genetic alteration of human neuroblastoma cells. Am J Hum Genet 1994; 55: 334–40.

133. Bown N, Cotterill S, Lastowska M, O'Neill S, Pearson AD, Plantaz D, Meddeb M, Danglot G, Brinkschmidt C, Christiansen H, Laureys G, Speleman F, Nicholson J, Bernheim A, Betts DR, Vandesompele J, Van Roy N. Gain of chromosome arm 17q and adverse outcome in patients with neuroblastoma. N Engl J Med 1999; 340: 1954–61.

134. Godfried MB, Veenstra M, Sluis von P, Boon K, Asperen von R, Hermus MC, Schaik von BDC, Voûte TPA, Schwab M, Versteeg R, Caron HN. The N-myc and c-myc downstream pathways include the chromosome 17q genes nm23-H1 and nm23-H2. Oncogene 2002; 21: 2097–101.

135. Islam A, Kageyama H, Takada N, Kawamoto T, Takayasu H, Isogai E, Ohira M, Hashizume K, Kobayashi H, Kaneko Y, Nakagawara A. High expression of

Survivin, mapped to 17q25, is significantly associated with poor prognostic factors and promotes cell survival in human neuroblastoma. Oncogene 2000; 19: 617–23.

136. Saito-Ohara F, Imoto I, Inoue J, Hosoi H, Nakagawara A, Sugimoto T, Inazawa J. PPM1D is a potential target for 17q gain in neuroblastoma. Cancer Res 2003; 63: 1876–83.

137. Milosevic J, Fransson S, Gulyas M, Gallo-Oller G, Olsen TK, Treis D, Wickström M, Elfman LH, Sveinbjörnsson B, Hertwig F, Bartenhagen C, Reinsbach S, Wilhelm M, Abel F, Javanmardi N, Thankaswamy-Kosalai S, Eissler N, Kock A, Shi Y, Tanino K, Hehir-Kwa JY, Mensenkamp A, Tytgat GA, Kanduri C, Holmberg J, Gisselsson D, Molenaar JJ, Jongmans M, Fischer M, Kool M, Sakaguchi K, Baryawno N, Martinsson T, Johnsen JI, Kogner P. PPM1D is a neuroblastoma oncogene and therapeutic target in childhood neural tumors. bioRxiv [Internet] 2020; 63: 2020.09.04.283648–1. Available from: https://www.biorxiv.org/content/10.1101/2020.09.04.283648v1

138. Amoroso L, Ognibene M, Morini M, Conte M, Di Cataldo A, Tondo A, D'Angelo P, Castellano A, Garaventa A, Lasorsa VA, Podestà M, Capasso M, Pezzolo A. Genomic coamplification of CDK4/MDM2/FRS2 is associated with very poor prognosis and atypical clinical features in neuroblastoma patients. Genes Chromosomes Cancer 2020; 59: 277–85.

139. Guimier A, Ferrand S, Pierron G, Couturier J, Janoueix-Lerosey I, Combaret V, Mosseri V, Thebaud E, Gambart M, Plantaz D, Marabelle A, Coze C, Rialland X, Fasola S, Lapouble E, Fréneaux P, Peuchmaur M, Michon J, Delattre O, Schleiermacher G. Clinical characteristics and outcome of patients with neuroblastoma presenting genomic amplification of loci other than MYCN. PLoS ONE 2014; 9: e101990.

140. Reifenberger G, Ichimura K, Reifenberger J, Elkahloun AG, Meltzer PS, Collins VP. Refined mapping of 12q13-q15 amplicons in human malignant gliomas suggests CDK4/SAS and MDM2 as independent amplification targets. Cancer Res 1996; 56: 5141–5.

141. Muthusamy V, Hobbs C, Nogueira C, Cordon-Cardo C, McKee PH, Chin L, Bosenberg MW. Amplification of CDK4 and MDM2 in malignant melanoma. Genes Chromosomes Cancer 2006; 45: 447–54.

142. Rader J, Russell MR, Hart LS, Nakazawa MS, Belcastro LT, Martinez D, Li Y, Carpenter EL, Attiyeh EF, Diskin SJ, Kim S, Parasuraman S, Caponigro G, Schnepp RW, Wood AC, Pawel B, Cole KA, Maris JM. Dual CDK4/CDK6 Inhibition Induces Cell-Cycle Arrest and Senescence in Neuroblastoma. Clinical Cancer Research 2013; 19: 6173–82.

143. Hart LS, Rader J, Raman P, Batra V, Russell MR, Tsang M, Gagliardi M, Chen L, Martinez D, Li Y, Wood A, Kim S, Parasuraman S, Delach S, Cole KA, Krupa S, Boehm M, Peters M, Caponigro G, Maris JM. Preclinical Therapeutic Synergy of MEK1/2 and CDK4/6 Inhibition in Neuroblastoma. Clinical Cancer Research 2017; 23: 1785–96.

144. Ong SH, Guy GR, Hadari YR, Laks S, Gotoh N, Schlessinger J, Lax I. FRS2 proteins recruit intracellular signaling pathways by binding to diverse targets on fibroblast growth factor and nerve growth factor receptors. Mol Cell Biol 2000; 20: 979–89.

145. Cross MJ, Lu L, Magnusson P, Nyqvist D, Holmqvist K, Welsh M, Claesson-Welsh L. The Shb adaptor protein binds to tyrosine 766 in the FGFR-1 and regulates the Ras/MEK/MAPK pathway via FRS2 phosphorylation in endothelial cells. Mol Biol Cell 2002; 13: 2881–93.

146. Amorim JP, Santos G, Vinagre J, Soares P. The Role of ATRX in the Alternative Lengthening of Telomeres (ALT) Phenotype. Genes 2016; 7

147. Koneru B, Lopez G, Farooqi A, Conkrite KL, Nguyen TH, Macha SJ, Modi A, Rokita JL, Urias E, Hindle A, Davidson H, Mccoy K, Nance J, Yazdani V, Irwin MS, Yang S, Wheeler DA, Maris JM, Diskin SJ, Reynolds CP. Telomere Maintenance Mechanisms Define Clinical Outcome in High-Risk Neuroblastoma. Cancer Res 2020; 80: 2663–75.

148. Wang X, Fu X, Hoffman RM. A new patient-like metastatic model of human lung cancer constructed orthotopically with intact tissue via thoracotomy in immunodeficient mice. Int J Cancer 1992; 51: 992–5.

149. Grasse S, Lienhard M, Frese S, Kerick M, Steinbach A, Grimm C, Hussong M, Rolff J, Becker M, Dreher F, Schirmer U, Boerno S, Ramisch A, Leschber G, Timmermann B, Grohé C, Lüders H, Vingron M, Fichtner I, Klein S, Odenthal M, Büttner R, Lehrach H, Sültmann H, Herwig R, Schweiger MR. Epigenomic profiling of non-small cell lung cancer xenografts uncover LRP12 DNA methylation as predictive biomarker for carboplatin resistance. Genome Med 2018; 10: 55.

150. Speicher MR, Carter NP. The new cytogenetics: blurring the boundaries with molecular biology. Nat Rev Genet 2005; 6: 782–92.

151. Sato-Otsubo A, Sanada M, Ogawa S. Single-nucleotide polymorphism array karyotyping in clinical practice: where, when, and how? Semin Oncol 2012; 39: 13–25.

152. Volchenboum SL, Li C, Li S, Attiyeh EF, Reynolds CP, Maris JM, Look AT, George RE. Comparison of primary neuroblastoma tumors and derivative early-passage cell lines using genome-wide single nucleotide polymorphism array analysis. Cancer Res 2009; 69: 4143–9.

153. Ianevski A, He L, Aittokallio T, Tang J. SynergyFinder: a web application for analyzing drug combination dose-response matrix data. Bioinformatics 2017; 33: 2413–5.

154. Braekeveldt N, Wigerup C, Tadeo I, Beckman S, Sandén C, Jönsson J, Erjefält JS, Berbegall AP, Börjesson A, Backman T, Øra I, Navarro S, Noguera R, Gisselsson D, Påhlman S, Bexell D. Neuroblastoma patient-derived orthotopic xenografts reflect the microenvironmental hallmarks of aggressive patient tumours. Cancer Lett 2016; 375: 384–9.

155. Braekeveldt N, Wigerup C, Gisselsson D, Mohlin S, Merselius M, Beckman S, Jonson T, Börjesson A, Backman T, Tadeo I, Berbegall AP, Øra I, Navarro S, Noguera R, Påhlman S, Bexell D. Neuroblastoma patient-derived orthotopic xenografts retain metastatic patterns and. Int J Cancer 2015; 136: E252–61.

156. Kreso A, O'Brien CA, van Galen P, Gan OI, Notta F, Brown AMK, Ng K, Ma J, Wienholds E, Dunant C, Pollett A, Gallinger S, McPherson J, Mullighan CG, Shibata D, Dick JE. Variable clonal repopulation dynamics influence chemotherapy response in colorectal cancer. Science 2013; 339: 543–8.

157. Semenza GL. Hypoxia. Cross talk between oxygen sensing and the cell cycle machinery. Am J Physiol Cell Physiol 2011; 301: C550–2.

158. Janoueix-Lerosey I, Lequin D, Brugières L, Ribeiro A, de Pontual L, Combaret V, Raynal V, Puisieux A, Schleiermacher G, Pierron G, Valteau-Couanet D, Frebourg T, Michon J, Lyonnet S, Amiel J, Delattre O. Somatic and germline activating mutations of the ALK kinase receptor in neuroblastoma. Nature 2008; 455: 967–70.

159. Mossé YP, Laudenslager M, Khazi D, Carlisle AJ, Winter CL, Rappaport E, Maris JM. Germline PHOX2B mutation in hereditary neuroblastoma. Am J Hum Genet 2004; 75: 727–30.

160. Eleveld TF, Schild L, Koster J, Zwijnenburg DA, Alles LK, Ebus ME, Volckmann R, Tijtgat GA, van Sluis P, Versteeg R, Molenaar JJ. RAS-MAPK Pathway-Driven Tumor Progression Is Associated with Loss of CIC and Other Genomic Aberrations in Neuroblastoma. Cancer Research 2018; 78: 6297–307.

161. Lasorsa VA, Formicola D, Pignataro P, Cimmino F, Calabrese FM, Mora J, Esposito MR, Pantile M, Zanon C, De Mariano M, Longo L, Hogarty MD, de Torres C, Tonini GP, Iolascon A, Capasso M. Exome and deep sequencing of clinically aggressive neuroblastoma reveal somatic mutations that affect key pathways involved in cancer progression. Oncotarget 2016; 7: 21840–52.

162. Caprini E, Bresin A, Cristofoletti C, Helmer Citterich M, Tocco V, Scala E, Monopoli A, Benucci R, Narducci MG, Russo G. Loss of the candidate tumor suppressor ZEB1 (TCF8, ZFHX1A) in Sézary syndrome. Cell Death and Disease 2018; 9: 1178.

163. Ammazzalorso F, Pirzio LM, Bignami M, Franchitto A, Pichierri P. ATR and ATM differently regulate WRN to prevent DSBs at stalled replication forks and promote replication fork recovery. EMBO J 2010; 29: 3156–69.

164. Kimura Y, Katoh A, Kaneko T, Takahama K, Tanaka H. Two members of the IgLON family are expressed in a restricted region of the developing chick brain and neural crest. Dev Growth Differ 2001; 43: 257–63.

165. Sanz RL, Ferraro GB, Girouard M-P, Fournier AE. Ectodomain shedding of Limbic System-Associated Membrane Protein (LSAMP) by ADAM Metallopeptidases promotes neurite outgrowth in DRG neurons. Sci Rep 2017; 7: 1–11.

166. Coccaro N, Zagaria A, Tota G, Anelli L, Orsini P, Casieri P, Cellamare A, Minervini A, Impera L, Minervini CF, Brunetti C, Mestice A, Carluccio P, Cumbo C, Specchia G, Albano F. Overexpression of the LSAMP and TUSC7 genes in acute myeloid leukemia following microdeletion/duplication of chromosome 3. Cancer Genetics 2015; 208: 517–22.

167. Zhang L, Yuan Y, Lu KH, Zhang L. Identification of recurrent focal copy number variations and their putative targeted driver genes in ovarian cancer. BMC Bioinformatics 2016; 17: 222–12.

168. Smida J, Xu H, Zhang Y, Baumhoer D, Ribi S, Kovac M, Luettichau von I, Bielack S, O'Leary VB, Leib-Mösch C, Frishman D, Nathrath M. Genome-wide analysis of somatic copy number alterations and chromosomal breakages in osteosarcoma. Int J Cancer 2017; 141: 816–28.

169. Petrovics G, Li H, Stümpel T, Tan S-H, Young D, Katta S, Li Q, Ying K, Klocke B, Ravindranath L, Kohaar I, Chen Y, Ribli D, Grote K, Zou H, Cheng J, Dalgard CL, Zhang S, Csabai I, Kagan J, Takeda D, Loda M, Srivastava S, Scherf M, Seifert M, Gaiser T, McLeod DG, Szallasi Z, Ebner R, Werner T, Sesterhenn IA, Freedman M, Dobi A, Srivastava S. A novel genomic alteration of LSAMP associates with aggressive prostate cancer in African American men. EBIOM 2015; 2: 1957–64.

170. Nobusawa S, Hirato J, Kurihara H, Ogawa A, Okura N, Nagaishi M, Ikota H, Yokoo H, Nakazato Y. Intratumoral heterogeneity of genomic imbalance in a case of epithelioid glioblastoma with BRAF V600E mutation. Brain Pathol 2014; 24: 239–46.

171. Kühn MWM, Radtke I, Bullinger L, Goorha S, Cheng J, Edelmann J, Gohlke J, Su X, Paschka P, Pounds S, Krauter J, Ganser A, Quessar A, Ribeiro R, Gaidzik VI, Shurtleff S, Krönke J, Holzmann K, Ma J, Schlenk RF, Rubnitz JE, Döhner K, Döhner H, Downing JR. High-resolution genomic profiling of adult and pediatric core-binding factor acute myeloid leukemia reveals new recurrent genomic alterations. Blood 2012; 119: e67–75.

172. Pasic I, Shlien A, Durbin AD, Stavropoulos DJ, Baskin B, Ray PN, Novokmet A, Malkin D. Recurrent focal copy-number changes and loss of heterozygosity implicate two noncoding RNAs and one tumor suppressor gene at chromosome 3q13.31 in osteosarcoma. Cancer Res 2010; 70: 160–71.

173. Kresse SH, Ohnstad HO, Paulsen EB, Bjerkehagen B, Szuhai K, Serra M, Schaefer K-L, Myklebost O, Meza-Zepeda LA. LSAMP, a novel candidate tumor

suppressor gene in human osteosarcomas, identified by array comparative genomic hybridization. Genes Chromosomes Cancer 2009; 48: 679–93.

174. Karis K, Eskla K-L, Kaare M, Täht K, Tuusov J, Visnapuu T, Innos J, Jayaram M, Timmusk T, Weickert CS, Väli M, Vasar E, Philips M-A. Altered Expression Profile of IgLON Family of Neural Cell Adhesion Molecules in the Dorsolateral Prefrontal Cortex of Schizophrenic Patients. Front Mol Neurosci 2018; 11: 27–12.

175. Koido K, Traks T, ev RBOTS, Eller T, Must A, Koks S, Maron E, ru ITO, Shlik J, Vasar V, Vasar E. Associations between LSAMP gene polymorphisms and major depressive disorder and panic disorder. 2019 Apr 24. doi: 10.1038/tp.2012.74: 1–5.

176. Sherr CJ, Beach D, Shapiro GI. Targeting CDK4 and CDK6: From Discovery to Therapy. Cancer Discovery 2016; 6: 353–67.

177. Oliner JD, Saiki AY, Caenepeel S. The Role of MDM2 Amplification and Overexpression in Tumorigenesis. Cold Spring Harbor Perspectives in Medicine 2016; 6

178. Tweddle DA, Pearson ADJ, Haber M, Norris MD, Xue C, Flemming C, Lunec J. The p53 pathway and its inactivation in neuroblastoma. Cancer Letters 2003; 197: 93–8.

179. Carr-Wilkinson J, O'Toole K, Wood KM, Challen CC, Baker AG, Board JR, Evans L, Cole M, Cheung NKV, Boos J, Kohler G, Leuschner I, Pearson ADJ, Lunec J, Tweddle DA. High Frequency of p53/MDM2/p14ARF Pathway Abnormalities in Relapsed Neuroblastoma. Clinical Cancer Research 2010; 16: 1108–18.

The mind is not a vessel to be filled but a fire to be kindled.

Plutarch