Embryonic origin and treatment of disseminated neuroblastoma

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COLOFON

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Embryonic origin and treatment of disseminated neuroblastoma

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(met een samenvatting in het Nederlands)

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CHAPTER 1

General introduction

NEUROBLASTOMA

Sympathetic nervous system

Neuroblastoma is a common pediatric malignancy of the developing autonomic nervous system.[1-5] The autonomic nervous system regulates functions such as heart rate, the rate of breathing, blood pressure, bowel movement and the well-known "fight or flight' response. The autonomic nervous system consists of sympathetic and parasympathetic branches. The sympathetic system is activated in stressful situations, it is characterized by myelinated preganglionic neurons which originate in the central nervous system and end in prevertebral ganglia, paravertebral ganglia and in the adrenal gland (Figure 1).[6]

The paravertebral sympathetic ganglia are also referred to as sympathetic side chain. The sympathetic (non-myelinated) postganglionic neurons in the prevertebral and paravertebral ganglia act on target tissues via release of norepinephrine.[6,7] In the adrenal medulla the postsynaptic cells are not neurons, but chromaffin cells, which are hormone releasing cells. Chromaffin cells act via the release of epinephrine to the bloodstream and are important in the "fight or flight" response. The parasympathetic system is activated during resting periods and is characterized by preganglionic (myelinated) neurons originating in the central nervous systems and ending in the terminal ganglia, which are located near target organs. The postganglionic neurons activate target cells via the excretion of acetylcholine (Figure 1). Both chromaffin cells and paravertebral sympathetic ganglia arise from precursor cells of the neural crest.[6] These cells are considered the precursor cells of neuroblastoma.

Neuroblastoma pathology and epidemiology

Neuroblastoma tumors are histologically part of the so-called *small blue round cell tumors*. Within neuroblastoma, three degrees of differentiation are recognized: neuroblastoma (mainly composed of undifferentiated neuroblasts), ganglioneuroblastoma (combination of undifferentiated neuroblasts, mature ganglion cells, and at least 50% mature stroma), and ganglioneuroma (mature ganglion cells and mature stroma).[8] Ganglioneuroma is considered a benign tumor, while ganglioneuroblastoma and neuroblastoma are considered malignant (hereafter grouped together as 'neuroblastoma'). Neuroblastoma tumors are regarded developmental tumors. They are thought to arise as a result of developmental defects in neural crest cells.[1-5,9] As a result, tumors typically arise in early childhood. It is the most frequently diagnosed malignancy in infants, with an incidence of 65 per million person years in children <1 year old.[10] The incidence declines with age, to 1 per million children aged 10-14 years old.[10] Overall incidence during childhood (0-14 years) is estimated at 12 per million person years.[10] The median age at diagnosis is 18 months and 90% percent of neuroblastoma patients are diagnosed before the age of 10 years.[11,12] Overall long term survival of neuroblastoma is estimated at approximately 75%. However, prognosis largely varies between different risk groups. Half of the patients is

diagnosed with a high-risk tumor, with a 5 year overall survival estimated around 50%, while patients with a low-risk tumor have a 5 year overall survival of >90%.[13,14] In all stages, children < 18 months at diagnosis fare a more favorable course of disease than older children, for reasons not yet fully understood.

Clinical symptoms

Patients with localized tumors are often asymptomatic and tumors can be detected on imaging made for other medical reasons or on prenatal ultrasounds. The most common localization of neuroblastoma primary tumors is the adrenal gland, accounting for 50% of primary tumors, followed by the abdominal and thoracic sympathetic side chain, accounting for 24% and 15% of primary tumors, respectively.[15] Abdominal or thoracic tumors can be large before symptoms arise. Symptoms of large abdominal tumors typically are pain and abdominal distension. Hypertension can occur as a result of high circulating levels of catecholamines, but also by tumor compression of the renal artery. Paravertebral neuroblastomas have a tendency to grow into the intervertebral foramina or extend into the spinal canal, causing symptoms associated with spinal cord compression.[16] Thoracic tumors can cause Horner syndrome, a combination of unilateral ptosis (droopy eyelid), miosis (constricted pupil) and anhidrosis (lack of sweat). A small fraction of 2-3% of neuroblastoma patients presents with opsoclonus myoclonus syndrome, a paraneoplastic neurological disorder, which is likely caused by an autoimmune reaction in the cerebellum.[16,17]

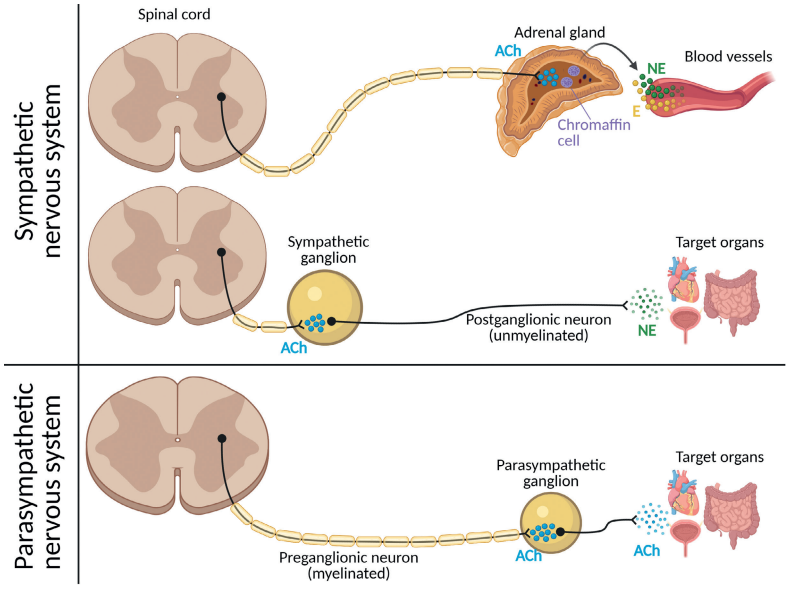

Figure 1 Sympathetic and parasympathetic nervous system.
Abbreviations: Ach: acetylcholine, NE: norepinephrine, E: epinephrine

Patients with metastasized tumors present mainly with symptoms caused by the metastases. Metastases are mainly found in bone marrow (74% of stage M patients) and bone (58%), followed by distant lymph nodes (29%) and liver (18%). Metastases in skin, lung or central nervous system are identified in <5% of stage M patients.[18] Bone marrow and bone invasion can cause pain and symptoms related to reduced blood formation: anemia with associated paleness and fatigue, low platelets with increased risk of bleeding (mostly petechiae and nose bleeds) and low immune cells with associated fever and infection. Typical for neuroblastoma are bone metastases in the orbits which present with periorbital ecchymosis and look like a black eye or so-called "Raccoon eyes".

A special pattern of dissemination is seen in stage 4S or MS ('S' for special). Patients with stage MS (metastatic special) can present with blue skin metastases and an enlarged liver.

Diagnosis and staging

Diagnosis of neuroblastoma is based on elevated urinary excretion of catecholamines, and on histopathological prove of tumor tissue or tumor cells in bone marrow. Magnetic resonance imaging (MRI) is the golden standard for assessing the primary tumor, and nuclear I^{123}-MIBG (metaiodobenzylguanidine) SPECT (single-photon emission computed tomography) scans for assessing metastatic spread. MIBG is a norepinephrine analog and is being accumulated in 90% of neuroblastoma tumors.[19] In ^{123}I-MIBG non-avid tumors, ^{18}FDG (fluorodeoxyglucose) PET/CT (positron emission tomography/computed tomography) scans can be used as an alternative. Staging and stratifications occurs according to the INRG (International Neuroblastoma Research Group) staging system[13], previously the International Neuroblastoma Staging System (INSS)[20]. The INSS is a post-surgical staging system, while the INRG is a diagnostic staging system which uses image defined risk factors for surgery (IDRFs). The INRG staging system recognizes 4 stages: locoregional tumors with the absence (stage L1) or presence (stage L2) of IDRFs, metastatic tumors (stage M), and "metastatic special" tumors (stage MS). Stage MS patients are characterized by an age at diagnosis younger than 18 months, and metastases limited to skin, bone marrow (<10% infiltration) and liver.[20-22] Patients aged 18 months or older and patients with metastases at other locations are excluded from stage MS and staged as stage M.

Prognosis and risk stratification

Based on several clinical and molecular factors, tumors are stratified and treated according to three risk categories: low, intermediate and high-risk. The American "Children's Oncology Group" (COG)[13,] (Table 1) and the European "Société International d'Oncologie Pédiatrique European Neuroblastoma" (SIOPEN) (Table 2) use a largely similar risk stratification model, with the following risk factors:

1) Stage of disease: patients with a more advanced stage have a worse course of disease.[13,22]

2) Age: patients younger than 18 months have a more favorable course of disease, are more often diagnosed with low stage disease and have higher degrees of tumor maturation and regression. Age is therefore thought to be a surrogate for tumor biology.[12,13,23]
3) Tumor morphology: approximately 85% of tumors are classified as neuroblastoma (NB), 14% as ganglioneuroblastoma (GNB) and <1% as ganglioneuroma (GN).[24]
4) *MYCN* amplification status: *MYCN* amplification is detected in about 20% of patients, and strongly correlated with an aggressive course of disease. All patients with *MYCN* amplification are stratified as high-risk patients, regardless of stage and age.[13,16,25]

Additional risk factors used in the INRG risk stratification[13] are:
1) Degree of differentiation: within NB morphology further morphology subgroups can be recognized. Undifferentiated neuroblastoma is associated with the most aggressive tumors, followed by poorly differentiated neuroblastoma morphology. Differentiating NB is associated with a slightly less aggressive tumor growth.[24]
2) Aberrations at chromosome 11q: Loss of 11q is found in 15-44% of tumors and is associated with more aggressive tumors.[26,27]
3) Ploidy: diploid tumors are associated with an unfavorable course of disease and are associated with more segmental chromosomal gains and losses, while hyperdiploid tumors harbor more often whole chromosomal gains and losses.[25,28]

The SIOPEN does not examine ploidy, or specific chromosomal aberrations (loss of 11q), but uses the global pattern of chromosomal aberrations as an additional risk factor. Tumors with only numerical chromosomal aberrations (NCA; whole gains and losses) fare a more favorable course of disease than tumors with one or more segmental chromosomal aberrations (SCA). [29-31]

Table 1 Risk stratification Children's Oncology Group (COG)

INRG stage	Age (months)	Histology	Grade of differentiation	MNA	11q aberration	ploidy	Risk group
L1/ L2	any	GN maturing, GNBi	any	no	any	any	Very low
L1	any	GNBn, NB	any	no	any	any	Very low
L1	any	GNBn, NB	any	yes	any	any	High
L2	<18	GNBn, NB	any	no	no	any	Low
L2	<18	GNBn, NB	any	no	yes	any	Intermediate
L2	≥18	GNBn, NB	differentiating	no	no	any	Low
L2	≥18	GNBn, NB	differentiating	no	yes	any	Intermediate
L2	≥18	GNBn, NB	poorly diff/undiff	no	any	any	Intermediate
L2	≥18	GNBn, NB	poorly diff/undiff	yes	any	any	High
M	<18	any	any	no	any	Hyperdiploid	Low
M	<12	any	any	no	any	Diploid	Intermediate
M	12-18	any	any	no	any	Diploid	Intermediate
M	<18	any	any	yes	any	any	High

Table 1 Continued.

INRG stage	Age (months)	Histology	Grade of differentiation	MNA	11q aberration	ploidy	Risk group
M	≥18	any	any	yes	any	any	High
MS	<18	any	any	no	no	any	Very low
MS	<18	any	any	no	yes	any	High
MS	<18	any	any	yes	any	any	High

Abbreviations: diff: differentiated, GN: ganglioneuroma, GNB(i/n): ganglioneurblastoma (intermixed/nodular), L1: localized 1, L2: localized 2, M: metastatic, MNA: MYCN amplification, MS: metastatic special, NB: neuroblastoma, undiff: undifferentiated

Table 2 Risk stratification Société International d'Oncologie Pédiatrique European Neuroblastoma (SIOPEN)

INRG stage	Age (months)	MYCN	Chromosomal abberations	Risk group
L1	any	no	any	Low
L1	any	yes	any	High
L2	≤18	no	NCA	Low
L2	≤18	no	SCA	Low
L2	>18	no	any	Intermediate
L2	>18	yes	any	High
M	<12	no	any	Intermediate
M	12-18	no	NCA	Intermediate
M	12-18	no	SCA	High
M	>18	no	any	High
M	>18	yes	any	High
MS	≤12	no	any	Low
MS	≤12	yes	any	High

Abbreviations: L1: localized 1, L2: localized 2, M: metastatic, MNA: MYCN amplification, MS: metastatic special, NCA numerical chromosomal aberrations, SCA structural chromosomal aberrations.

Treatment

Treatment of patients with neuroblastoma is determined by the risk-group the patient is stratified to. Patients with low-risk tumors are prone to spontaneous tumor regression and can be followed with a watchful waiting approach. In case of symptomatic disease or tumor progression, two to four courses of chemotherapy are required. With this approach 5-yr event free survival is estimated at 87% and 5-yr overall survival (OS) at 95%.[13] In the Netherlands, patient with less than complete remission after four courses of chemotherapy and patients with medium-risk tumors will receive treatment according to the medium risk protocol, consisting of six courses of induction chemotherapy and local treatment (tumor resection, often complemented by radiation treatment),

followed by four courses of maintenance chemotherapy. High-risk tumors are treated with intensive multimodality treatment. All international high-risk treatment protocols consist of an induction phase with combination chemotherapy and tumor resection, a consolidation phase with high dose chemotherapy followed by stem cell rescue and irradiation treatment and a post-consolidation or maintenance phase.[14] Unfortunately, after the consolidation, approximately 50% of patients in remission will develop relapse of tumors. To prevent these relapses, the maintenance phase has been developed. The maintenance phase currently exists of six courses of isotretinoin (vitamin A acid/Retinoic acid) in combination with five courses of anti-GD2 antibody based immunotherapy. The high-risk treatment protocol results in a 5-yr OS of approximately 50%.[14]

Familial neuroblastoma

An estimated 1-2% of neuroblastomas occur in families. Familial neuroblastoma cases are identified at a younger age and patients are more often diagnosed having multiple primary tumors. In 2004, it was discovered that patients with Hirschsprung's disease and congenital hyperventilation syndrome, caused by a loss-of-function mutation in PHOX2B, had a 5-10% chance to develop neuroblastoma.[32,33] Subsequently, it was discovered that loss of PHOX2B function increases the risk of neuroblastoma.[32,33] Still only 6-10% of familial neuroblastoma cases could be explained by germline PHOX2B mutations. In 2008, a second gene involved in familial neuroblastoma was identified.[34-36] Gain-of-function germline mutations of the Anaplastic Lymphoma Kinase (ALK) gene were found in 80% of familial neuroblastoma cases.[34-36] Interestingly, the three hotspot mutation in germline ALK alterations, R1192P, R1275Q and G1128A, are not found in sporadic neuroblastoma. Similarly, the hotspot somatic mutation, F1174L, is not identified in familial neuroblastoma.[37] This mutation, F1174L in germline, has only been identified in one patient, suffering from neonatal multifocal neuroblastoma and fatal severe non-epileptic encephalopathy.[38]

Neuroblastomas can also develop in patients with more general cancer predisposition syndromes. These include neurofibromatosis type 1 (NF1), Li-Fraumeni syndrome (TP53), Costello syndrome (HRAS), Noonan syndrome (PTPN11, KRAS), Beckwith-Wiedemann syndrome (chromosome 11) and Fanconi Anemia. Identification of additional neuroblastoma susceptibility genes is ongoing and mostly part of whole genome sequencing and genome-wide association studies (GWAS) in sporadic neuroblastoma.

Recurrent somatic mutations

Somatic mutations are relatively rare in pediatric tumors compared to adult cancers.[39,40] In neuroblastoma, the most common recurrent mutations are: ALK (7-10%), ATRX (3-6%), PTPN11 (3%), PHOX2B (2%), NRAS (1%).[41-43] The most frequently identified somatic ALK mutation is F1174L, a gain-of-function mutation.[36] ATRX mutations and small deletions are almost exclusively found in children ≥12 years old, these alterations are associated with

an indolent but fatal course of disease.[44] Whole genome and exome sequencing (WGS/WES) studies identified recurrent somatic mutations in genes involved in (neural crest) development indicating a role for these genes in the oncogenesis of neuroblastoma. Molenaar et al. found multiple somatic mutations involved in neuronal growth cones (PTPRD, ODZ3, ODZ2, SCMD1) and the regulation of these growth cones by the Rac/Rho signalling (TIAM1, DLC1, ARHGAP10, ATRX) in almost exclusively stage L2 and M tumors.[42] However, these recurrent mutations were not all confirmed in a larger genomic study.[43] Sausen et al. found ARID1A and ARID1B as recurrent somatic mutations, which were also found in the large genomic study of Pugh et al.[25,45]. These genes are members of the SWI/SNF neuronal progenitor specific chromatin-remodeling BAF complex, which is essential for the self-renewal of multipotent neural stem cells.[46] Bellini et al. looked specifically at genes involved in chromatin remodeling and epigenetic modifications and found that these processes were often mutated in neuroblastoma.[47] A recent study observed an enrichment of telomerase activity and the RAS and p53 pathways in patients with aggressive neuroblastoma.[48] This study has proposed to base the risk classification on telomerase activation, and mutations in the RAS or p53 pathways.[48] Of these recurrently mutated genes, only ALK mutations are being used for treatment decision making. In the current first-line ANBL1531 study of the COG (NCT03126916) and the HR-NB2 study of the SIOPEN (NCT04221035), newly diagnosed patients with an ALK mutation or amplification will receive Lorlatinib, a third generation ALK-inhibitor.

Recurrent copy number aberrations

Neuroblastoma tumors are characterized by specific patterns of copy number aberrations. Gain of chromosome 17q is observed in 90-95% of neuroblastoma tumors, loss of chromosome 1p in 41-59%, loss of chromosome 11q in 46%, predominantly seen in high-risk tumors. Others chromosomal regions associated with frequent gain or loss in neuroblastoma are 2p (gained in 25-33%, amplified in 25-43%), 3p (loss in 21-24%) and 4p (loss in 14-17%).[29] Research focused on the smallest regions of overlap identified candidate genes involved in tumorigenesis.[49,50] However, the precise role of the gained and lost regions remains elusive.

In addition to the larger chromosomal regions, amplifications of smaller regions, involving one or a few genes, are also identified. Tumors with amplifications are associated with a poor prognosis. Amplification of MYCN, at chromosome 2p24.3, occurs frequently (20% of patients).[13,23] Amplification of other genes occur infrequent and often in patient that also harbor MYCN amplification.[51,52]

Neural crest development

Neuroblastoma tumors arise from neural crest cells in the trunk region of the embryo. The neural crest cells are formed during early embryonic development as multipotent and highly migratory cells. Tissues originating from neural crest cells are among others

the chromaffin cells of the adrenal gland, sympathetic neurons of the sympathetic side chain, neurons in the dorsal root ganglia, Schwann cells and melanocytes.[9,53]

Trunk neural crest cells migrate from the dorsal part of neural tube along three pathways: the dorsolateral, ventrolateral and ventromedial pathways.[9] Cells following the dorsolateral pathway will mainly form skin melanocytes.[54,55] Cells following the ventrolateral pathway migrate to the dorsal root ganglia (DRG), here the Schwann cells precursors are formed.[54,55] The Schwann cell precursors continue to migrate to more distal parts of the body along the nerves coming from the DRG. Despite their name, they form approximately 80% of the chromaffin cells of the adrenal gland, and 10% of the neurons of the sympathetic side chain.[56,57] Cells following the ventromedial pathway aggregate near the dorsal aorta, where they specify into semi-committed precursor cells.[54] These precursors cells subsequently migrate to the adrenal anlagen (primitive adrenal gland) and the sympathetic side chain to further develop in respectively 10% of fully differentiated chromaffin cells and 90% sympathetic neurons.[56,57]

Connections between NB and NC development

It is widely accepted that neuroblastoma tumors arise from neural crest cells. However, it remains unknown how cells evolve into (pre)malignant cells, at what point in development malignant transformation takes place and which factors affect this process. Of interest in this discussion are the different clinical patterns observed in neuroblastoma tumors. High-risk tumors are highly aggressive and even with intensive multimodality treatment outcome of patients remains poor. In addition, high-risk tumors have more mutations and segmental gains or losses than low-risk tumors, and show high expression of genes involved in metabolic processes, also expressed in adult cancers.[58] On the other end of the spectrum are the stage L1 and MS tumors which can regress spontaneously. Interestingly, during neural crest and autonomic nervous system development, apoptosis is widely used to shape the body and remove cells in an orderly fashion. Neurons are formed in excess and neurons that do not reach a NGF secreting target undergo apoptosis.[59] Chromaffin cells are also made in excess. During embryonic development the Organ of Zuckerkandl is formed as an embryonic source for catecholamines. Postnatal, this organ is not needed anymore and undergoes apoptosis and regression. The organ of Zuckerkandl involutes completely by the age of 3 years.[60] Could this mean that the low-risk tumors are not truly malignant, but an erroneous variation of normal development? This could suggest that different clinical subgroups of neuroblastoma are the result of different oncogenic processes and possibly of different cells-of-origin.

SCOPE OF THIS THESIS

Part I: Embryonic origin and subgroups of disseminated neuroblastoma

Part one of this thesis has a focus on neuroblastoma as a developmental tumor. Neuroblastoma derives from neural crest cells, but the cell-of-origin and time-of-origin of neuroblastoma ontogeny remains elusive. This part aims to explain clinical neuroblastoma patterns as a reflection of the normal neural crest development. Specifically, it is aimed to elucidate some of the uncertainties about the origin of neuroblastoma tumors, and why some tumors behave benign, while others are highly aggressive. In **Chapter 2** we review the current literature on normal neural crest development, with a focus on the genes that are important during normal development and describe the similarities and differences to neuroblastoma development. Subsequently, different subgroups of neuroblastoma are being discussed and we hypothesize about their potential embryological origin in view of normal development. In **Chapter 3**, we describe the regression of stage 4S/MS tumors. These tumors are known for their spontaneous regression. However, it is not clear how much time is needed for the process of regression and it remains also unknown if complete regression is always reached, or if some patients remain in partial regression. In addition, we describe risk factors for progressive disease. In **Chapter 4** we describe a cohort of patients with a chemotherapy insensitive tumors who fare a chronic course of disease with long-term survival without tumor regression. We describe clinical characteristics of these patients in order to enable recognition of these patients soon after diagnosis, to prevent them from overtreatment. In addition, we try to place this entity within the well-known disseminated entities stage MS and stage M tumors. Subsequently, we hypothesize on the cell-of-origin of this specific tumor pattern. In **Chapter 5**, we analyze the role of *ZFP42*, a pluripotency marker of embryonic stem cells and potential gene in neuroblastoma development. We identified a patient who developed two tumors in childhood and who has a germline rearrangement positioning a region positive for epigenetic enhancer marks (H3K27ac and H3K4me1) near the promoter of *ZFP42*. The rearrangement alters this region and potentially activates this gene to become involved in tumor development. The chapter discusses the potential role of *ZFP42* in the tumors.

Part II: Progress in the treatment of (high-risk) neuroblastoma

Part two of this thesis has a focus on the progress made in treating neuroblastoma, especially in treating high-risk neuroblastoma. Over time, many changes and additions in treatment have been made, all aimed to improve survival. To understand the effect of these treatment changes we analyze in **Chapter 6** the incidence and survival of all neuroblastoma patients, diagnosed between 1990 and 2014. By analyzing survival data for different subgroups of patients and over different periods, we can estimate the effect of treatment changes on the prognosis of patients with neuroblastoma. In

Chapter 7, we analyze the specific effect of anti-GD2 immunotherapy on the (long-term) survival of high-risk neuroblastoma patients. We compared patients treated with anti-GD2 immunotherapy between 2009-2015 to a historical control group (1999-2009). We compared our data with other published cohorts.

At the end of this thesis, in **Chapter 8** a discussion of the main findings as well as future implications are enclosed. The discussion summarizes the insights from our studies and those of others combined to give direction towards potential progress and fields of interest for future studies.

In **Chapter 9** an English and Dutch summary (Nederlandse samenvatting) can be found. Finally, as an appendix, a list of publications is enclosed, together with a biography and acknowledgments (dankwoord).

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PART I

Embryonic origin and subgroups of disseminated neuroblastoma

CHAPTER

Neuroblastoma, a developmental malignancy of neural crest cells

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ABSTRACT

Neuroblastoma is an embryonic malignancy of neural crest cells. It is a very heterogeneous tumor with low-risk tumors that have survival rates of >85% without treatment, and high-risk tumors with survival rates <50% despite intensive multimodal treatment. Low-risk tumors are associated with sympathetic side chain tumors, while high-risk tumors are associated with adrenal gland tumors. Both the sympathetic neurons of the sympathetic side chain and the chromaffin cells of the adrenal gland are formed by neural crest cells. The neural crest is a multipotent transient population of cells only present during embryonal development. Recently in-depth studies have outlined the transcriptome of mouse and human neural crest cells and their derivatives. This led to the discovery of two main precursor cells: the sympathoadrenal progenitor (SA progenitor) which mainly forms the sympathetic neurons of the side chain and the Schwann cell precursor (SCP) which mainly forms the chromaffin cells of the adrenal gland. This marked difference in precursor cells and neuroblastoma subtypes could indicate that different neuroblastoma subtypes arise from different precursor cells. More knowledge of the transcriptome and fate decisions of SA progenitors, SCPs and chromaffin is needed to identify the cell(s)-of-origin of neuroblastoma.

INTRODUCTION

The neural crest (NC) is a temporary embryonic structure that develops from neuroectodermal cells and is regarded as a fourth embryonic layer. Neural crest cells (NCCs) are multipotent and highly migratory. They differentiate into multiple cell types and tissues, among which melanocytes, chromaffin cells, sympathetic neurons, Schwann cells and smooth muscle cells.[1-3] The NCC fate strongly depends on the regions of the NC they arise from: cranial, vagal, trunk, and or sacral neural crest.[1] In this review we will focus on the trunk neural crest in relation to neuroblastoma development. Neuroblastoma is a pediatric malignancy of the developing sympathetic nervous system.[4] It has an incidence of 12 per million children aged <15 years. It effects mostly young children, the median age at diagnosis is 18 months and 95% of patients are diagnosed before the age of 10.[5,6] Tumors arise mainly in the adrenal gland, and the thoracic and abdominal sympathetic side chain, which are derivatives of the trunk NC. Although it is widely accepted that neuroblastoma tumors originate from NCCs, the precise stage of development from which the tumors originate remains unclear. Recent technologies have provided novel insights in the developing sympathetic nervous system on a single cell level. This review aims to combine the latest insights in neural crest development and clinical and molecular knowledge on neuroblastomas to elucidate the possible cells of origin of neuroblastoma.

NEUROBLASTOMA

The clinical course of neuroblastoma varies from low-risk disease with survival rates of >85% without treatment, to high-risk disease with <50% long term survival despite intensive multimodal treatment.[7-9] Low-risk tumors are defined by the International Neuroblastoma Research Group classification system[7] as stage L1 (localized tumors) or disseminated MS (metastatic special) disease, age at diagnosis below 18 months and no amplification of the *MYCN* oncogene.[7] These tumors are associated with whole chromosomal gains and losses, and few genetic mutations.[10] Patients with stage MS neuroblastoma are young (below 18 months of age at diagnosis) with dissemination limited to liver, skin and bone marrow (<10% infiltration).[7,11,12] Despite the multifocal tumor nodules, spontaneous regression is expected.[13-15] Low-risk stage L1 primary tumors are most often located in the sympathetic side chain in the neck and thorax (50%) and less often in the adrenal gland (28%) or other abdominal locations (15%).[4,16,17] Patients with thoracic sympathetic side chain tumors have higher survival rates than patients with adrenal primary tumors, even when corrected for age, *MYCN* amplification status and stage.[4]

High-risk neuroblastoma is associated with stage M (metastatic) disease, older age at diagnosis (≥18 months), *MYCN* amplification, segmental chromosomal gains and losses (gain of 17q, LOH of 1p and loss of 11q), and other genetic alterations.[7,10] Stage M primary tumors are most often located in the adrenal gland (60%) and other abdominal locations (22%), while tumors located in the neck or thorax are found in only 9% of patients.[4] Recurrent mutations are found in *ATRX* and *TERT* (both involved in telomere lengthening), the Rac/Rho pathway, and the RAS-MAPK pathway (Table 1).[18-23] Genes with high expression in high-risk tumors and associated with poor prognosis are *TrkB* (*NTRK2*)[24], *ALK*[18,25,26], *LGR5*[27], *DUPS5*[28], *TERT*[29], *GATA3*[30], *ASCL1*[31] and *MYCN*[7].

Intermediate risk neuroblastoma are stage L2 (localized tumors with risk factors on imaging modalities for incomplete resection) tumors in children > 18 months and clinically varied and genetically less well defined.[7,10]

Neuroblastoma tumors can be detected by measuring homovanillic acid (HVA) and vanillylmandelic acid (VMA) in urine. Based on this simple test, screening studies were started in multiple countries. The aim was to detect high-risk tumors at an early phase of disease. During the screening studies, incidence of stage L1 tumors increased 4-fold, but the incidence of stage M tumors and mortality remained the same, subsequently all screenings were ceased.[17,32,33] In line, autopsy studies on fetal adrenal glands have shed light on the existence of neuroblastoma like nodules, "in situ neuroblastoma", in 40x more cases than expected based on neuroblastoma incidence. The "in situ neuroblastoma" most likely undergo spontaneous involution, before the diagnosis of a clinically relevant/detectable neuroblastoma is made.[34] The screening studies suggested that stage L1 and stage M tumors are two different entities and that stepwise evolution from premalignant to low stage tumors to high stage tumors, as in adult carcinomas, does not apply to neuroblastoma.[35,36]

Table 1 Recurrent gene mutations associated with somatic neuroblastoma

Gene	Location	Percentage	Cohort	Related Gene/ Pathway	Function	Referencer
Epigenetic processes						
ARID1A	1p36	5,63%	all	SWI/SNF complex	chromatin remodeler	[21]
ARID1B	6q25	1-7%	all	SWI/SNF complex	chromatin remodeler	[18,21]
ATRX	Xq21	2-5%	HR	SWI/SNF complex	chromatin remodeler	[18,20,22]
CHD9	16q12	4.04%	mainly HR	PPARA	chromatin remodeler	[20]
KMT2C/ MLL3	7q36	1.82%	all	ASCOM complex	epigenetic transcriptional activation	[22]
PBRM1	3p21	1-2%	all	SWI/SNF complex	chromatin remodeler	[18,22]
Developmental processes						
ALK	2p23	6-14%	HR	MAPK	receptor tyrosine kinase	[18-21,23]
NEB	2q23	4.04%	mainly HR		cytoskeleton	[20]
TIAM1	21q22	3.45%	all	RAC1, Rho-like GTPases	guanin nucleotide exchange factor	[19]
VANGL1	1p13	2.22%	all	RhoA pathway	cell polarity	[21]
ZHX2	8q24	2.22%	all		cell polarity	[21]
PTK2	8q24	2.02%	mainly HR	RhoA pathway, MAPK	protein tyrosine kinase, cell migration and cell cycle progression	[20]
COL6A6	3q22	3.03%	mainly HR	VWF	cell cell interactions	[20]
HTRA1	10q26	3.03%	mainly HR	FGF, IGF	protease	[20]
Tumorigenic processes						
MYCN	2q24	1.67%	HR		transcription factor	[18]
NRAS	1p13	0.83%	HR		GTPase	[18]
PTPN11	12q24	2.92%	HR	RhoA, MAPK	protein tyrosine phosphatase	[18]

Predisposition and susceptibility for neuroblastoma

Although rare, familial neuroblastoma is observed in 1-2% of patients. Patients with familial neuroblastoma tend to be younger than patients with a sporadic neuroblastoma, and often have multiple primary tumors.[37] Three genes are currently known to be involved in familial neuroblastoma: *ALK*[25,26], *PHOX2B*[38], and *KIF1B*[39].

Several general cancer predisposition syndromes, among which Li-Fraumeni syndrome and neurofibromatosis type I, are associated with neuroblastoma predisposition.[40] Genome-wide association studies (GWAS) in sporadic neuroblastoma indicated that low-risk and high-risk can be seen as different developmental entities (Table 2). Low-risk tumors are associated with single nucleotide polymorphisms (SNPs) in *IL31RA*, *HSD17B12*, *DUSP12*, and *DDX4*.[41] *HSD17B12* and *DDX* are important for embryonic development in general.[42-44] The role of *IL31RA* and *DUSP12* during development remains unknown.[45,46] High-risk tumors are associated with SNPs in genes involved in oncogenic processes (Table 2).[18,20,41,47-52] SNPs for high-risk tumors are identified in *ALK*, *LMO1*, *LIN28B*, and *MYCN*, which are involved in the neuroblastoma core regulatory circuit (CRC). [31,53-56] A CRC is a set of transcription factors able to collectively (auto)regulate the expression of a large number of genes and enhancers and thereby determine cell identity.[57] For high-risk tumors, SNPs are also identified in*TP53*, *BARD1*, *PALB2*, *CHEK2*, *HACE1*, and *PINK1*, which are putative tumor suppressors of several cancer types.[58-65]

THE ORIGIN OF THE NEUROBLASTOMA IN RELATION TO THE NC DEVELOPMENT

1. Neural Crest Cells

Neural crest cells (NCCs) are formed at the dorsal borders of the neural tube, in between the neural plate and the non-neural ectoderm.[66-73] Influenced by the morphogens Wnt and BMPs,[1,74,75] NCCs undergo epithelial to mesenchymal transition (EMT) which enables delamination from the neural tube and subsequent migration to fate organs. EMT is marked by changes in gene expression of cell adhesion molecules, signal transduction molecules/proteins, and transcription factors (Figure 1).[76-79] As EMT genes are essential for migration, many remain expressed in the migrating NCCs. [2,71,79-85] The Rho family of small GTPases (among others *Rac1* and *RhoB*) are activated in NCCs to guide migration.[86,87]In addition, surrounding structures guide the migrating NCCs by forming physical barriers and trails (Figure 2) and by expressing repelling and attracting signaling molecules such as Semaphorins, and Slit/Robo. [88-91] Recurrent somatic mutations in the Rac/Rho pathways are found in high-risk neuroblastoma (Table 1).[19-21] In contrast, *VANGL2* and *PRICKLE1* are highly expressed in low-risk neuroblastoma tumors,[92] they are part of the planar cell polarity (PCP) signaling pathway.[87] This pathway remodels the cytoskeleton of NCCs and defines polarity.[77,87,93-95]

Table 2 SNPs associated with neuroblastoma susceptibility

Gene	Location	Percentage	Snp	Pt-group	Related Pathway/gene	Function	Technique used	Reference
Epigenetic processes								
ARID1A	1p36	1.82%	rs369118235	all	SWI/SNF complex	chromatin remodeler	WGS/WES	22
ARID1B	6q25	9.09%	rs374755339	all	SWI/SNF complex	chromatin remodeler	WGS/WES	22
ATRX	Xq21	3.64%	focal deletion	all	SWI/SNF complex	chromatin remodeler	WGS/WES	22
KMT2D/MLL2	12q13	3.64%	rs1028524252	all	ASCOM complex	epigenetic transcriptional activation	WGS/WES	22
KMT2C/MLL3	7q36	3.64%	rs200152380	all	ASCOM complex	epigenetic transcriptional activation	WGS/WES	22
SMARCA4	19p13	1.82%	rs768522438	all	SWI/SNF complex	chromatin remodeler	WGS/WES	22,170
Developmental processes								
SPAG16	2q34		rs1033069	HR*		microtubular backbone of cilia	GWAS	50
NEFL	8q21		rs1059111		RET	neurofilament	GWAS	171
ALK	2p23	0.83%	rs113994087	HR	MAPK	receptor tyrosine kinase	WGS/WES	18
IL31RA	5q11		rs10055201	LR	STA3/STAT5	cytokine receptor	GWAS	41
HSD17B12	11p11		rs11037575	LR		dehydrogenase	GWAS	21
CPZ	4p16		rs3796727			metallocarboxypeptidase	GWAS	172
LMO1	11p15		rs2168101	HR	GATA	transcription factor	GWAS	21,41,48-50
MMP20	11q22		rs10895322			proteinase	GWAS	48
Tumorigenic processes								
CASC14/CASC15	6p22		rs6939340			cell cycle control	GWAS	41,48,50,173
DUSP12	1q23		rs1027702	LR	MAPK	phosphatase	GWAS	41
HACE1	6p16		rs4336470	HR?	RAC1	tumor suppressor	GWAS	47
LIN28B	6p16		rs17065417	HR?	MYCN	suppressor of miRNA biogenesis	GWAS	47
MLF1	3q25		rs6441201		CDKN1B	transcription factor	GWAS	172

Gene	Location	Percentage	Snp	Pt-group	Related Pathway/gene	Function	Technique used	Reference
CDKN1B	12p13		rs34330			cell cycle control	GWAS	174
KIF15	3p21		rs80059929		MAPK, MHC1 processing		GWAS	175
AXIN2	17q24	7.69%		all	APC, beta-catenin	tumor suppressor	WES	20
BARD1	2q35	1-4%	rs6435862	HR	BRCA1	tumor suppressor	WGS/WES/GWAS	18,41,48,50,51
CHEK2	22q12	1-6%	rs17879961	HR	BRCA1	cell cycle control	WGS/WES	18,20
PALB2	16q12	0.42%	rs747785029	HR	BRCA2	tumor suppressor	WGS/WES	18,52
TP53	17p13	0.42%	rs879253894	HR		tumor suppressor	WGS/WES	18
DDX4	5q11		rs2619046	LR		piRNA biogenesis	GWAS	41
PINK1	1p36	0.83%	rs28940285	HR	PTEN	protein kinase mitochondria	WGS/WES	18
Familial neuroblastoma								
ALK	2p23		rs863225281	familial	MAPK	receptor tyrosine kinase	Targeted sequencing	23,25,26,176
PHOX2B	4p13			familial		transcription factor	Targeted sequencing	38,177

* African ancestry

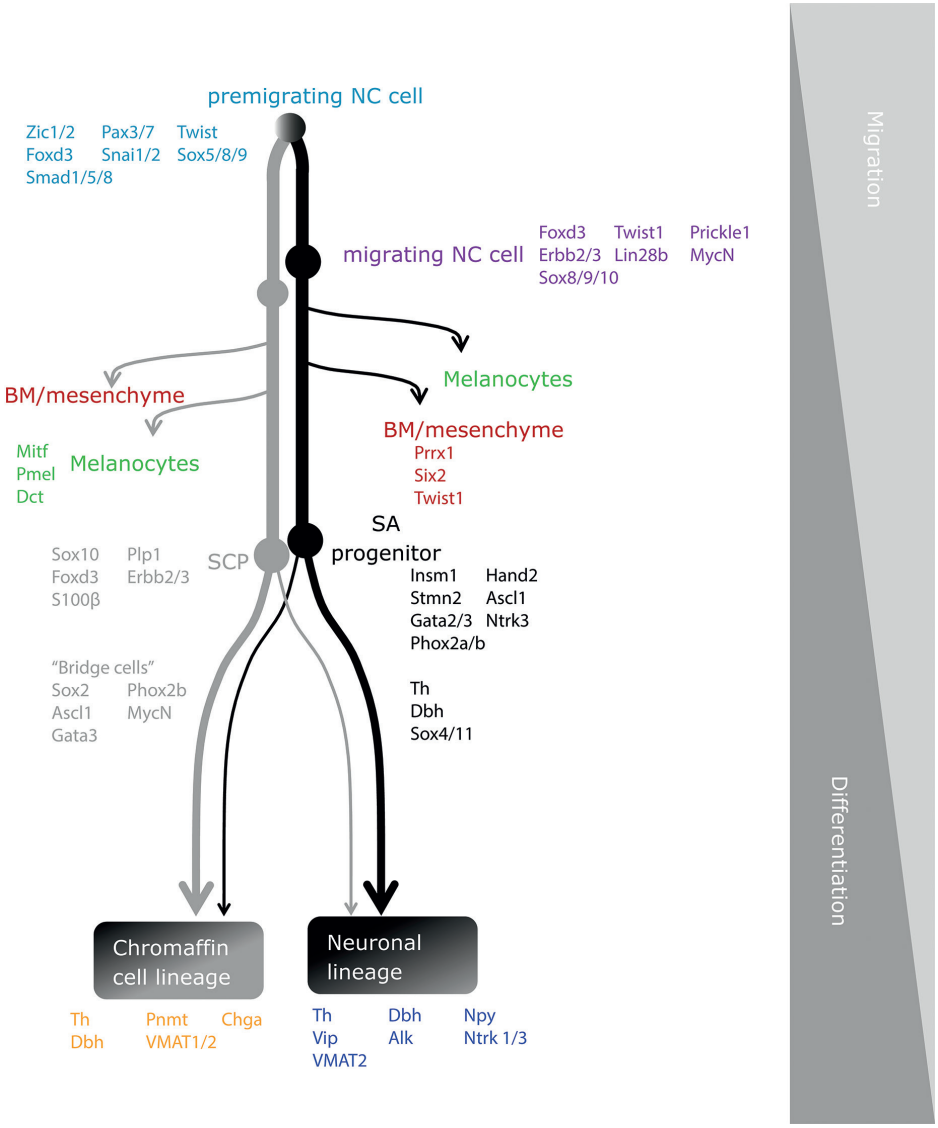


Figure 1 NC development with marker genes expressed by NCCs during migration and differentiation
Schematic view of neural crest (NC) differentiation as a time line from early (top) to late (bottom) NC migration and differentiation. Marker genes expressed at the different differentiation stages of neural crest (NC) migration and differentiation are given per differentiation stage.
For a more extensive version with refs to the different genes: see supplemental Figure S1

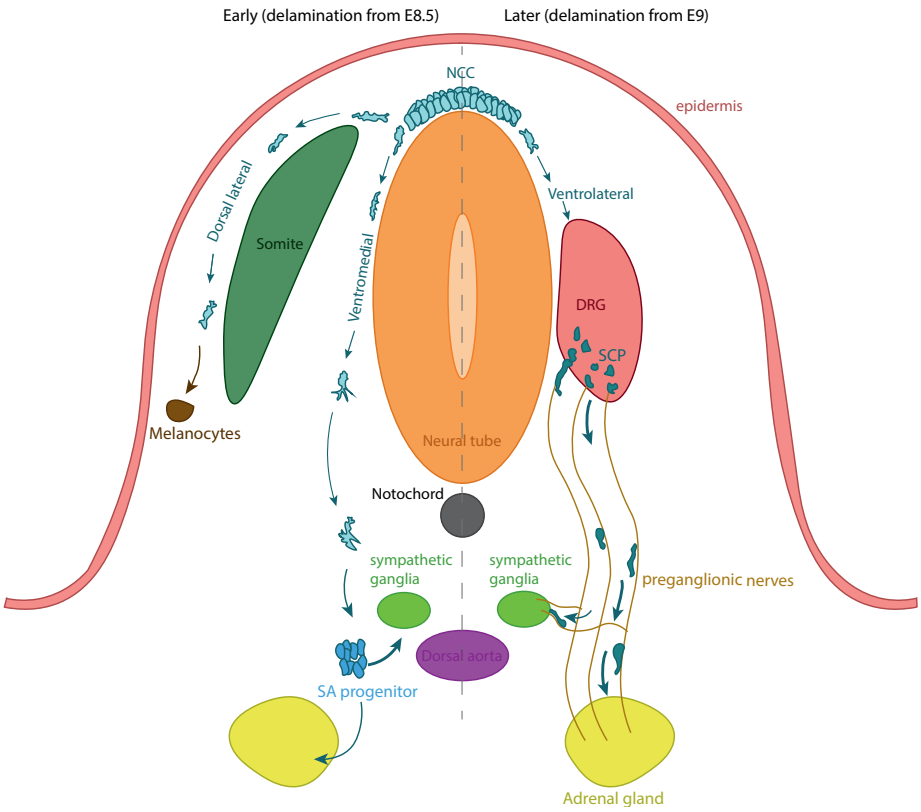


Figure 2 Trunk Neural crest migration.
The three main pathways followed by migrating neural crest cells (in blue). The dorsal lateral pathway is taken by NCC destined to become melanocytes. NCCs that follow the ventrolateral pathway aggregate near the dorsal aorta where they differentiate into the SA progenitor to subsequently migrate to the sympathetic ganglia and adrenal gland. NCCs that follow the ventrolateral pathway aggregate in the dorsal root ganglia, where they differentiate into SCPs (Schwann cell precursors), subsequently they migrate along preganglionic nerves towards their target organs: the adrenal gland and the sympathetic ganglia.

2. Migration pathways

Trunk NCCs migrate along three pathways (Figure 2): 1) the dorsolateral pathway, which gives mainly rise to melanocytes; 2) the ventromedial pathway, which gives rise to cells in the sympathetic ganglia, adrenal medulla and posterior region of the enteric nervous system; 3) the ventrolateral pathway which gives rise to neurons of the dorsal root ganglia and Schwann cells.[89,96] Between these well-established pathways some cross over takes place, an example being Schwann cell precursors who develop into melanocytes.[97] Migration starts at embryonic day 8.5 (E8.5) in mice, and around the third to fourth week of gestation in humans, with the delamination of the NCCs following the dorsolateral

and ventromedial pathway.[98] Delamination is initiated in rostrocaudal direction, and continues until E9.5 for NCCs following the ventromedial pathway and until E10.5 for NCCs following the dorsolateral pathway. NCCs following the ventrolateral pathway start delaminating after E9 and continue through E10.5.[98] Migrating NCCs can be recognized by expression of *Sox10* and *FoxD3*.[2,99]

3. Common sympathoadrenal progenitor

Trunk NCCs that follow the ventromedial pathway aggregate in the vicinity of the dorsal aorta to form the primary sympathetic ganglia.[100] Here, the semi-committed progenitors (sympathoadrenal (SA) progenitors) are further exposed to signaling factors (e.g. BMPs) necessary for their specification. Subsequently, the SA progenitors migrate towards the adrenal anlagen and the sympathetic side chain.[101,102] Upon arrival at their final destination, the cells differentiate into chromaffin cells and secondary sympathetic ganglia (Figure 2).[103]

Transcription factors directing the specification of the SA progenitor are *Phox2a*, *Phox2b*, *Insm1*, *Stmn2*, *Ascl1*, *Gata2*, *Gata3* and *Hand2*.[104-110] The SA progenitor matures and starts expressing genes that indicates further commitment. These genes are also expressed in immature sympathetic neurons and chromaffin cells (*Th*, *Dbh*, *Ntrk1, Ntrk3*, *Notch1*, *Phox2a*, *c-RET*, *Nf*, *Nse*, *Pgp9.5* and *HNK*).[66,99,109-117]*Phox2b* is considered the master regulator of specification.[111] As mentioned, PHOX2B alterations are involved in familial neuroblastoma, typically as multifocal side chain tumors. *PHOX2B* and*STMN2* expression can be found in nearly all neuroblastoma tumors.[118] In addition, *Phox2b*, *Hand2*, *Ascl1,* and *Gata3* are part of the adrenergic neuroblastoma CRC.[31,119-122]*TH* is used as a diagnostic marker for neuroblastoma and both *Th* and *Dbh* are being used to generate animal models.[55,118,123-126]

To further differentiate into the sympathetic neuron lineage and chromaffin lineage, the SA progenitors start expressing *Sox4 and Sox11*.[110] From here on the SA progenitor pool segregates into two lineages, one group committed to the sympathetic neuron lineage and the other to the adrenal chromaffin lineage.[101,127] These two pools will form about 90% of the sympathetic neurons, 10% of the adrenal chromaffin cells and 55% of the extra-adrenal chromaffin cells in the Organ of Zuckerkandl (Figure 1).[69,85,128]

Interestingly, in neuroblastoma cell lines two separate neuroblastoma cell states, the adrenergic and mesenchymal state were identified.[119,120] Both states have their own CRC. The adrenergic CRC is characterized by genes important for SA progenitor specification (*Phox2b*, *Hand2*, *Tbx2*, *Isl1*, and *Gata3*), along with *LMO1*.[31,119-122] *MYCN* is thought to be an amplifier of this CRC.[129] The mesenchymal CRC is characterized by *PRRX1* and *FOSL1/2*.[120] *In vitro*, cell lines were able to switch between the adrenergic and mesenchymal state. In bulk neuroblastoma tumor samples, the adrenergic and mesenchymal subtypes can be identified on epigenetic and gene expression level. The mesenchymal subtype was enriched in the relapse samples and closely resembled expression of the Schwann cell precursor (SCP).[130]

3.1 Sympathetic differentiation of the SA progenitor

The differentiating sympathetic neurons can be identified by the expression of *SytI*, *Npy*, *Vip*, *Alk*, *Neurog1*, *Neurog2*, *Ntrk1* and *Ntrk3*, and *Vmat2*.[54,110,113,131-135] During differentiation, *MycN* is upregulated, followed by loss of expression in mature sympathetic neurons.[136] In neuroblastoma, high expression of *MYCN* is assured by amplification of *MYCN* in 20% of patients and a poor prognostic factor for survival.[137,138] *VIP* and *NPY* are often expressed in neuroblastoma, but do not correlate with stage or prognosis.[139-141] *VMAT2* is correlated with MIBG avidity and is inversely correlated with *MYCN* amplification.[142] High expression or amplification of *ALK* is associated with poor outcome and high-risk neuroblastoma.[18,23] Activating mutations of ALK are responsible for 80% of familial neuroblastoma cases.[25,26] The ALK^{F1174L} mutation is one of the few recurrent somatic mutations in neuroblastoma and is associated with a poor outcome.[23] ALK^{F1174L} is frequently used for the development of transgenic neuroblastoma mouse models.[125,126,143]

The expression of *Ntrk1* in combination with the NGF signal is important for the survival of the sympathetic neurons, as well as their ability to innervate distal targets.[134,144,145] They undergo apoptosis when exposed to insufficient amounts. In neuroblastoma, expression of *NTRK1 (TrkA)* and *NTRK3 (TrkC)* is associated with young age, a favorable clinical course, low stage of disease, and tumors located in the sympathetic side chain.[16,135,146-148] In contrast, expression of *NTRK2 (TrkB)* is associated with high-risk tumors and poor prognosis.[24] *Ntrk2* is not expressed by the SA progenitor or sympathetic neurons at any stage of development. Its expression level in adrenal chromaffin cells is unknown. It has been found to be expressed in motor and sensory neurons, Schwann cells and in the central nervous system. [134,149]

3.2 Chromaffin differentiation of the SA progenitor

The chromaffin lineage of SA progenitors starts expressing *Pnmt* and *Chga*, while downregulating the neuronal markers *Stmn2* and *Nf*.[99,111,113] Although glucocorticoids are essential for differentiation *in vitro*, ablation of the adrenal cortex, which produces glucocorticoids, *in vivo* resulted in only 50% reduction in chromaffin cell number. This could indicate a second population contributing to the chromaffin cells, or a degree of glucocorticoid independence.[150-152] *CHGA* is expressed in neuroblastoma and is used as immunohistochemistry marker at diagnosis.[120,153,154] *PNMT* is necessary for the synthesis of epinephrine. However, expression was not observed in a panel of neuroblastoma cell lines.[155] Chromaffin cells of the organ of Zuckerkandl undergo autophagy mediated cell death postnatally.[128,156] This is probably regulated by glucocorticoid signaling.

4. The Schwann cell precursor

Recent research has revealed a second important source of chromaffin cells and sympathetic neurons: the Schwann cell precursor. The ventrolateral migrating NCCs that start migrating at E9.5 first reach the dorsal root ganglia. At this developmental hub the NCCs start expressing Schwann cell markers and are characterized by expression

of *ERBB2/3, Plp1, and S100β*.[85,157] From this stage the NCCs are referred to as SCPs. Between E11.5 and E15.5, the SCPs migrate towards their target organs, using the axons of preganglionic nerves for migration.[157] The ventrolateral pathway was believed to generate dorsal root ganglia and Schwann cells only, but recent mouse lineage tracing studies revealed the multipotency of this migrating NCC population.[69,85,128,158] SCPs are currently known to contribute to chromaffin cells, sympathetic neurons, melanocytes, endoneurial fibroblasts and Schwann cells (Figure 1). [3,69,84,85,97,128,133,158,159]

During differentiation of SCPs to chromaffin cells an intermediate cell type can be recognized, indicated as "bridge cell".[85] Bridge cells express markers such as *Sox2*, *Gata3*, *Htr3a*, *Phox2b*, *Ascl1* and *MycN*, largely similar to the SA progenitor and the adrenergic neuroblastoma CRC. [31,85,119-122] Chromaffin cells of SCP origin – like of SA progenitor origin – are characterized by expression of *Gata2*, *Hand2*, *Chgb*, and *Th* (Figure 2).[85,136] SCPs populate an estimated 78% of the adrenal medulla, 10% of sympathetic ganglia and 45% of the organ of Zuckerkandl.[85,128]

5. Current directions of research

A single cell fate decisions study revealed that migrating NCCs lose their multipotency and become lineage restricted through a process of multiple bifurcations.[69] In mice, the first bifurcation splits the sensory lineage from a common progenitor of autonomic and mesenchymal branches. The second bifurcation splits the autonomic neuronal fate from mesenchymal differentiation, this step is proceeded by coactivation of both programs.[69] Fate restriction becomes overt by expression of *Phox2b* (autonomic lineage) or *Prrx1* (mesenchyme lineage). This research was performed in NCCs migrating at a more cranial level than NCCs that differentiate into adrenal chromaffin cells. When the fate restriction to chromaffin cells occurs was therefore not within the scope of the study.

After the initial studies on the SCP origin of chromaffin cells,[69,85,128] many have confirmed the origin of chromaffin cells from SCPs and built upon it. Kameneva et al.[160] and Hanemaaijer et al.[161] showed that mouse SCPs formed bridge cells, which were able to differentiate into both chromaffin cells and neuroblasts. Kameneva et al. showed that human sympathoblasts could differentiate into chromaffin cells, whether this trajectory is reversible needs to be investigated in further research. In their mouse study chromaffin cells could differentiate to sympathoblasts. Jansky et al.[162] discovered that human fetal adrenal gland contained connecting progenitor cells, a yet undescribed population, which spanned the transcriptional space between bridge, chromaffin and neuroblast populations. These cells were present 7 and 8 weeks after conception, but not at later analyzed stages.

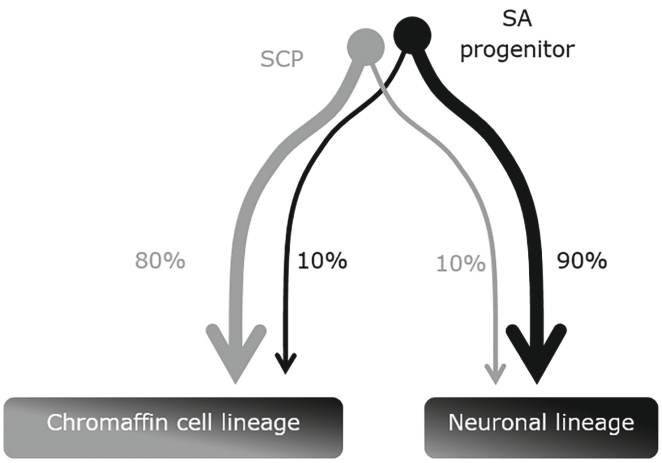
Kameneva et al. found that mature sympathoblast signature, as well as the SCP signature were associated with better prognosis in MYCN-non-amplified tumors. Hanemaaijer et al. showed that the SCP signature was associated with better survival. The SCP signature overlapped with Schwannian stroma cells in the Kameneva study, so it could be that this association is based on higher degree of invasion by non-tumorous

Schwannian stroma. Kildisiute et al.[163] revealed that neuroblastoma tumor cells, when compared to normal adrenal cell types, resembled sympathoblasts. High-risk tumors showed a weakened sympathoblast signature compared to low-risk tumors, possibly driven by distinct cancer specific processes. Jansky et al. found that MYCN-amplified tumors were most similar to neuroblasts 7-8 weeks post-conception, while the proportion of late neuroblasts increased in MYCN-non-amplified high-risk and low-risk tumors. They concluded that high-risk tumors arise earlier in development or show higher degrees of dedifferentiation than low-risk tumors.

Interestingly, in the single cell sequencing studies, the mesenchymal cell state as found in neuroblastoma cell lines could not be identified. In line, Durbin et al. found high levels of H3K27 acetylation, suggesting active transcription, of the adrenergic CRC members in nearly all neuroblastoma cell lines, regardless of adrenergic/mesenchymal cell state. This indicates a general requirement for these genes in regulating cell growth and survival in neuroblastoma *in vitro*, also in mesenchymal neuroblastoma cells.[121] In line, Gartlgruber et al. found adrenergic signatures in all neuroblastoma subgroups. However, they identified a mesenchymal signature, which was enriched in relapse samples and present regardless of coexpression of the adrenergic CRC.[130]

DISCUSSION

NC development is closely associated with neuroblastoma development. Recent studies of high-risk neuroblastoma tumors seem to pinpoint to the SCP to chromaffin cell development with the closely related neuroblast as the cell-of-origin for neuroblastoma. However, the variation in clinical presentation and course of disease suggests that there are possibly two neuroblastoma entities (Figure 3). The SA progenitor being the dominant source of sympathetic ganglia, suggests a role of the SA progenitors in the origin of low-risk sympathetic side chain tumors. Likewise, the SCP is a dominant source of chromaffin cells and therefore the most likely origin of high-risk adrenal tumors (Figure 4). The role of the SA progenitors as a source for low risk or side chain neuroblastomas is supported by several molecular similarities. Maybe the most prominent being the quintessential high *NTRK1 (TrkA)* expression which has been a hallmark of low-risk neuroblastoma cells. High *NTRK1* expression illustrates dependence of both sympathetic ganglia and low-risk neuroblastoma cells on NGF.[135]



Characteristic	Adrenal tumors	Thoracic tumors
MYCN amplified	23-24%	3%
Stage 4	50-54%	19-22%
Age ≥18 months	47-57%	39-49%
High-risk	53%	15%
LOH 1p	30%	10%
11q loss	20-26%	11-14%

Figure 3 Tumor characteristics by primary tumor localization.
The Schwann cell precursor (SCP) and sympathoadrenal (SA) progenitor contribute unevenly to the chromaffin cell lineage and the neuronal lineage. The table below the figure shows the percentage of adrenal and thoracic tumors, respectively, with a specific characteristic. Data from Vo 2014[4] and the CHOP GWAS cohort described by Oldridge 2019[169].

In the center, the development of the neural crest (NC) cells is presented as a time line. In black, the sympathoadrenal (SA) progenitor as a precursor cell is depicted, in grey the Schwann cell precursor (SCP). In the boxes left to the graph, the origin of the different tumor entities are postulated. Stage 4S originates before or during the migration of NC cells to the skin and bone marrow. Stage 1 and 2 tumors originate later, after arrival of the mNCCs in the target organ regions. Stage 4 seems to originate later in/after normal development of the target organs.

There is some overlap between the genes expressed by premigratory and migrating NCCs when compared to neuroblastoma. However, their expression is also implicated in many other diseases and tissues, indicating a broad use of these genes.[164-166] In contrast, the characteristic expression of *Phox2b*, *Th* and *Chga* in neuroblastoma indicates that neuroblastomas resemble a more differentiated phenotype than that of the migrating NCCs.

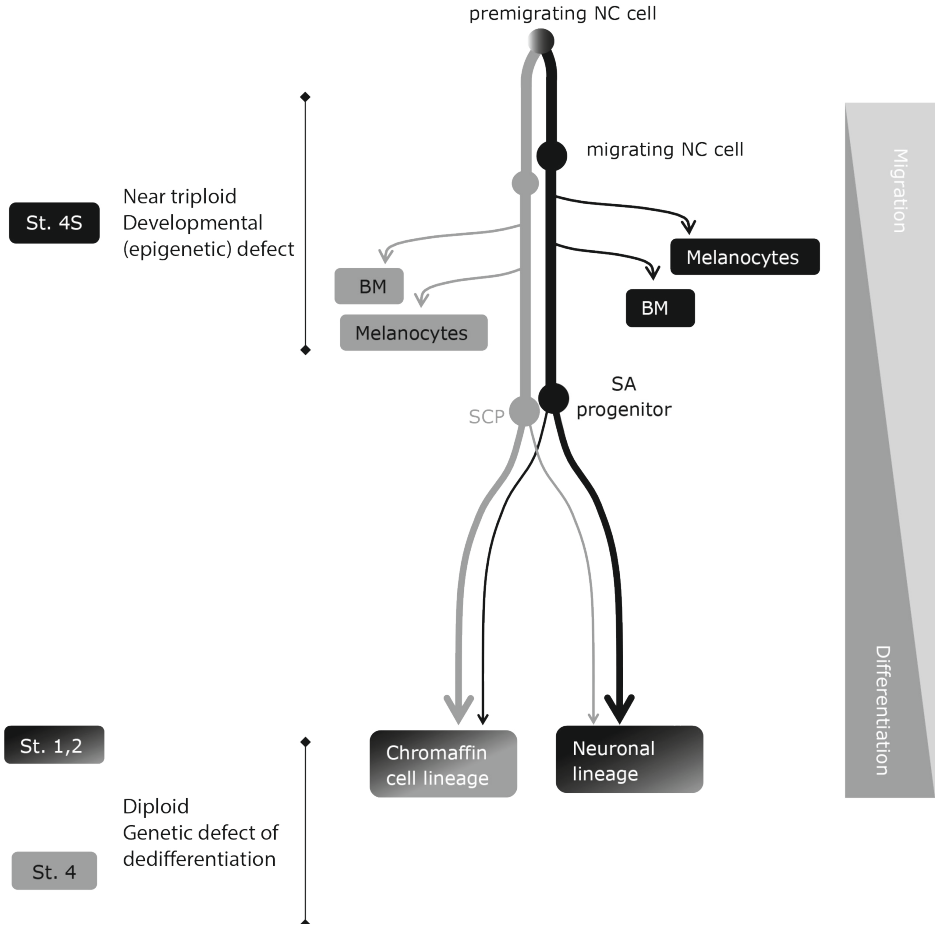


Figure 4 Neuroblastoma stages related to the embryonic development of neural crest cells
The right-sided bar indicates the relative role of migration and differentiation of the NC cells during embryonic development.

An important question in identifying the origin of neuroblastoma is the timing of malignant conversion. Stage L1 and MS tumors are diagnosed early in life, predominantly in the first 2 years of age. The pattern of tumor nodule spread and the unusual high frequency of bilateral tumors in stage MS tumors suggests that these tumors have their malignant hit/differentiation arrest very early in embryonic development, before the NCCs start delaminating at either the left or right side of the embryo and before their target organs are formed (Figure 4).[167]

The early hit is in line with the findings of the "in situ neuroblastoma" and the findings in screening studies, with higher incidence of low-risk tumors in screened populations, while the incidence of high-risk tumors was not altered.[17,32,33] The spontaneous tumor regression of low-risk tumors also resembles normal development. The parallels

between NC development and stage L1 and MS neuroblastoma raise the question if these neuroblastoma tumors are truly malignant or if they are developmental defects. The metastatic spread of stage MS tumors is therefore more likely to being multifocal seeding of NC precursor cells following normal cell migration. The SNPs associated with low-risk neuroblastoma are found in genes important for early embryonic development, rather than NC development. Could this indicate that the first hit in low-risk tumors occurs very early? Does this also imply that the tumors in high-risk patients arise later in development or even postnatal? If so, this could explain why the scRNA seq studies were incapable of identifying the cell of origin for neuroblastoma. Alternatively, the premalignant tumors of future high-risk patients could arise during embryonic development but need extra hits to become truly malignant (Figure 4).

When neuroblastoma is compared to normal development, the general transforming processes, such as cell cycle deregulation, should be considered. An integrative genomic analysis including all clinical subtypes of neuroblastoma, showed that low-risk tumors have high expression genes involved in normal SA development (*TH*, *DBH*, *PHOX2A*, *GATA3*, *NTRK1*), while high-risk tumors were enriched for genes involved in cellular metabolism, nucleic acid, protein and macromolecule biosynthesis, RNA processing and ribosomal turnover.[168] This suggests that low-risk tumors remain quite similar to their precursor cell equivalents, while high-risk tumors deviate more from their precursor cell equivalents. This further complicates efforts to ascertain the cell of origin through scRNA seq studies.

The multiple-cell-of-origin model presented here has uncertainties. The adrenergic CRC is shared between all neuroblastoma risk groups and some genetic factors are not restricted to either high-risk or low-risk tumors. An example of such is *NTRK1* expression, which seems more important in survival of sympathetic neurons than in chromaffin cells, still *NTRK1* expression is also found in chromaffin cells.[135] Nevertheless, there are evident differences between low-risk and high-risk neuroblastomas that can better be clarified with a multiple cells-of-origin tumor model, rather than with a one cell-of-origin model.

In conclusion, with the contribution of the SCP and SA progenitor to the sympathetic lineage and chromaffin lineage recently discovered, and with the two neuroblastoma entities having asymmetric division over these lineages, it seems plausible that different neuroblastoma entities (high-risk and low-risk) arise from different NC populations (SCPs and SA progenitors, respectively). With little information about the fate decisions and transcriptome of the SA progenitors, there is a need for more detailed analysis before conclusions can be drawn. Further research might provide a better comparison of embryonic cell states and tumors, enabling a cleaner view on the relationship between NC development and neuroblastoma tumor types.

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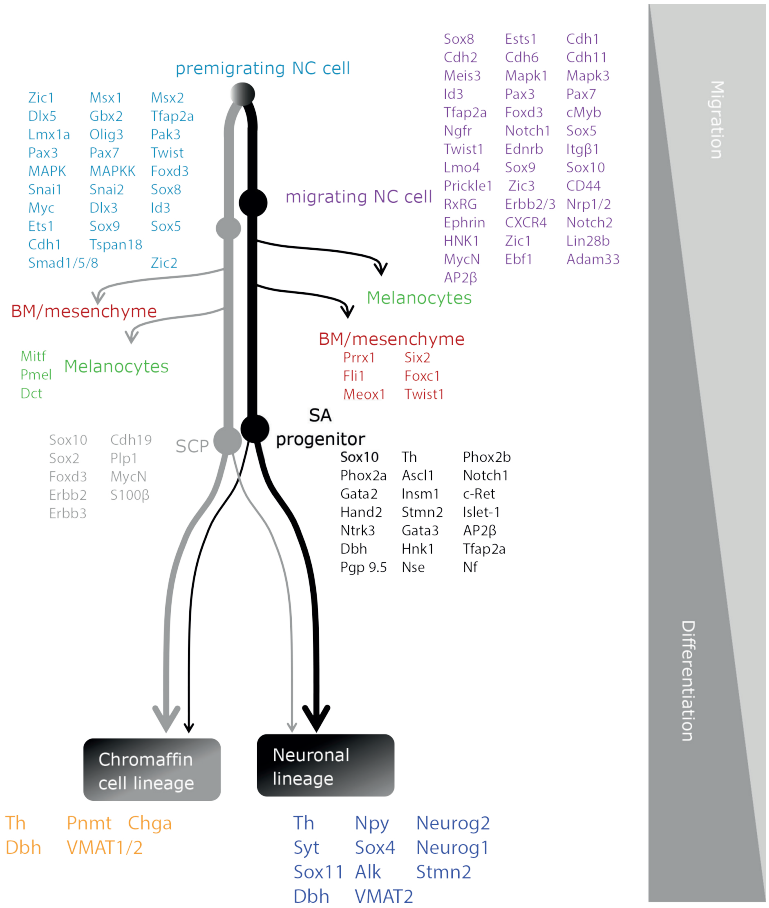
SUPPLEMENTAL MATERIAL



Supplemental Figure S1 NC development with marker genes expressed by NCCs during migration and differentiation

Schematic view of neural crest (NC) differentiation as a time line from early (top) to late (bottom) NC migration and differentiation. Marker genes expressed at the different differentiation stages of neural crest (NC) migration and differentiation are given per differentiation stage.

All genes are listed alphabetically with references below:

Adam33[79], Alk[54,132], Ascl1[66,111,113], Cd44[178,179], Cdh1/2[77,79], Cdh6[180], Cdh11[69,79,181], ChgA[110], cMyb[182]c-Ret[111], CXCR4[183] Dbh[110,111,113], Dct[69], Dlx3[71], Dlx5[69], Ebf1[80], Ednrb[184,185], Eph/Ephrin[186], Erbb2/3[82,83,187], Ets1[69,72], Fli1[69], Foxc1[69], FoxD3[69,71,188,189], Gata2[110,111], Gata3[66,110,111], Gbx2[68,70], Hand2[66,110,111], HNK1[76,112,115,190], Id3[71,191], Insm1[66,109,110], Islet-1[110], ItgB1[192], Lin28B[80], Lmo4[80,193], Lmx1a[69], Mapk[71], Mapkk[71], Mapk1/3[194], Meis3[195], Meox1[69], Msx1/2[66,69,189], Mtif[69], Myc[196], MycN[136], Neurog1/2[133], Ngfr[76], Nf[111-114], Notch1[116,197], Plp1[69,85,189] Notch2[197], Npy[131], Nse[113], Ntrk3[66], Olig3[69], Pak3[69], Pax3[70,183,198,199], Pax7[72,80,198,200], Pgp9.5[113], Phox2a[111,113], Phox2b[111,113,127], Pmel[69], Pnmt[110,112], Prickle1[77], RxRG[80,201], Sema3a/Nrp1[127,202], Six2[69],Smad1/5/8[71], Snai1/2[203], Sox4[110], Sox5[73], Sox8[70,81], Sox9[69,72,73,81,204,205], Sox10[66,80,81,112,189], Sox11[110], Stmn2[99,110-113], Sytl[110], Tfap2a[66,67,76,117], Tfap2b[110,127], Th[110,111,113], Tspan18[206], Twist1[69,189,207], Vip[131], Vmat1/2[110], Zic1[66,70,189,199], Zic2[189]Zic3[69].

CHAPTER

Neuroblastoma stage 4S: Tumor regression rate and risk factors of progressive disease

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ABSTRACT

Background

The clinical course of neuroblastoma stage 4S or MS is characterized by a high rate of spontaneous tumor regression and favorable outcome. However, the clinical course and rate of the regression are poorly understood.

Methods

A retrospective cohort study was performed, including all patients with stage 4S neuroblastoma without *MYCN* amplification, from two Dutch centers between 1972 and 2012. We investigated the clinical characteristics, the biochemical activity reflected in urinary catecholamine excretion, and radiological imaging to describe the kinetics of tumor regression, therapy response and outcome.

Results

The cohort of 31 patients reached a 10 year overall survival of 84 ±7% (median follow-up 16 years, range 3.3-39). During the regressive phase, liver size normalized in 91% of the patients and catecholamine excretion in 83%, both after a median of two months (liver size: range 0-131; catecholamines: range 0-158). The primary tumors completely regressed in 69% after 13 months (range 6-73), the liver architecture normalized in 52% after 15 months (range 5-131). Anti-tumor treatment was given in 52% of the patients. Interesting, regression rates were similar for treated and untreated patients. Four of seven patients <4 weeks old died of rapid liver expansion and organ compression. Three patients progressed to stage 4, 3-13 months after diagnosis, all had persistently elevated catecholamines.

Conclusion

Patients <4 week old with neuroblastoma stage 4S are at risk of fatal outcome caused by progression of liver metastases. In other patients, tumor regression is characterized by a rapid biochemical normalization that precedes radiological regression.

INTRODUCTION

Neuroblastoma stage 4S (S for 'Special') defined patients <12 months old with primary tumors stage 1 or 2 and dissemination limited to specific sites: liver, bone marrow (<10% invasion) and skin.[1] In the International Neuroblastoma Risk Group Staging System (INRGSS), the age limit was extended to 18 months, and primary tumors stage was not taken into account anymore.[2] In all staging systems, osteomedullary tracer uptake on ^{99}Tc bone scans or ^{123}I-MIBG scans defines stage 4. Stage 4S is characterized by a favorable course of disease and a high rate of spontaneous tumor regression.[1-3] Long term survival rates are estimated between 65% and 92%.[4-7] In general, neuroblastoma 4S can be managed by active surveillance in expectation of spontaneous tumor regression. In patients with symptoms of organ compression, anti-tumor treatment (chemotherapy or low dose radiotherapy to the liver) is advised. Although the tumor regresses in most patients, very young patients are at risk of early and rapid progression of liver metastases, causing life threatening compression of lungs, kidneys, inferior vena cava, normal liver tissue and intestines[8]. Tumor progression to true stage 4 or high risk disease is seen occasionally. This can occur before complete regression and is different from late onset recurrent disease.

Previously, we explained regression as a process of delayed differentiation of tumor nodules.[9] We consider stage 4S as a multifocal developmental disease with an onset in the early stage of neural crest development. (Pre)migratory neural crest cells suffer from a defect and spread to different target organs of the neural crest (skin, liver, bone marrow, adrenal glands, sympathetic side chain) to form proliferative tumor nodules. The tumor regression reflects a delayed step of cellular differentiation and apoptosis.[9] The process and clinical regression rate have not been studied for the expected time to normalization on different modalities. Also, it is not clear if anti-tumor treatment (chemotherapy, radiotherapy) has any effect on the initiation of the regression process.

Here, we studied the survival rates, frequency and rate of tumor regression in a retrospective cohort of patients with neuroblastoma stage 4S. We describe risk factors of progressive disease. Finally, we evaluated the effect of therapy on disease regression.

METHODS

Patient cohort

The Erasmus Medical Center's and Amsterdam University Medical Centers' Pediatric Oncology databases were reviewed to identify all patients diagnosed with neuroblastoma stage 4S[1] or MS[2] between 1972 and 2012. Patients diagnosed with stage 4 aged 12-18 months were reviewed for MS criteria. The term 4S was used for this study, since all patients met these criteria. Shallow whole-genome sequencing was performed as described previously.[10]

Clinical outcome

Tumor response was evaluated according to the International Neuroblastoma Response Criteria (INRC)[1] at last moment of follow-up. For the purpose of this study, we classified three types of progression: A: fast initial tumor progression with increase of liver mass, B: tumor progression to stage 4 neuroblastoma <5 years from diagnosis, C: late recurrent disease after >5 years . Initial treatment was according to local protocols, all protocols were based on a wait-and-see approach, unless life-threatening symptoms or complications occurred. Chemotherapy regimens differed over time but in general consisted of cyclophosphamide and vincristine. From 2009 onwards all treated patients are given the combination of cyclophosphamide, vincristine and doxorubicin. Used chemotherapeutics are listed per patient in Supplemental Table S3. Patients with progression to stage 4 aged <1 year at time of progression were upstaged to medium-risk protocols, and patients aged ≥1 year to high-risk protocols. To compare the therapy effect on regression, regression kinetics of treated patients were compared to untreated patients. Surgery only patients were analyzed in the untreated group, because surgery will not have influenced regression of metastases.

Radiological evaluation

All radiological and nuclear scan reports were reviewed (MT, BdK). For the liver, both size and architecture were evaluated. Liver size was compared to age-related reference levels.[11] No strict intervals for follow-up were defined until 2009. Ultrasounds in patients with hepatomegaly were performed biweekly or monthly in the first two months, expanding to every two months until six months after diagnosis, followed by undefined regular follow-up. ^{123}I-MIBG and MRI scans were performed every two months from 2009 onwards, and without defined follow-up intervals before 2009. CT scans were rarely performed, or in low dose in combination with ^{123}I-MIBG scanning. Patients with an event (progression, death) were excluded from the long term regression analysis.

Metabolic evaluation

Urinary homovanillic acid (HVA) and vanillylmandelic acid (VMA) were measured and reported (fold change of the upper limit for age (defined as Mean +2SD)) as described previously.[12] Time from diagnosis to last abnormal value was used instead of time to normalization. This probably represented a more accurate time to normalization beause the lack of standardized intervals occasionally resulted in long periods between last abnormal (but slightly elevated values) and first normal values. Additionally, a few patients maintained elevated HVA (n=4) or VMA (n=3) excretion levels even after years of follow-up. By using time to first normal measurement we would have to exclude these patients, causing information bias.

Statistical analysis

Statistical analysis was performed using the statistical program SPSS 25 (IBM Corp., Armonk, NY). Estimated 10-year overall survival (OS) and event-free survival (EFS) were calculated according to Kaplan-Meier survival analysis and are reported ±SE. Follow-up time was defined as time of diagnosis to any event, death or last follow-up. Fisher exact test and the Mann-Whitney *U* test were used for comparisons between treated and untreated patients. *P* values of <0.05 were considered statistically significant.

RESULTS

Patient characteristics

We identified 35 patients with the clinical diagnosis of neuroblastoma stage 4S, representing 8% of total neuroblastoma diagnoses. After exclusion of patients with *MYCN* amplification (n=4), 31 patients were included (Supplemental Table S1). The median age at diagnosis was 70 days (range 1-228 days). Seven (23%) patients were <4 weeks old. The primary tumor was located in the adrenal gland in 23 (74%) (five patients had bilateral adrenal tumors), in the sympathetic side chain in 5 (16%) patients, and remained unknown in 3 (10%) patients. All patients except one had liver metastases, 5 (16%) had skin metastases. Bone marrow was evaluated in 28 patients, and infiltrated in 7 (25%) (Table 1). Anti-tumor treatment was given in 16 (52%) patients: 12 (39%) patients received chemotherapy, 7 (23%) received Iodine-131-Metaiodobenzylguanidine (^{131}I-MIBG) therapy, 6 (19%) received radiotherapy and the primary tumor was surgically removed in 5 (16%) patients (Supplemental Table S1). One patient was presented in a case report previously.[13]

Survival and response

The 10-year OS and EFS were 84 ±7% and 69 ±9%(Figure 1A), respectively. At last follow-up, 18 (58%) patients had reached complete remission (CR), 8 (26%) (very good) partial remission ((VG)PR) and 5 (16%) had died of the neuroblastoma. This was similar in treated and untreated patients (Table 1).

Table 1. Patients characteristics at diagnosis and outcome, compared between untreated and treated patients.

	Untreated n/N (%)	Treated n/N (%)	Sign (p)
Patient characteristics at diagnosis			
Gender: male	9/15 (60.0)	7/16 (43.8)	0.48
Age <1 month	5/15 (33.3)	2/16 (12.5)	0.22
Age (median days, range)	70 (1-228)	78 (2-186)	0.29
Primary tumor	11/15 (73.3)	12/16 (75.0)	NA
adrenal	2/15 (13.3)	3/16 (18.8)	
Sympathetic side chain	2/15 (13.3)	1/16 (6.3)	
unknown			
Liver metastases	15/15 (100)	15/16 (93.8)	NA
Skin metastases	2/15 (13.3)	3/16 (18.8)	NA
Bone marrow metastases	2/12 (16.7)	5/16 (31.3)	0.66
Fold change of HVA at diagnosis (median)	3.67 (n=12)	13.35 (n=10)	0.12
Fold change of VMA at diagnosis (median)	4.61 (n=12)	13.33 (n=10)	0.12
MYCN amplification status unknown	5/15 (33.3)	4/16 (25.0)	0.70
LOH1p (n=21)	2/9 (22.2)	3/12 (25.0)	NA
Outcome characteristics			
Progression	12/15 (80.0)	10/16 (62.5)	0.60
no progression	2/15 (13.3)	2/16 (12.5)	
Type A	1/15 (6.7)	2/16 (12.5)	
Type B	0/15 (0.0)	2/16 (12.5)	
Type C			
INRC at last follow up	9/15 (60.0)	9/16 (56.3)	NA
CR	4/15 (26.7)	4/16 (25.0)	
(VG)PR	2/15 (13.3)	3/16 (18.8)	
PD*			
Primary tumor	6/8 (75)	5/8 (62.5)	NA
Complete regression	2/8 (25)	3/8 (37.5)	
Residual lesion			
Liver	6/12 (50.0)	5/11 (45.5)	NA
Normalization	6/12 (50.0)	6/11 (54.5)	
Parenchymal aberrations and/or hepatomegaly			
Last abnormal HVA (months, median)	1.2 (n=12)	3.5 (n=12)	0.32
Last abnormal VMA (months, median)	0.3 (n=12)	2.2 (n=12)	0.14
First normal HVA (months, median)	6.7 (n=11)	6.2 (n=9)	0.67
First normal VMA (months, median)	5.4 (n=11)	6.3 (n=10)	0.62

Abbreviations: NA: not applicable, HVA: homovanillic acid, VMA: vanillylmandelic acid, LOH: loss of heterozygosity, INRC[1], CR: complete remission, (VG)PR: (very good) partial remission, PD: progressive disease.

* All patients with progressive disease at last follow up died of disease.

Tumor regression

Adrenal/sympathetic side chain tumor: Evaluation of primary tumor regression was feasible in 16 of 31 patients. For 15 patients follow-up was not feasible because of progressive disease (n=7), resection of the primary tumor (n=5) or an unknown primary tumor (n=3). Complete regression was achieved in 11 (69%) of 16 patients, after a median time of 13 months (range 6-73 months; Figure 2), while residual lesions persisted in 5 (31%) patients (Table 1). Primary tumor regression was comparable in treated and untreated patients (63 vs. 75%%, 15 vs. 9 months, respectively; Table 1). Interestingly, complete regression was achieved in 92% of the adrenal tumors, while in 0% of the sympathetic side chain tumors ($p<0.01$).

Liver: Regression of liver metastases was studied in 23 of 30 patients with liver metastases. For seven patients follow-up was not possible because of progressive disease. Liver size normalized in 21 (91%) patients, after a median of two months (range 0-131) (Figure 2). No difference was observed between the treated and the untreated patients (91% vs. 92%, and 10 vs. 2 months $p=0.31$). The liver architecture normalized in 12 (52%) patients, after a median time of 14.5 months (range 5-131 months). Again, no difference was observed between the treated and the untreated patients (45% vs. 58%, $p=0.68$; 17 vs. 14 months $p=0.45$). In 11 (48%) patients, both the size and architecture of the liver completely normalized.

Metabolic regression: Regression of the catecholamines HVA and VMA was studied in 24 of 31 patients. In the other seven patients excretion levels were not available, because of rapid progression (n=4) or before this was standard of care (n=3). HVA levels normalized in 20 (83%) patients, VMA in 21 (88%) patients. Last abnormal values were measured after a median of 2.2 months for HVA (range 0-158) and 1.6 months for VMA (range 0-158) (Figure 2), which was comparable between treated and the untreated patients ($p=0.32$ and $p=0.14$) (Table 1).

Progressive disease

Patients with progressive disease were subcategorized in three types of progression. Four patients died of fast initial tumor progression (type A). Three patients progressed to stage 4 disease <5 years from diagnosis (type B), one of them died of disease and two are in CR after additional treatment. Two patients suffered from late relapses (type C): one with stage 4 disease without *MYCN* amplification who is in complete remission after high-risk treatment; the other with a localized ganglioneuroma, which has been stable without further treatment.

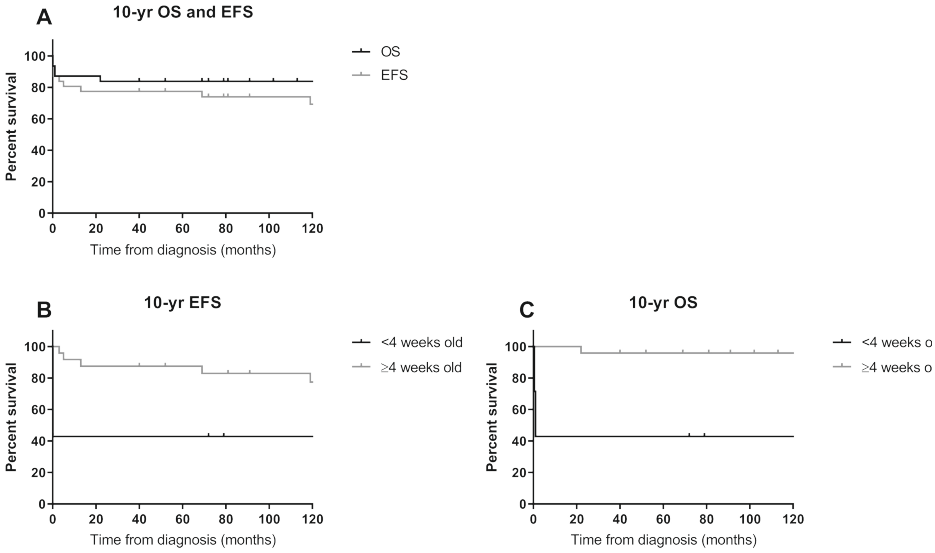

Figure 1. Kaplan-Meier estimates of survival of the neuroblastoma stage 4S patients.
10-yr EFS and the OS of all patients; (A) 10 year EFS (grey line; 69±9%) and OS (black line; 84±7%). (B) 10-yr EFS according to age groups (black line <4 weeks old at diagnosis (n=7) 43±19%; grey line ≥4 weeks (n=24) 77±9%; p=0.02). (C) 10-yr OS according to age groups (black line <4 weeks old (n=7) 43±19%; grey line ≥4 weeks (n=24) 96±4%; p<0.001).

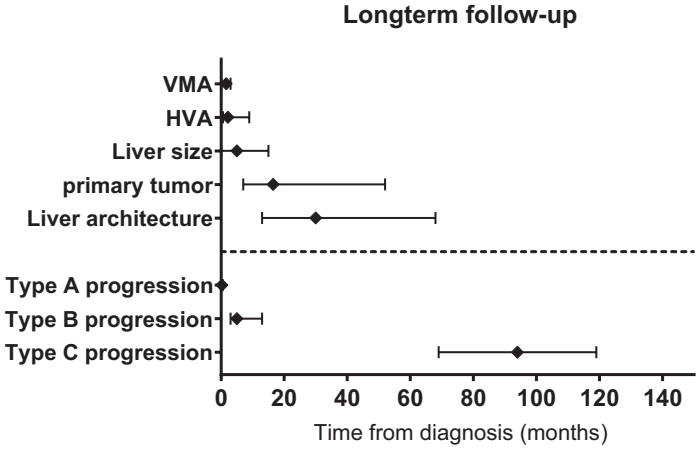

Figure 2. Time to normalization according to clinical assessment and events.
Median time from diagnosis to normalization or event in months, with 95% confidence intervals, according to respectively urinary catecholamines, radiological assessment and clinical events, respectively.

Type A progression: Fast initial tumor progression with increase of the liver mass: Liver metastases and hepatomegaly were present in respectively 30 and 21 patients. Six patients (4 patients <4 weeks old) developed respiratory distress requiring mechanical ventilation (Figure 1B). All four very young patients died within three weeks after diagnosis of multiple organ failure due to organ compression. Two of them were treated with cyclophosphamide and vincristine without effect on liver mass or respiration. The other two patients <4 weeks old did not receive anti-tumor treatment, because of poor clinical condition, they died within 2 days after admission. The two older patients, 6 weeks and 6 months old, survived. In total, four of seven patients <4 weeks old (58%) died of Type A progression, resulting in a significant higher risk of dying due to Type A progression for younger than for older patients (Figure 1C) (log rank p<0.001).

Type B progression: Progression to stage 4 disease <5 years from diagnosis: Type B progression occurred in 3 patients – 3, 8 and 13 months after initial diagnosis – and was fatal in one patient despite high-risk treatment (Table 1). The remaining two patients are in CR, 99 and 108 months after progression. In none of the patients with Type B progression catecholamine excretion normalized prior to the progression. In contrast, patients with no progression and finally CR, the catecholamines normalized after 1.2 months (range 0-8.9 months) for HVA and 0.7 months (range 0-4.2 months) for VMA (Figure 3; Type B progression vs CR: p=0.02 for HVA, p<0.01 for VMA).

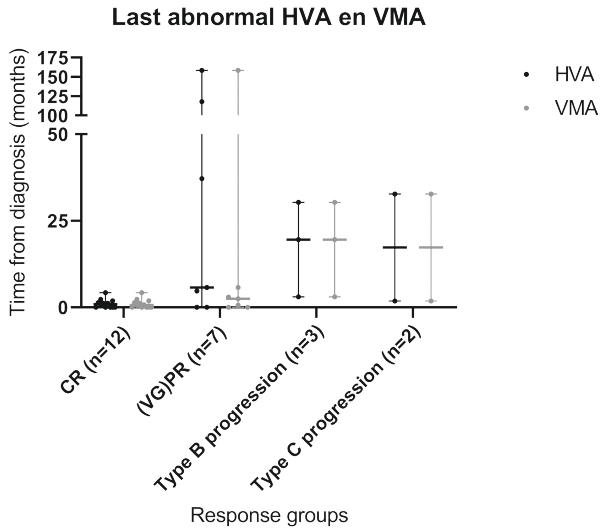

Figure 3 Last abnormal HVA and VMA for different response groups.
The last abnormal values (measured from diagnosis) for HVA and VMA are depicted in months (y-axis) for the response groups. HVA, black dots/ lines; VMA grey dots/lines, whiskers depict the range. CR group: median 1.2 months (range 0-8.9 months) for HVA and 0.7 months (0-4.2 months) for VMA. (VG)PR group: 5.7 months for HVA (0-159 months) and 2.5 months for VMA (0-159 months). Type B progression: 19.5 months for HVA and VMA (3.0-30.3 months). Type C progression: 17.3 months for HVA and VMA (range 1.8-32.7 months).
CR: complete remission, (VG)PR: (very good) partial remission.

Type C progression: Late recurrent disease after >5 years: Type C progression occurred in two patients: one suffered from recurrent stage 4 disease, without *MYCN* amplification, 69 months after initial neuroblastoma 4S diagnosis. This patient is currently, 97 months after progression, in CR after high-risk treatment. The other patient developed a new tumor nodule in a previously unaffected site, 119 months after initial neuroblastoma 4S diagnosis. Biopsy revealed a ganglioneuroma and the tumor remained stable without treatment until last follow-up 143 months later.

Copy number analysis

Twenty tumors were evaluated for numerical chromosomal aberrations (NCA: whole chromosome gains and losses) and for segmental chromosomal aberrations (SCA: partial gains and losses).[14] No tumor material was available for patients with Type A progression. Two of three patients with Type B progression had isolated SCA, while none of the progression free patients had isolated SCA (Supplemental Table S2). The patient with type C stage 4 progression had both NCA and SCA at time of 4S diagnosis. In this limited cohort significant differences were observed in event-free survival when

the patients were classified in the different genomic pattern groups. Patients with only NCA had a 10-yr EFS of 89 ±11%, patients with both NCA and SCA had a 10-yr EFS of 86 ±13%, patients with only SCA had a significant poorer 10-yr EFS 0% (log-rank p<0.01, Supplemental Figure S1).

DISCUSSION

We performed a retrospective cohort study investigating the regression kinetics and risk factors of progression in patients with stage 4S neuroblastoma. In this cohort of 31 patients the 10-yr OS was 84 ±7%. Catecholamine excretion and liver size normalization occurred in 83% and 91% of the patients after a median of two months. Primary tumors and liver architecture normalized in 69% and 52% after a median of 13 and 15 months, respectively (Figure 2). Patients <4 weeks old are at risk of a fatal outcome due to massive progression of liver metastases and subsequent organ compression.

Normalization of liver architecture has been reported by Levitt et al. and French et al., they describe liver architecture normalization in 42% and 48% of 28 and 15 patients, respectively.[15,16] One paper described liver size normalization in <12 months in two patients.[17] Early expansion of liver metastases caused respiratory distress requiring mechanical ventilation in 19% of patients in the first weeks after diagnosis. It was only fatal in children <4 weeks old. In our cohort, 58% of the patients <4 weeks died of type A progression, while none of the patients ≥4 weeks did (p<0.001). In six previous studies [6,8,18-21] with a total of 506 neuroblastoma stage 4S patients, 45 patients died of what we describe as type A progression. Of these 45 children 93% was <2 months old at diagnosis. A recent report of patients with symptomatic disease and/or unfavorable histology, showed a 13 times increased risk of fatal outcome in patients younger than 40 days compared to patients older than 47 days.[22] These and our study lead to the conclusion that children <2 months at diagnosis are at risk of dying of type A progression. They should be monitored closely and starting anti-tumor treatment should be considered at onset of symptoms. Older children are hardly at risk for a fatal outcome due to type A progression, even if the liver nodules show initial progression.

Anti-tumor treatment is given in patients with type A progression in an attempt to induce tumor regression to prevent clinical complications of organ compression. However, its effectiveness has never been proven. Here, the commonly used combination of vincristine and cyclophosphamide did not result in arresting the progression in the two treated young patients. Additionally, we observed no difference in the outcome or the regression rate of patients with or without treatment. Although this is a retrospective study with a limited number of patients, which hampers a proper analysis of base line differences in tumor load, the question is raised whether anti-tumor therapy influences the outcome or regression in very young children with this type of early progression, and if anti-tumor treatment is needed in older stage 4S patients. Moreover, a recent large prospective study could not establish an effect of chemotherapy on tumor regression or

outcome.[22] Considering the concept of delayed differentiation of early neural crest cells and subsequent differentiation,[9] the question is valid if chemotherapy can accelerate the differentiation and regression process or if it can halt the progression. Therefore it would be best to study (early) response kinetics in detail in a prospective cohort.

The retrospective design and the long inclusion period are obvious limitations of the study. In this 40-year period, improvements have been achieved in the quality and availability of imaging techniques and other diagnostics. Supportive care and treatment protocols have also been improved, our current treatment protocol contains doxorubicin in addition to cyclophosphamide and vincristine. Still, one of the patients that died of type A progression was diagnosed in 2011, when all modern imaging modalities were present and our current treatment protocol was standard care.

All patients with type B progression to stage 4 disease retained elevated catecholamine excretion until the tumor progression was observed, suggesting that these tumors are biologically different than tumors that regress. A different biology is also suggested by the higher number of patients with structural chromosomal aberrations.[23] This is consistent with earlier reports that tumors with segmental aberrations have a more aggressive disease.[14,23,24] Because CNA was tested in only a small number of patients in this study, we cannot draw definitive conclusions. However, in the ongoing European LINES trial, the effect of the CNA in this group is being prospectively studied. Recent research concluded that the combination of telomere maintenance activity and aberrations in the RAS and TP53 pathways can predict unfavorable outcome in low-risk patients.[25] It would be interesting to see if these parameters are helpful in making treatment decisions.

In conclusion, patients with neuroblastoma stage 4S have a favorable outcome as a result of spontaneous tumor regression. Biochemical regression precedes radiological normalization and is usually reached within the first months. Children <2 months old at diagnosis are at risk of fatal outcome in the first weeks after diagnosis due to tumor progression and it is strongly recommended to watch them closely and consider treatment. The remainder of patients has a small chance to progress to high-risk disease, especially when they have normalized biochemical activity and no segmental gains and losses in their tumor genome.

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Supplemental Table S1 Detailed patients characteristics at diagnosis, with genetic prognostic factors, clinical outcome, biochemical evaluation and radiological outcome.

Patient characteristics at diagnosis						Genetic prognostic factors		Clinical outcome			Time to normalization of catecholamines				Radio-logical outcome	
Patient ID	Gender	Age (mo.)	Primary tumor	Meta-stases	Therapy	MYCN ampli-fication	LOH1p	Response	Out-come	Follow-up (mo.)	Last ab-normal HVA (mo.)	Last ab-normal VMA (mo.)	First normal HVA (mo.)	First normal VMA (mo.)	Primary tumor	liver
93	M	0	adrenal both sides	liver	No	NA	NA	PD	DOC	0					PD	PD
1	F	0	adrenal L	liver	No	neg	neg	CR	ANED	72	1,8	1,8	5,4	5,4	CR	IP
83	M	0	adrenal L	liver	No	NA	NA	PD	DOC	0					PD	PD
20	M	0	adrenal L	liver	No	neg	neg	CR	ANED	137	2,5	0	13,2	0	CR	CR
89	M	0	adrenal both sides	liver	No	NA	NA	CR	ANED	79	0	0	2,6	0	CR	CR
10	M	1	adrenal L	liver	No	neg	neg	ST 4	ANED	102	3	3	5,2	5,2	CR	CR
86	F	1	adrenal R	liver	No	NA	NA	CR	ANED	259	0	0	17,7	17,7	CR	CR
85	M	2	unknown	liver	No	NA	NA	CR	ANED	215					NA	CR
91	F	2	thoracic	liver, BM	No	neg	neg	VGPR	AWD	81	0	0	6,7	6,7	SD	IP
5	M	3	adrenal both sides	liver	No	neg	neg	CR	ANED	203	0,6	0,6	3	3	CR	HM
88	M	3	adrenal R	liver, skin, BM	No*	NA	pos	PR	AWD	160	158,1	158,1			resected	IP
17	F	3	sympathetic chain	liver, skin	No	neg	pos	PR	AWD	199	117,7	3	130,6	5,2	SD	CR
12	F	4	unknown	liver	No	neg	NA	CR	ANED	203	0	0	17,4	17,4	NA	IP
21	M	7	adrenal L	liver	No	neg	neg	CR	ANED	189	0	0	6,5	6,5	CR	CR
84	M	0	adrenal both sides	liver, skin, BM	CT	NA	NA	PD	DOC	1					PD	PD
87	F	0	adrenal L	liver	CT	NA	NA	PD	DOC	1					PD	PD
16	M	1	adrenal L	liver	CT, RT, MIBG	neg	pos	Relapse st 4	ANED	166	1,8	1,8	5	5	CR	IP
90	F	1	sympathetic chain	liver, skin	CT, MIBG	neg	NA	PR	AWD	91	2,5	2,5	4,7	4,7	SD	CR
3	F	1	unknown	liver, BM	CT, MIBG	neg	neg	CR	ANED	208	1,4	1,4			NA	CR

Supplemental Table S1 Detailed patients characteristics at diagnosis, with genetic prognostic factors, clinical outcome, biochemical evaluation and radiological outcome.

Patient characteristics at diagnosis						Genetic prognostic factors		Clinical outcome			Time to normalization of catecholamines				Radio-logical outcome	
Patient ID	Gender	Age (mo.)	Primary tumor	Meta-stases	Therapy	MYCN ampli-fication	LOH1p	Response	Out-come	Follow-up (mo.)	Last bnormal HVA (mo.)	Last ab-normal VMA (mo.)	First normal HVA (mo.)	First normal VMA (mo.)	Primary tumor	liver
6	M	1	adrenal R	skin	CT, S	neg	NA	relapse GN	AWD	262	32,7	32,7	33,6	33,6	resected	CR
81	F	2	adrenal both sides	liver, BM	CT, RT, S	NA	NA	CR	ANED	468					CR	IP
7	F	2	adrenal R	liver	MIBG	neg	neg	CR	ANED	40	1,2	0,7	6,2	1,2	CR	HM/IP
11	F	2	adrenal R	liver	S	neg	neg	VGPR	AWD	205	37	3	109,2	37	resected	IP
4	F	2	adrenal L	liver	MIBG	neg	pos	ST 4	ANED	113	109,5	30,2		32,2	resected	IP
2	M	3	sympathetic chain	liver, pleura	CT	neg	neg	PR	AWD	69	0	0	24,2	24,2	SD	CR
15	M	3	adrenal R	liver	RT, S	neg	NA	CR	ANED	272	4,2	4,2	5,4	5,4	resected	CR
9	F	4	adrenal R	liver	MIBG	neg	pos	CR	ANED	221	1,2	1,2	4,4	4,4	CR	CR
92	M	4	adrenal R	liver, BM	CT	neg	neg	VGPR	AWD	52	5,7	5,7	11,9	11,9	SD	IP
82	F	5	adrenal L	liver	CT, RT, S	NA	NA	CR	ANED	393					resected	IP
8	F	6	adrenal R	liver, BM	CT, RT, MIBG	neg	neg	CR	ANED	198	8,9	0	12	7,1	CR	IP
19	M	6	sympathetic chain	liver	CT, RT	neg	pos	ST 4	DOP	22	19,5	19,5			SD	HM/IP

Detailed baseline and outcome characteristics of the cohort. Response to therapy was evaluated according to the International Neuroblastoma Response Criteria (INRC).[1]

Abbreviations: mo.: months, LOH: loss of hyterozygosity, HVA: homovanillic acid, VMA: vanillylmandelic acid, M: male, F: female, BM: bone marrow, CTx: chemotherapy, RT: radiotherapy, ^{131}I-MIBG: ^{131}I-MIBG therapy, S: surgery, NA: not available, US: ultrasound, MR: magnetic resonance imaging, ^{123}I-MIBG: ^{123}I-MIBG SPECT scan, AXR: abdominal X-ray, CXR: chest X-ray, SXR: skeletal survey X-rays, ^{111}In SMS: ^{111}In somatostatin scan, ^{99}Tc: ^{99}Tc bone scan, CT: computed tomography scan, resp, respiratory symptoms, clotting: clotting disorder, GI: gastro-intestinal problems, neg: negative, pos: positive, DOD: died of disease, CR: complete remission, VGPR: very good partial remission, PR: partial remission, IP: inhomogeneous parenchyma, HM: hepatomegaly

* the adrenal tumor of patient #88 was resected 17 months after diagnosis for diagnostic and not therapeutic reasons (see ref 13)

† in patients with progression types B and C we only analyzed the treatments given before the progression.

Supplemental Table S2: Comparison of overall genomic patterns and progression types.

Progression type	Chromosomal aberrations		
	NCA only	SCA only	SCA+NCA
No progression	8	0	7
Type B progression	1	2	0
Type C progression	0	1	1

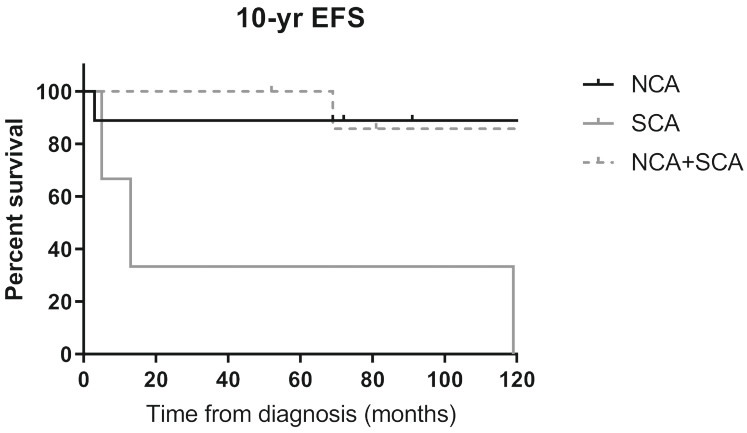
Overall genomic patterns as described by Janoueix-lerosey et al. [14] compared to different progression types. Abbreviations: NCA: numerical chromosomal aberrations (whole chromosome gains and losses); SCA: segmental chromosomal aberrations. Fisher's Exact: p=0.01.

Supplemental Table S3. Chemotherapy courses per patient.

patient #	chemotherapy
2	2x VCR/Dox/CPM
3	1x VCR/CPM/Pred
6	1x CPM, 1x VCR/Carbo/Dox
8	1x VCR/CPM
16	1x VCR/TOPO
19	1x VCR/Pred, 1xVCR
81	2x VCR/CPM/MTX, 2x CPM
82	4x VCR/CPM/Pred
84	1x VCR/CPM
87	1x VCR/CPM
90	2x VCR/Dox/CPM
92	3x VCR/Dox/CPM

Chemotherapy regimens used in the patients who received chemotherapy.
Abbreviations: VCR: vincristine; Dox: doxorubicin; CPM: cyclophosphamide; pred: prednisolone; Carbo: carboplatin; TOPO: topotecan; MTX: methotrexate.

Supplemental Figure S1 Coverage plot displaying the structural variations in primary tumors
Shallow whole genome sequencing data (above the black line) with coverage calculated for 0.1 Mb bins, normalized to the mean coverage of lymphocyte DNA of 19 healthy persons. For the normal whole genome sequencing data (below the black line) this was calculated for 1 Mb bins, normalized to the coverage in patient lymphocyte DNA. Color intensity reflects the magnitude of the gains or losses (blue=loss, red=gain).



Supplemental Figure S2. 10-yr EFS by genomic pattern of the tumor.
10-yr EFS by overall genomic pattern as described by Janoueix-Lerosey et al.[14]. 10-yr EFS was 89 ±11% for patients with NCA only (n=9), 86 ±13% for patients with both NCA and SCA (n=8) and 0% for patients with SCA only (n=3) (log-rank p<0.01). NCA= numerical chromosomal aberrations; SCA segmental chromosome aberrations.

CHAPTER

Refractory stage M ganglioneuroblastoma with bone metastases and a favorable, chronic course of disease; description of a patient cohort

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ABSTRACT

Refractory stage M neuroblastoma is associated with a poor prognosis and a progressive course of disease. Here, we describe a unique group of patients with a discrepant clinical course. Seven histologically confirmed ganglioneuroblastoma (n=6) and differentiating neuroblastoma (n=1) patients were identified who were diagnosed with stage M disease based on ^{123}I-MIBG avid bone metastases. Six patients started on high-risk treatment, without tumor response (stable disease). Treatment was discontinued before the start of consolidation treatment due to refractory response in all patients. Unexpectedly, after cessation of treatment no progression of disease occurred. In two patients, the primary tumors expanded (>25%) very slowly during 1.5 and 3 years, and remained stable thereafter. Metabolically, a slow decrease of urinary HVA and VMA levels and ^{123}I-MIBG avidity was observed. All patients are alive with presence of metastatic disease after a median follow-up of 17 years (range 6.7-27 years). Interestingly, at diagnosis, six patients were asymptomatic, six patients had ganglioneuroblastoma morphology and five patients had meningeal metastases. These are all features seen in only a small minority of stage M patients. This ganglioneuroblastoma entity illustrates the clinical heterogeneity of neuroblastic tumors and can be used to further study the developmental origin of different neuroblastoma subtypes.

INTRODUCTION

Neuroblastoma is a pediatric malignancy with a variable clinical course. Even in metastatic disease, patient groups with good to dismal prognosis can be recognized based on histopathology, age, dissemination pattern, and genetic aberrations (e.g. amplification of the *MYCN* oncogene).[1] High-risk tumors are characterized by stage M and age at diagnosis older than 18 months, or by amplification of *MYCN*.[1] Despite intensive multimodality treatment, the clinical course is unfavorable, with 5 year overall survival (OS) estimated around 50%.[1,2] A rare phenotype of metastatic disease is the low-risk, stage MS (metastatic special) tumor, with the potential of spontaneous regression after limited or no treatment, and a 5 year event free survival (EFS) of >85%.[1,3] By definition stage MS is diagnosed in patients younger than 18 months old, with metastases limited to liver, skin and bone marrow (<10% infiltration), and lacking bone metastases.

Response to induction treatment is an important prognostic factor for patients with stage M neuroblastoma. Patients who reach complete response (CR) or very good partial response (VGPR) at the end of induction have a better outcome than patients with a partial response (PR) or less (no response, mixed response or progression).[4] Patients with refractory disease to chemotherapeutic induction are considered ultra-high-risk patients.[5] A retrospective study estimated the median time to progression of refractory patients at 23.5 months and the median OS at 30.3months. The 5-yr EFS and OS were estimated at 0%.[5]

Here, we describe a cohort of patients with refractory stage M ganglioneuroblastoma (GNB) (n=6) and differentiating neuroblastoma (NB) (n=1) with an unexpected chronic clinical course and excellent long-term outcome. From this cohort, we identified patient characteristics for future early clinical detection. In addition, we discuss the heterogeneous course of metastatic neuroblastic tumors from a cell-of-origin perspective.

PATIENTS AND METHODS

Patients

The clinical courses of disease of patient 1 and 2 were exceptional and based on these index patients we reviewed all Dutch patients diagnosed with a stage 4 [International Neuroblastoma Staging System (INSS) staging] or M [International Neuroblastoma Risk Group (INRG) staging] GNB and NB between 1990 and 2014. The criteria for retrospective inclusion in this cohort were 1) the presence of GNB or NB stage M disease, 2) refractory disease under treatment or stable disease without treatment and 3) survival of at least 5 years. The following data were collected from the original medical records: symptoms at diagnosis, response to treatment, histological, radiological, nuclear imaging [^{123}I-MIBG (metaiodobenzylguanidine) scans] and biochemical (urinary catecholamine excretion) characteristics. Patients were classified using the INRG staging system.[1]

Metabolic and Radiologic response

Urinary catecholamine excretion [homovanillic acid (HVA), vanillylmandelic acid (VMA), dopamine, epinephrine, norepinephrine, metanephrine, normetanephrine and 3-methoxytyramine (3MT)] were reported as fold change of the upper limit for age (defined as mean +2 SD) as described previously.[6] Ultrasound, computed tomography (CT) and magnetic resonance imaging (MRI) scan reports, as well as ^{123}I-MIBG single photon emission computed tomography (SPECT) whole body/CT scan reports were reviewed retrospectively. Response to treatment at last follow-up was determined using the 2017 revision of the International Neuroblastoma Response Criteria.[7]

Pathology and Shallow whole genome sequencing

All available tumor material was reviewed by an expert pediatric pathologist (R.v.D.). DNA was isolated from formalin-fixed paraffin-embedded (FFPE) archived pathology material of the primary tumors or lymph node metastases. Areas with highest tumor cell percentage were selected for DNA isolation. Shallow whole genome sequencing was performed as described previously.[8] Whole genome sequencing was performed in two tumors as described previously.[9] (Shallow) whole genome sequencing was

visualized in the R2: Genomics Analysis and Visualization Platform (https://r2.amc.nl). *MYCN* amplification status was determined by fluorescent in situ hybridization (FISH) or Southern blot.

RESULTS

Patient characteristics and outcome

Between 1990 and 2014, 306 patients with stage M GNB or NB were diagnosed.[10] Seven patients were identified who met the inclusion criteria. The median age at diagnosis was 18 months (range: 5 to 47 months). Dissemination was observed in bone (n=7), bone marrow (n=4), meninges (n=5), lymph nodes (n=4), liver (n=1), skin (n=1), pleura (n=1), muscle (n=1) and ovary (n=1) (Table 1). Six patients had GNB morphology, one patient had differentiating neuroblastoma morphology. At presentation, six patients were asymptomatic, one patient (#2) had a persistent fever without focus. Six patients were regarded as high-risk patients and five were initially treated according to high-risk protocols (Supplemental Table S1). For patient #2 (below) initial high-risk treatment was not available in her native country, but started in the Netherlands, after disease progression. Patient #6 was never treated with systemic therapy. At diagnosis, clinical history revealed indications of presence of the metastatic indolent neuroblastoma for two years. In all six treated patients, high-risk treatment was discontinued before the start of consolidation due to refractory disease status and at the time limited alternative treatment available. After cessation of treatment, disease progression with fatal outcome was expected, but stable disease was observed. In the current INRG risk classification, patient #2, 3 and 4 would have been classified as intermediate-risk, because of an age younger than 18 months, the other four patients would still classify as high-risk. After induction treatment, all patients had a refractory response. In current times they would subsequently continue treatment according to the VERITAS (NCT03165292), BEACON trial (NCT02308527) or equivalent trials. All patients are alive and well after a median follow-up of 17 years (range: 6.7 to27 y). In the complete cohort of 306 stage M patients, the 5-yr OS was 35±3%. For patients with GNB morphology (n=16) 5-yr OS was 69±12%, compared to 33±3% for NB morphology (n=287). Study patient characteristics are summarized in Table 1 and the clinical course of two index patients is described in more detail below.

Table 1 Patient characteristics and tumor response

Patient number	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Diagnosis							
Age at Dx (mo)	26	14	11	5	18	47	18
Presenting symptoms	Cervical mass	Persistent fever	Racoon eyes	Skin nodules	Swelling above the eye	Swelling frontal bone and above the eye	Abdominal mass
Primary tumor	Thorax	Adrenal left	Organ of Zuckerkandl	Adrenal bilateral	Adrenal right	Adrenal left	Thorax
Metastases	Bone, BM, LN, pleura	Bone, meninges, LN	Bone, BM	Bone, meninges, LN, liver, skin,ovary	Bone, meninges	Bone, meninges, muscle, LN	Bone, BM, meninges
Histology	GNBn	GNB NOS	GNB NOS	GNBi	GNBi	GNBi	NB diff.
DNA copy number	NA	NA	NCA and SCA	NCA	NCA	ND	ND
MNA	Neg	Neg	Neg	Neg	Neg	ND	Neg
Response							
Treatment	CT, MIBG	CT, MIBG, S, RA	CT, S	CT, S	CT, S	S	CT, MIBG, S
Urinary catecholamines							
HVA normalized	yes	yes	no	yes	no	no	no
VMA normalized	yes	yes	yes	yes	yes	yes	yes
INRC	PD	MR	PD	MR	PR	MR	MR
Primary tumor response	PD	CR	PD	PR	CR	CR	PR
Tumor Response at Metastatic Sites	PD	SD	PD	CR	PR	SD	SD
BM metastases response	SD	CR	CR	SD	CR	CR	CR
Follow-up (yr.)	16.9	20.1	26.8	24.2	10.2	11.7	6.7

Abbreviations: BM: bone marrow; CR: complete response; CT: chemotherapy; diff.: differentiating; Dx: diagnosis; GNB: ganglioneuroblastoma; i: intermixed; INRC: international neuroblastoma response criteria; LN: lymph nodes; MIBG: ^{131}I-MIBG treatment; MNA: *MYCN* amplification; mo: months; MR: minimal response; n: nodular; NA: not available; NB: neuroblastoma; NCA: numerical chromosomal aberrations; ND: not done; Neg: negative; NOS: not otherwise specified; PD: progressive disease; PR: partial reponse; RA: retinoic acid; S: surgery; SCA: structural chromosomal aberrations; SD: stable disease.

Patient 1

A two-year-old male presented with a right-sided cervical lymph node mass. Chest X-ray, ^{123}I-MIBG and CT scans revealed a large thoracic neuroblastic tumor (Figs 1A, B), multiple involved cervical lymph nodes, a pleural lesion, and multiple skeletal metastases. Urinary excretion of HVA, dopamine and 3MT was elevated, VMA excretion was normal (Supplemental Figure S1). Histology was defined as GNB nodular (GNBn), without *MYCN* amplification (Figs 1C, D). High-risk treatment (Supplemental Table S1) was initiated, but no response was observed. On second-line chemotherapy, the disease remained stable and treatment was discontinued. During the first 2 years after diagnosis the primary tumor expanded slowly (>25%), and remained stable over the following 15 years. A new ^{123}I-MIBG avid bone lesion was observed 1.5 years after diagnosis, but subsequently ^{123}I-MIBG avidity gradually decreased for all lesions, without reaching complete remission (Figure 1A). Urinary HVA, dopamine and 3MT excretion slowly decreased and normalized 7, 10 and 7 years after diagnosis, respectively (Supplemental Figure S1).

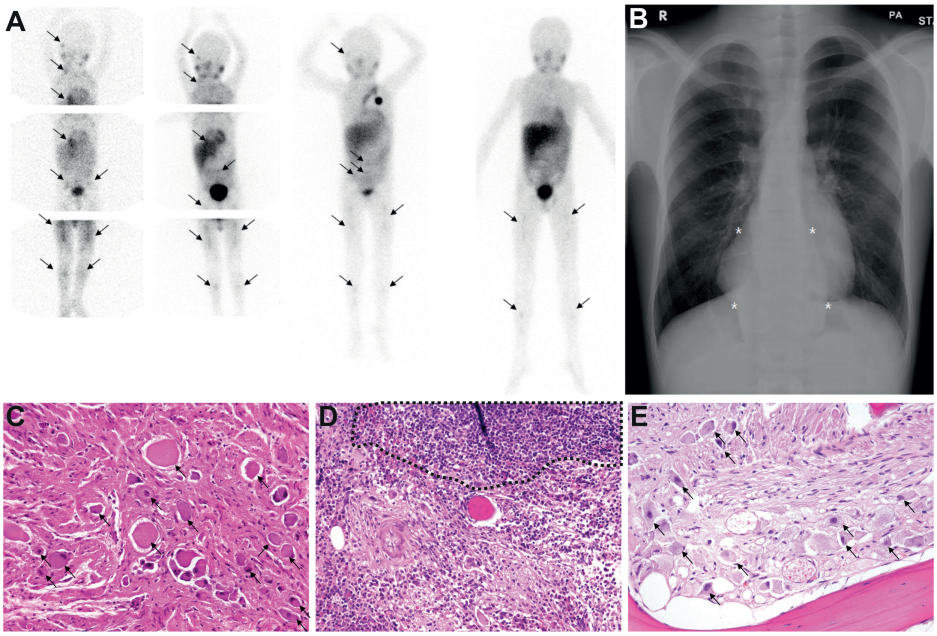

Figure 1 Imaging and pathological follow-up of patient 1

Figure 1A)^{123}I-MIBG scans of patient 1 made 0, 1, 4 and 6 years after diagnosis. Pathological uptake is marked by the arrows.1B) Chest X-ray made 17 years after diagnosis showing the large thoracic primary tumor, marked by four asterisks. 1C-E) Hematoxylin-Eosin staining of 1C) the primary tumor at diagnosis showing large ganglion cells (arrows) with mature stroma, classified ads ganglioneuroblastoma nodular subtype (without a nodule in this sample); 1D) bone marrow at diagnosis show infiltration of neuroblasts (within dashed line) in a background of fibrotic bone marrow and 1E) bone marrow at latest follow-up, 2 years after diagnosis, showing large ganglion cells (arrows) with mature Schwannian stroma.

Patient 2

A one-year-old female was diagnosed with a metastatic neuroblastic tumor in the Democratic Republic of the Congo, and the primary adrenal tumor was resected. Three years later she immigrated to the Netherlands, with ^{123}I-MIBG avid metastases in bone, meninges and lymph nodes (Figure 2A). Biopsy of bone marrow and a lymph node revealed GNB, without *MYCN* amplification or loss of heterozygosity of chromosome 1p. During a year of follow-up the mass of the zygomatic bone and an asymptomatic meningeal mass at the level of C5-C7 progressed. This metastasis was exclusively located in the meninges and not intraspinal extension of a prevertebral tumor. She was subsequently treated with four courses of chemotherapy, two courses of ^{131}I-MIBG therapy, and six months of isotretinoin (Supplemental Table S1), without response to any of the treatment modalities. In the following 10 years, the meningeal metastasis remained stable. However, the zygomatic bone metastasis slowly expanded (Figs 2B, 2C). ^{123}I-MIBG avidity slowly decreased (Figure 2A). The patient developed a chondrosarcoma of the left distal fibula, which was completely resected and she is alive and well after a follow-up time of 23 years (4.3 y for the chondrosarcoma).

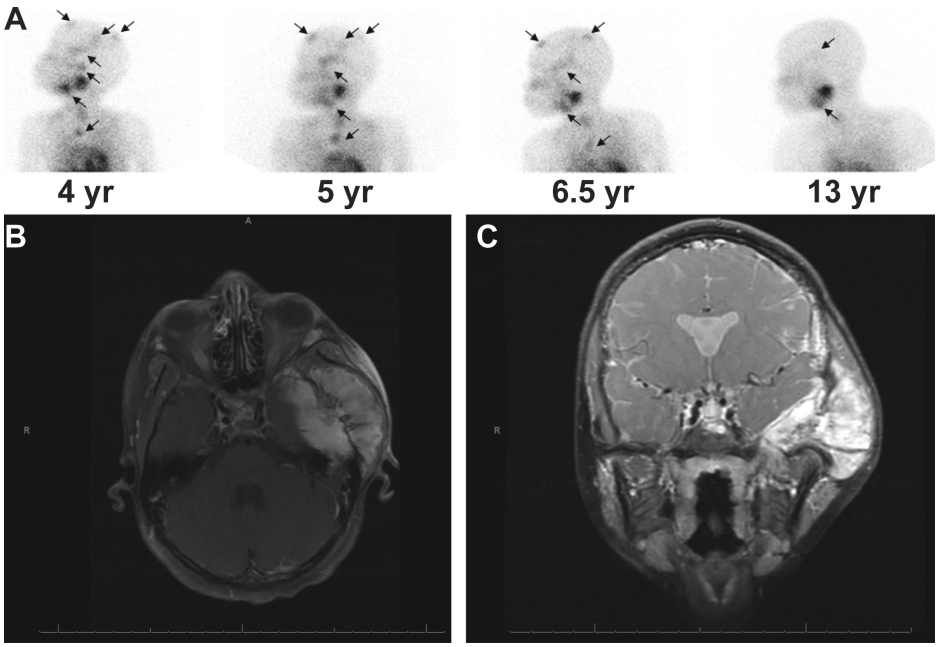

Figure 2 Imaging follow-up of patient 2

Figure 2A)^{123}I-MIBG scans of patient 2, performed 4, 5, 6.5 and 13 years after diagnosis. Pathological uptake is marked by arrows. At the last ^{123}I-MIBG scan there was still pathological uptake at the left temporal bone, right parietal bone and both femora. Suspected uptake was seen at the right mandibular angle and at a rib. Figure 2B/C) T1 (2B) and T2 (2C) weighted MRI with gadolinium contrast showing a large metastasis in the left temporal bone, 11.5 years after diagnosis.

Meningeal metastases

An unexpected number of rare metastatic sites was observed, including meningeal metastases in 5 patients, a muscle, a pleural and an ovarian metastasis. These metastases were diagnosed on MRI scans, none of these metastases have been histologically proven, but were radiologically suspect for meningeal involvement. These metastases are normally detected in less than 1% of patients.[11] In addition to the meningeal metastases, all seven patients had metastases in the facial bones, causing facial deformation in patient #2 (Figs 2B, C) and #5. In patient #2, the meningeal metastasis slowly expanded approximately four years after diagnosis, and remained stable thereafter (see above). In patient #4, a tentorial meningeal metastasis expanded during four months of treatment, and was detected as a cystic lesion with calcifications on MRI, 13 months after diagnosis. Her other meningeal metastases slowly regressed over the course of 5.5 years. In patient #5, the meningeal metastases showed very slow progression in the first 3.5 years after diagnosis and remained stable during the following 5 years. In patient #6, the meningeal metastases (Figure 3) were not followed by imaging.

Histopathology and genomic patterns

The tumors were classified as differentiating NB (n=1) and GNB (n=6). In cohorts where histology is classified by the Shimada criteria, 13% of stage M are reported to be GNB.[12] In the Dutch stage M cohort (n=306) diagnosed between 1990-2014, only 16 patients (5%) were diagnosed with GNB morphology. Seven of these 16 patients are included in this study. This affects the difference in outcome between NB and GNB patient, of 69±12%, compared to 33±3% for NB morphology (n=287). Of the GNBs, three were classified as the favorable GNBi, one as unfavorable GNBn, and two tumors could not be further classified. Differentiation towards ganglioneuroma (GN) was observed in patient #4: a tumor nodule resected six years after diagnosis revealed GN. Trephine biopsies remained positive in two patients (#1 and 4), with the most recent biopsy of patient #1 showing ganglion cells with mature stroma and no undifferentiated neuroblasts (Figure 1E).

Genetically, no amplification of *MYCN* was detected in six tested tumors, for one patient no result was available. Loss of heterozygosity of chromosome 1p36 was determined in four patients, and absent in all. (Shallow) whole genome sequencing was performed on tumor FFPE material of five patients. Two patients (#4 and 5) had a favorable profile with only numerical chromosomal aberrations, one patient (#3) had an unfavorable profile with both numerical and segmental chromosomal aberrations. In two patients (#1 and 2), no conclusions could be drawn, due to low tumor content. (Supplemental Figure S1).

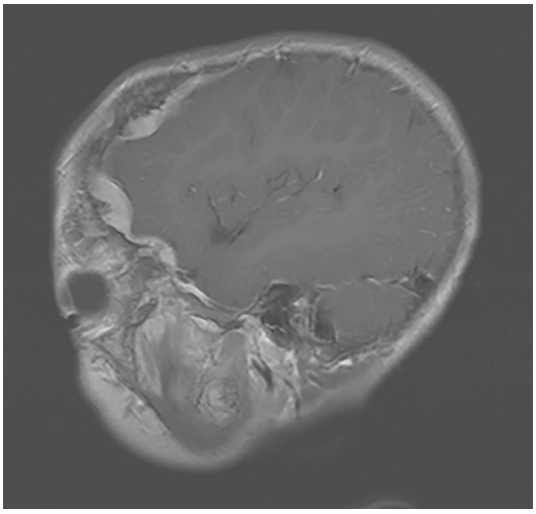

Figure 3 Meningeal metastases of patient 6
Sagittal T1 weighted MRI of patient 6 at diagnosis, showing meningeal and bone metastases.

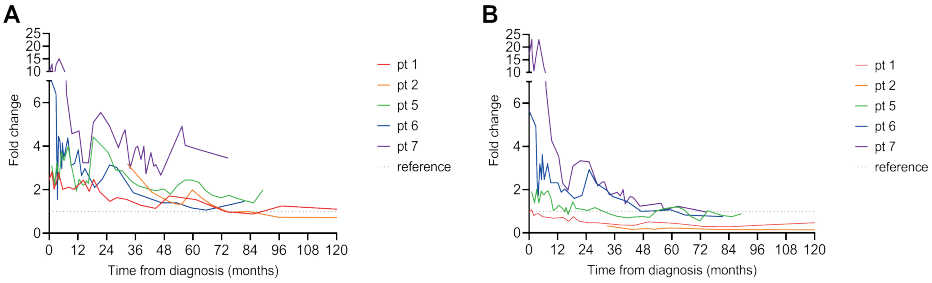

Figure 4 Fold change of HVA and VMA over time
Homovanillic acid (HVA) (A) and Vanillylmandelic acid (VMA) (B) per patient as fold change of age-related upper limit. On the x-axis, time from diagnosis is given.

Clinical course and long-term follow-up

The clinical course was typical with main characteristics being chemotherapy insensitivity and long-term survival with stable disease. During long-term follow-up, the primary tumors of patients #1 and #3 expanded slowly (>25%). Patient #1 developed a new ^{123}I-MIBG avid metastasis 1.5 year after diagnosis, patient #3 developed new ^{123}I-MIBG avid lesions after 1, 3 and 5 years. Despite these new lesions, the long-term follow-up of all patients was characterized by a very slow decrease in ^{123}I-MIBG avidity (Figs 1A, 2A). Normalization of ^{123}I-MIBG uptake was seen in only one patient (#4), after resection of a remaining ^{123}I-MIBG-avid skin lesion. Urinary excretion of the catecholamines HVA and VMA tended to decrease to (near) normal levels over time (Figure 4). HVA remained elevated in 4 patients after a follow-up of 6-14 years while VMA normalized in all patients

after a median of 2.8 years (range: 0 to 6.2 y) (Table 1, Figure 4). Dopamine and 3MT levels were measured in two patients (#1 and #7). Dopamine normalized 10 years after diagnosis in patient #1, in patient #7 it remained 3x elevated at last follow-up, 6.2 years after diagnosis. 3MT levels normalized after 7 years in patient #1, and remained 3x elevated at last follow-up of patient #7.

DISCUSSION

We present a cohort of patients with stage M GNB (n=6) and differentiating NB (n=1) with an unexpected chronic course of disease and an excellent outcome. The patients showed refractory disease after induction chemotherapy, but all remained alive with metastatic disease after a median follow-up of 17 years. The available histological samples and metabolic changes strongly suggest that these tumors differentiated from NB to GNB and GN, without signs of regression.

GNB morphology was seen in 6 of 7 patients. GNBs are considered the more differentiated subgroup of NB. Nonetheless, morphology does not influence risk-stratification of metastatic tumors.[1] GNBs can be further subclassified in the favorable GNB intermixed (GNBi) and the unfavorable GNB nodular (GNBn).[12] In localized cases, GNBi has a similar clinical course to GN.[13-16] Metastatic GNB is almost exclusively classified as GNBn, which has a similar clinical course to NB .[17] In the literature, metastatic GNBi was diagnosed in only 5 patients in four larger cohorts with a total of 443 GN/GNBi patients, all 5 survived.[13-16] In our cohort, three patients were diagnosed with metastatic GNBi and one patient with metastatic GNBn. In two patients differentiation to GN was observed, as has been described in a patient, similar to this cohort, but without bone metastases, who did not receive systemic treatment.[18]

We identified 16 cases in literature, who match the description of a chronic stage M NB, GNB or GN with bone metastases as described in this paper (Table 2).[19-30] As we observed differentiation to ganglioneuroma in patient #1 and 4, we included also patients with GN in this literature overview. We believe that these fall within the spectrum of this cohort, but are diagnosed after full differentiation of the neuroblastic tumor. In line with our patients, chemotherapy, given in 5/16 described patients, did not result in objective responses. Two patients developed new metastases over time, and five patients suffered expansive growth of tumors. None of these patients died of the (G)NB/GN. These and our data suggest a difference in clinical behavior compared to true NB, although most high-risk treatment protocols will include GNB histology patients.

OS of Dutch stage M GNB patients was 69±12% (n=16), compared to 33±3% for NB patients (n=287). This challenges the current risk stratification, which does not take morphology into account for metastatic tumor patients. In addition, a recent publication by Tao et al. suggest a different treatment strategy for GN than for NB. In their study GNs showed to be dependent on the AKT pathway and subsequently sensitive to mTOR inhibitors, while NBs are dependent on the well-defined adrenergic core regulatory

circuit of PHOX2B, PHOX2a, TFAP2B, GATA3 and ISL1.[31] It could be discussed if the chemotherapy insensitivity of the tumors in this cohort is caused by being less dependent to the adrenergic core regulatory circuit and more dependent on the AKT pathway. For future patients, treatment with a mTOR inhibitor could be considered.

Of interest is the presence of suspected meningeal metastases in 5/7 patients in this cohort. Meningeal metastases are exceptionally rare in primary diagnosis and occur more often at relapse.[11,32-34] In most studies, central nervous system (CNS) metastases (parenchymal and meningeal) are grouped together as 'CNS metastases'. The 5-yr OS of neuroblastoma patients with CNS metastases at diagnosis is 35%, little is known on the long-term outcome. The 5-yr OS of patients with a CNS relapse is less than 5%.[32-34] Since the outcome of patients with CNS disease is generally poor, the finding of suspected meningeal metastases in 5/7 patients is puzzling and unexplained. The high incidence and good outcome in the presented cohort raises the question of this being a biologically different subgroup of metastatic disease. In addition, all patients in this cohort and at least 12/16 of the literature cases had metastases in the facial bones. Orbital metastases occur in around 25% of metastatic neuroblastoma cases and is associated with decreased 5yr OS in patients >18 months at diagnosis. Metastases to other facial bones are less common.[35] Both the facial bones and frontal meninges are of cranial neural crest origin.[36,37] The origin of the spinal meninges remains elusive, but histology of human embryos suggests a neural crest origin for the pia mater, and a somitic mesoderm origin for the dura mater.[38]

In general, neuroblastoma tumors arise from trunk neural crest cells. During neural crest development, the trunk neural crest cells form the sympathoadrenal (SA) progenitor and Schwann cell precursor (SCP), subsequently these cells form the chromaffin cells in the adrenal medulla and neurons in sympathetic ganglia (Figure 5).[39,40]

Table 2 Literature cases

Gender	Age at diagnosis	Morph-ology	Primary tumor	Metastases at diagnosis	Treatment	Response to treatment	Clinical course	Outcome	FU	Reference
NR	0mo	NR	unknown	Bone, orbit, skin	CT	no	SD	AWD	11yr	Hayes[23]
NR	1mo	NB	adrenal gland	Liver, skin, lung, LNs	CT, RT, BCG vaccine, injected irradiated tumor cells	no	New bone metastases after 8 mo, expansive growth during 4 yrs, 10yr SD, two new skin lesions after 14 yrs	AWD	15yr	Hayes[23]
f	4mo	GN	adrenal gland	Long bones, pelvic bones, vertebrae, LNs	S	NA	SD	AWD	2yr	Garvin[20]
m	6mo	NB	adrenal gland	Skull bones, vertebrae, LNs	RT	NA	SD, regression skull mets	DOC	21yr	Visfeldt[30]
f	9mo	undiff NB	abdominal	BM and mandible	CT	no	SD	AWD	15yr	Bhattacharyya[21]
f	2yr	GNB	thoracic	Lung, multiple vertebrae and ribs	RT	NA	SD	DOC	6yr	Kissane[28]
m	3yr	NB	abdominal	Os frontale, vertebrae, acetabulum, LNs	vit B12	NA	New bone mets	AWD	5yr	Goldman[25]
NR	3yr	GNB	thoracic	Facial bones, vertebrae	no	NA	SD	AWD	6.5yr	Hayes[23]
NR	3yr	GN	abdominal	Multiple bones, orbit, maxilla	CT, RT	no	Periods of growth during 7 yrs	DOC	8yr	Hayes[23]
m	3yr	GN?	adrenal gland	Multiple bone metastases in skull and large bones	S, CT, RT	NR	Slow expansive growth	AWD	11yr	Mitcherling[19]

Table 2 Continued.

Gender	Age at diagnosis	Morph-ology	Primary tumor	Metastases at diagnosis	Treatment	Response to treatment	Clinical course	Outcome	FU	Reference
NR	4yr	NR	abdominal	Facial bones, skull, vertebrae, pelvic bones, humerus	no	NA	Expansion skull lesions first two years of FU	AWD	23yr	Hayes[23]
f	12yr	GNB	adrenal gland	Mandible, femurs	S	NA	SD	AWD	4yr	Patterson[22]
f	15yr	GN	unknown	Mandible	S	NA	SD	AWD	18yr	Chou[26]
f	17yr	GN	adrenal gland	long bones, ribs, vertebrae	no	NA	Short FU	AWD	2mo	Mithofer[24]
m	21yr	GN	Unknown	Mandible	No	NA	Slow expansive growth	AWD	35yr	Oeppen[29]
f	82yr	GN	unknown	Mandible, meningeal?	S	NA	SD/very slow progression	DOC	2yr	Hustin[27]

Abbreviations: AWD: alive with disease, BCG: Bacillus Calmette-Guérin, BM: bone marrow, CT: chemotherapy, DOC: died of other causes, f: female, FU: follow-up, GN: ganglioneuroma, GNB ganglioneuroblastoma, LNs: lymph nodes, m: male, mets: metastases, mo: months, NA: not applicable, NB: neuroblastoma, NR: not reported, RT: radiotherapy, S: surgery, SD: stable disease, undiff: undifferentiated, vit: vitamin, y: year.

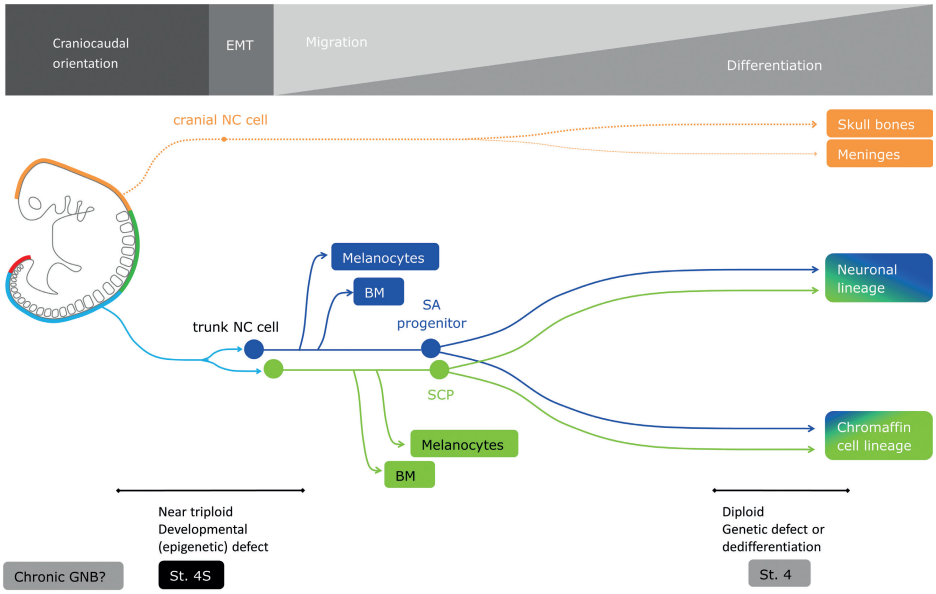

Figure 5 Metastatic neuroblastoma stages related to the embryonic development of neural crest cells
The upper bar indicates the relative role of craniocaudal orientation, EMT (epithelial to mesenchymal transition), migration and differentiation of the neural crest (NC) cells during embryonic development.
In the center, the development of the cranial (orange) and trunk (light blue) neural crest (NC) cells is presented as a time line. In dark blue, the sympathoadrenal (SA) progenitor as a precursor cells is depicted. In green, the Schwann cell precursor (SCP). In the boxes below the graph, the origin of the different tumor entities are postulated.
Abbreviations: EMT: epithelial to mesenchymal transition; NC: neural crest; BM: is bone marrow; SA progenitor: sympathoadrenal progenitor; SCP: Schwann cell precursor; GNB: ganglioneuroblastoma; st.: stage.

Previously we hypothesized that stage MS tumors appear to be derived from very early (pre)migrating neural crest cells and should be considered a multifocal developmental disease rather than a true metastatic cancer.[41] In the patients presented here with meningeal involvement, a similar question could be raised. The meningeal tumor spread could be interpreted as the result of a very early genetic hit, before separation of the cranial and trunk neural crest (Figure 5). Alternatively, the meningeal and facial sites could also be the result of metastases homing to neural crest tissues. The role of the neural crest development in neuroblastoma-genesis remains elusive, but the cell-of-origin of the developing neural crest may define the variable clinical courses.

In hindsight it could be concluded that a wait-and-see policy was indicated in these patients. Antitumor treatment was not effective and the outcome was favorable. Recognition of this entity can help in making treatment decisions and providing information to parents and patients about the clinical course that can be expected.

Recognition of the entity can be based on asymptomatic disease at diagnosis, a more mature morphology (GNB or differentiating NB), with meningeal and (facial) bone metastases and poor or absent chemotherapy response. In case of chemotherapy unresponsiveness, consecutive tumor sampling is advised to determine if the tumor has differentiated, treatment with a mTOR inhibitor could be considered or treatment could be stopped, in order to prevent from overtreatment.

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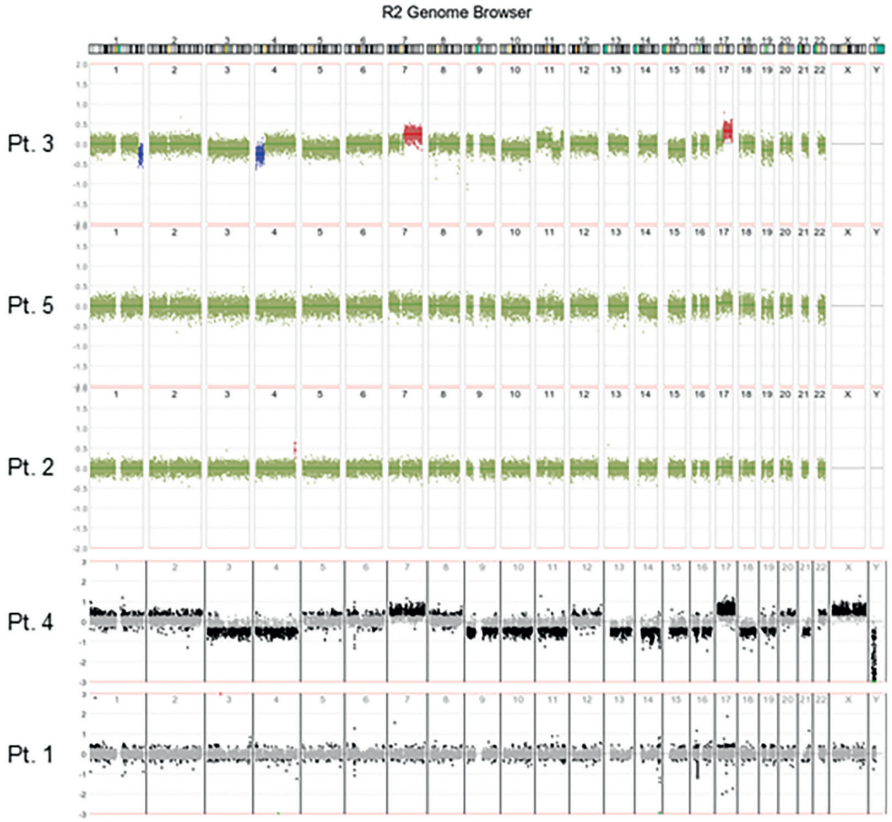
SUPPLEMENTAL MATERIAL

Supplemental Table S1 Treatment per patient

Patient number	1st treatment	2nd treatment	3rd treatment	4th treatment	5th treatment
1	2x MIBG	8x COJEC	2x CADO	NA	NA
2	Surgery	2x VECI	2x CADO	2x MIBG	6months RA
3	6x Cy/A	4x P1/VM	Surgery	NA	NA
4	4x Cy/A	Surgery	NA	NA	NA
5	5x CycloDox	Surgery	NA	NA	NA
6	Surgery	NA	NA	NA	NA
7	Surgery	POG9640 induction	2x MIBG	NA	NA

Abbreviations: MIBG: ^{131}I-MIBG therapy, RA: retinoic acid (isotretinoin)
VECI[42]: vincristine, carboplatin, and etoposide.
Rapid COJEC[43]: cisplatin, vincristine, carboplatin, etoposide, and cyclophosphamide.
CADO[44]: vincristine, cyclophosphamide and teniposide.
Cy/A, P1/VM[45]: cyclophosphamide with doxorubicin, and cisplatin with teniposide.
POG9640[46]: cisplatin etoposide, doxorubicin, vincristine, cyclophosphamide, ifosfamide, and carboplatin.



Supplemental Figure S1 Coverage plots of untreated ganglioneuroblastomas
Shallow whole genome sequencing data (in color) with coverage calculated for 0.1 Mb bins, normalized to the mean coverage of lymphocyte DNA of 19 healthy persons. Color intensity reflects the magnitude of the gains or losses (blue=loss, red=gain). For the normal whole genome sequencing data (in black-white) this was calculated for 1 Mb bins, normalized to the coverage in patient lymphocyte DNA. In patient 3 both segmental and numerical aberrations were observed, in patient 5 subtle gain of chromosome 7 and 17 and loss of chromosomes 14 and 19 can be observed. In patient 4 only numerical aberrations were observed and in patient 1 and 2 tumor content was too low to draw conclusions.
Abbreviation: pt.: patient

CHAPTER 5

ZFP42: a new tumor predisposition gene? Presentation of a patient with two neoplasms in childhood.

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Biomed J Sci & Tech Res 2020;27:21166-21172

ABSTRACT

Backgrounds

The transcription factor *ZFP42*, located on chromosome 4q35, is a pluripotency marker in early embryonic stem cells. *ZFP42* is expressed in embryonic stem cells, and downregulated in early development after exposure to retinoic acid. We present a patient who developed a neuroblastoma and a secondary peripheral atypical cartilaginous tumor during childhood. She has a germline rearrangement involving a genomic region close to the *ZFP42* gene.

Methods

Data was collected on clinical and tumor characteristics. (Shallow) whole genome sequencing (WGS) was performed on neuroblastoma and whole blood samples. *ZFP42* expression of in the cartilage tumor was analyzed using RT-PCR.

Results

WGS revealed a germline rearrangement resulting in the duplication of a 403kbp region and the relocation of a 154kbp region, in between the duplicated regions, on chromosome 4q35. Analysis of publicly available ChIP-seq data of neuroblastoma cell lines revealed the presence of the enhancer marks H3K27ac and H3K4me1 in the 154kbp region that is relocated in the patient. Due to the relocation, this active region is now positioned closer to *ZFP42*, suggesting "enhancer hijacking". In addition, *ZFP42* was detected in the cartilage tumor. Since ZFP42 is involved in the repression of retinoic acid induced differentiation of embryonic stem cells, we hypothesize that the activation of *ZFP42* in this patient resulted in a differentiation arrest and subsequent tumor susceptibility.

Conclusion

We present a patient, with a germline rearrangement upstream of *ZFP42*, who developed two neoplasms in childhood. We propose a role for *ZFP42* as a tumor predisposition gene in this patient.

INTRODUCTION

Neuroblastoma is a developmental malignancy of the neural crest.[1] Tumors typically arise in young children, with a median age of 18 months at diagnosis, and most frequently reside in the adrenal gland or sympathetic side chain.[2,3] The survival rates are high (>85% event free survival) for patients with localized disease, but low (<50%) for patients above 18 months with metastatic disease.[2] Most cases occur sporadically, but familial neuroblastoma is observed in 1-2% of patients.[4] Familial neuroblastoma cases are mostly (80%) attributed to *ALK* mutations.[5,6] The second most frequently mutated gene in familial neuroblastoma is *PHOX2B*, causing 6-10% of familial cases.[7] Genetic predisposition to neuroblastoma can also be caused by more general cancer predisposition syndromes, such as neurofibromatosis type 1 (*NF1*), Li-Fraumeni syndrome (*TP53*), Costello syndrome (*HRAS*), Noonan syndrome (*PTPN11, KRAS*), Beckwith-Wiedemann syndrome (chromosome 11) and Fanconi Anemia (FA pathway).[8]

Chondrosarcoma is a tumor showing cartilaginous differentiation. Chondrocytes, the cartilage producing cells, are partly neural crest derived. Chondrosarcoma is the second most frequent bone tumor after osteosarcoma, representing about 20% of bone tumors.[9] Tumors arise predominantly between the age of 20 and 60 years. Approximately 85% of chondrosarcomas are classified as conventional chondrosarcomas. These tumors develop in benign enchondromas (central chondrosarcoma) or osteochondromas (peripheral chondrosarcoma).[10] However, only 1-3% of osteochondromas will develop into secondary peripheral chondrosarcomas.[9] Peripheral chondrosarcoma occur in younger patients and generally behave less malignant than central chondrosarcomas.[11,12] Prognosis largely depends on histopathological grading based on tumor cell density, mitotic figures and cartilaginous matrix. Atypical cartilaginous tumors/grade I chondrosarcomas, are regarded locally aggressive and rarely metastasize, the 5-yr disease specific survival is estimated at 100% and 10-yr overall survival at 83%.[10,12,13] Grade II and III tumors have higher cell densities and less matrix and 10-yr OS is 64% and 29%, respectively.[10] Predisposition for the development of chondrosarcomas can be caused by germline inactivating mutations in the *EXT1* or *EXT2* genes, causing hereditary multiple osteochondromas.[14-16] Somatic mosaicism of mutations in *IDH1* or *IDH2* causes Ollier disease and Maffucci syndrome, which also predispose for development of chondrosarcoma.[17,18] In children, secondary peripheral chondrosarcomas are associated with radiation therapy.[19]

ZFP42 is a zinc finger transcription factor, a *YY1* transcription factor family member, located on chromosome 4q35, first recognized in 1989.[20,21] It was identified by its downregulation shortly after treatment with retinoic acid (RA) in F9 teratocarcinoma stem cells, which resulted in differentiation of these cells.[20] *ZFP42* was subsequently named *REX-1* (for reduced expression). Although some aspects of the function of *ZFP42* are still unclear, *ZFP42* is used as a marker of pluripotency for embryonic stem cells and induced pluripotent stem cells. Expression in mouse and human is restricted to embryonic stem cells, tissue specific stem cells and germ cells of the testis.[21-23] A

specific role for *ZFP42* in cancer is unknown. Being a stem cell marker, it is believed to be expressed in cancer stem cells. Expression has been found in some carcinoma cell lines and in glioblastoma multiforme.[20,21] *ZFP42* is positively regulated by *Nanog*, *Sox2*, *Oct3/4* and *Oct6*, also known markers for pluripotent stem cells. [24,25]

Here, we present a patient with two unrelated neoplasms in childhood, who carries a germline rearrangement, in a genomic region upstream of *ZFP42*.

MATERIAL AND METHODS

Patient

The patient's chart was reviewed for: patient characteristics, symptoms, treatment, pathology reports, radiological and nuclear medicine reports, and urinary excretion levels of homovanillic acid (HVA) and vanillylmandelic acid (VMA).

Shallow and whole genome sequencing

Shallow whole genome sequencing was performed on FFPE material of the neuroblastoma as described before.[26] DNA from a whole blood sample was isolated using the DSP DNA Midi kit according to manufacturer's protocol (Qiagen, Venlo, The Netherlands). Library preparation and sequencing were performed on the HiSeqX sequencing system (Illumina, San Diego, CA, USA), the BCL output was converted using the bcl2fastq tool (Illumina, v.2.20, San Diego, CA, USA) as described before.[27] For analysis and visualization, the "R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl)" was used.

Cartilage tumor RNA isolation

Twenty µm section of frozen tissue were collected in a tube and dissolved in 5 ml TRIzol. Homogenization was performed using the Ultra-Turrax (IKA, Staufen, Germany). After mixing for 1 min. with chloroform (1 ml), the sample was centrifuged for 30 min. at 15.000xg. A second phenol/chloroform extraction was performed similarly, followed by isopropanol precipitation. The RNA was dissolved in 30 µl MQ. cDNA was synthesized using RevertAid H Minus FirstStrand cDNA synthesis Kit (ThermoFisher) according to the recommendations of the manufacturer (372 ng RNA per 20 µl reaction).

RT-PCR and gel electrophoresis

Reverse transcriptase PCR (RT-PCR) was performed on cDNA using ReddyMix PCR Mastermix (ThermoFisher), and forward and reverse primers at 500 nM. Forward and reverse primer sequence of *ZFP42* were GGCCTTCACTCTAGTAGTGCTC and CTGGCTCATGTTTTCCTGCC, respectively. *UBC* was used as control gene to ensure cDNA

quality. Forward and reverse primer sequence of *UBC* were ATTTGGGTCGCGGTTCTTG and TGCCTTGACATTCTCGATGGT, respectively. The reaction was performed with heating for 3 min at 95°C, followed by 40 cycles of 30 sec. at 95°C, 30 sec. at 60°C and 30 sec. at 72°C, followed by a final extension step of 10 min at 72°C. Subsequently, RT-PCR products were visualized with gel electrophoresis.

Ethics

Written informed consent for the use of stored material was obtained from the patient.

CASE REPORT

In 1997, a 15-months old girl was diagnosed with neuroblastoma stage 4 in the Democratic Republic of the Congo and the primary tumor in the left adrenal gland was resected. In 2000, the patient immigrated to the Netherlands, still suffering from Iodine-123 Metaiodobenzylguanidine (^{123}I-MIBG) avid metastatic disease (Figures 1A and 1B), histology of a lymph node metastasis showed ganglioneuroblastoma morphology. After 1.5 years of wait-and-see, a ^{123}I-MIBG-avid meningeal metastasis showed progression. Chemotherapy was started, followed by ^{131}I-MIBG therapy, but all metastases were unresponsive. Next, treatment with isotretinoin (13-cis retinoic acid) was administered in an attempt to cause differentiation of the metastases. Chemotherapy resistance in patients with metastatic neuroblastoma is associated with a poor prognosis.[28] However, in this patient, no progression was observed, but a very slow metabolic response was observed by a decrease but not normalization in ^{123}I-MIBG uptake (Figure 1C and 1D) and normalization of HVA 6.25 years after the initial diagnosis. This is an unusual course of disease for a neuroblastoma, as aggressive growth with fatal outcome was expected. At the age of 12 years, the patient developed an osteochondroma, a benign cartilage tumor, at the surface of the left distal fibula (Figure 1E). Over the period of four years this tumor developed into a secondary peripheral chondrosarcoma/atypical cartilaginous tumor as the size of the cartilaginous cap exceeded 2 cm (Figure 1F-I).[9,29] Development of a secondary peripheral atypical cartilaginous tumor is exceptional for this age, especially since the patient was not treated with radiotherapy for the neuroblastoma.[16,30] The cartilage tumor was surgically resected (R0 resection) (Figure 1H), no adjuvant chemotherapy was administered. At last follow-up, 20 years after the neuroblastoma diagnosis and 4 years after the secondary peripheral atypical cartilaginous tumor diagnosis, the patient was alive and well. Because the patient developed two different neoplasms during childhood and because her neuroblastoma followed an aberrant indolent clinical course, we tested for germline aberrations.

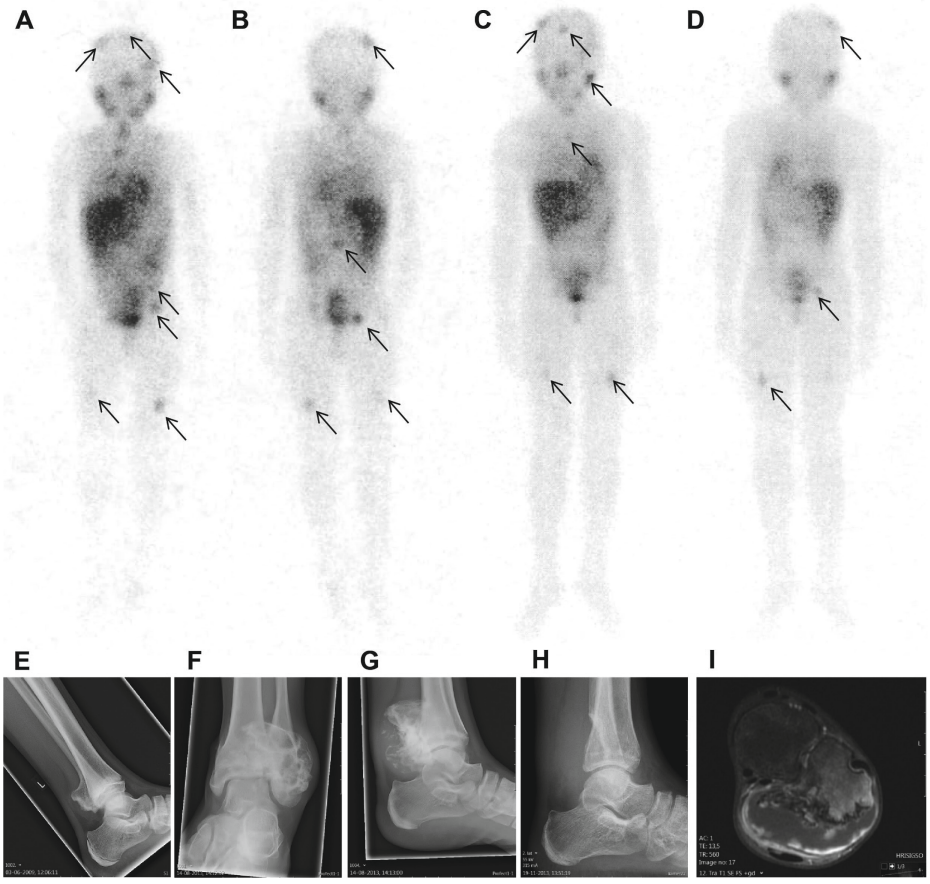

Figure 1 Radiological and nuclear imaging of the patient
Figure 1A-D) whole body ^{123}I-MIBG single photon emission computed tomography (SPECT) scans, the arrows indicate pathological ^{123}I-MIBG uptake. A/B) anterior and posterior scans 3 years after diagnosis; C/D) anterior and posterior scans, 6 years after diagnosis.
Figure 1E-H: conventional X-rays of the left ankle showing: E) an osteochondroma of the distal fibula at the age of 12 years, F/G) anterior/posterior and lateral X-ray at the age of 16 years, showing fast progression of the osteochondroma. H) lateral X-ray of the left ankle after resection of the cartilage tumor. I) T1 weighted MRI at the age of 16 years, showing an osteochondroma of the distal fibula metaphysis with a cartilage cap of 2.6 cm maximum diameter and gadolinium enhancement at the border of the cartilage cap, suspected for secondary peripheral chondrosarcoma development.

WGS of germline DNA identifies a relocation and duplication near *ZFP42*

Whole genome sequencing (WGS) on whole blood of the patient, revealed a germline heterozygous rearrangement on chromosome 4q (Figure 2). In the rearrangement, a 403 kbp region was duplicated and a 154 kbp region was relocated in between the duplicated regions (Figures 2A and 2B). The duplicated region contained no protein coding genes. The relocated region caused breakpoints in the *C4orf47, CCDC110*, and *SORBS2* genes. These genes are not known as tumor suppressor genes. Interestingly, the rearrangement was located near the *ZFP42* gene, a pluripotency marker for embryonic stem cells. Due to lack of parental blood samples, it could not be established if the rearrangement was inherited or de novo. No other germline alterations of relevance were identified. Shallow whole genome sequencing of the neuroblastoma tumor showed a flat copy number variation plot, with the germline duplication on chromosome 4q35 as only structural aberration (Figure 2A). This could either indicate that the tumor content was too low, or that no other chromosomal aberrations were present in the neuroblastoma.

Next, we investigated the epigenetic status of the rearranged regions in a publicly available ChIP-seq dataset (GSE138314) of neuroblastoma cell lines for the enhancer marks H3K27ac and H3K4me1 using the R2 genomics analysis and visualization platform (http://r2.amc.nl). ChIP-seq data of these neuroblastoma cell lines revealed that the 403 kbp region that is duplicated in this patient had near absent levels of H3K27ac and H3K4me1 histone modification marks. However, the 154 kbp region that is relocated in this patient showed higher levels of H3K27ac and H3K4me1 in these cell lines (Figure 2B/C). Due to the relocation, a region positive for enhancer marks was positioned near the promoter of *ZFP42* in this patient. Therefore, we hypothesized that this could result in activation of *ZFP42*, which is normally not expressed, by enhancer hijacking.

***ZFP42* is expressed in chondrosarcoma of the patient**

Neuroblastoma tissue was not available for expression analysis of *ZFP42*. Therefore, we tested *ZFP42* expression in the cartilage tumor. From the fresh frozen tumor tissue RNA was isolated and *ZFP42* expression was measured using reverse transcriptase polymerase chain reaction (RT-PCR). With RT-PCR, expression of *ZFP42* was found in the cartilage tumor (Figure 3). However, expression was low compared to the D341med medulloblastoma cell line, which served as a positive control. Sanger sequencing of the RT-PCR product confirmed the *ZFP42* sequence.

Figure 2 Germline rearrangement

Figure 2A) Copy number variation (CNV) plot of chr4q35 of the patient in the shallow WGS neuroblastoma sample, showing a duplication upstream of *ZFP42*. 2B) schematic overview of *ZFP42* in normal configuration (upper overview) and as found in the germline of the patient (lower overview), blue is the relocated region (A), red the duplicated region (B), green the *ZFP42* gene. Chromosomal localization is indicated in kbp resolution. 2C) ChIP-seq results of H3K27ac (red peaks, left) and H3K4me1 (purple peaks, right) in neuroblastoma cell lines (GSE138314 dataset). The blue box indicates the relocated region, the red box the duplicated region.

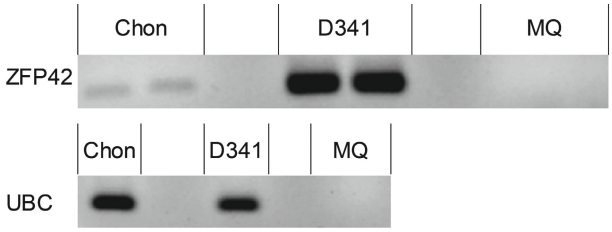

Figure 3 *ZFP42* expression in the cartilage tumor
Gel electrophoresis of the RT-PCR product of the *ZFP42* gene (top, in duplo) and of the *UBC* housekeeping gene (bottom), performed on the cartilage tumor (chon), D341med cell line (positive control) and MQ (negative control).

DISCUSSION

We present a patient who developed two neoplasms in childhood, a disseminated neuroblastoma at 1 year of age with an unexpected indolent course of disease and a secondary peripheral atypical cartilaginous tumor at 16 years of age. We identified a germline rearrangement in chromosome 4q35, resulting in the positioning of a region positive for enhancer marks near the promoter of the *ZFP42* gene, a human stem cell marker.[31,32] *ZFP42* was expressed in the cartilage tumor. The combination of two childhood neoplasms, the aberrant course of neuroblastoma disease, and the germline rearrangement affecting a pluripotency marker suggests that the *ZFP42* gene is involved in the development of these tumors.

ZFP42 is a stem cell marker which is normally downregulated in early development and which is overexpressed in glioblastoma multiforme and invasive cervical squamous cell carcinoma. [31-34] In glioblastoma downregulation of *ZFP42* resulted in a decreased proliferation and increased differentiation.[34] However, this result was not confirmed in embryonic stem cells.[32,35] In embryonic stem cells, *ZFP42* had a role in inhibiting retinoic acid induced differentiation. Knock-out of *ZFP42* resulted in higher degree of differentiation and growth inhibition after treatment with retinoic acid. Overexpression of *ZFP42* resulted in less differentiation and an increase in growth.[22] Neuroblastomas are sensitive to retinoic acid, which is used as differentiation treatment in patients.[36-38] Chondrosarcomas undergo growth inhibition after treatment with retinoic acids or selective agonists of RARγ (retinoic acid receptor gamma) in vitro and in vivo.[39-42] However, retinoic acid is not part of the standard chondrosarcoma treatment protocol, as it is in high-risk neuroblastoma treatment.[37,39]

Neuroblastoma is a developmental tumor of the neural crest. Tumors arise from a common precursors of the adrenal chromaffin cells and sympathetic neurons. Chondrosarcomas develop from cells committed to cartilaginous differentiation, which are partly neural crest derived and partly mesoderm derived. The precursors of both tumor types are influenced by retinoic acid for differentiation.[43] Based on literature, we

propose that *ZPF42* inhibits the effect of retinoic acid on differentiation, resulting in a differentiation arrest in the precursor cells. Avoiding differentiation is a crucial step in cancer development.[44,45] However, more research is needed to confirm the role of *ZFP42* in the inhibition of differentiation and the potential role in cancer development.

CONCLUSION

In conclusion, the patient presented here developed two neoplasms in childhood, a neuroblastoma and a cartilage tumor, of which the precursor cells depend on retinoic acid for differentiation. The patient has a germline rearrangement upstream of ZFP42, a repressor of retinoic acid induced differentiation. We propose a role for ZFP42 as a tumor predisposition gene in this patient.

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PART II

Progress in the treatment of (high-risk) neuroblastoma

CHAPTER

Neuroblastoma between 1990 and 2014 in the Netherlands: Increased incidence and improved survival of high-risk neuroblastoma.

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ABSTRACT

Purpose

Long-term trends in neuroblastoma incidence and survival in unscreened populations are unknown. We explored trends in incidence, stage at diagnosis, treatment and survival of neuroblastoma in the Netherlands from 1990 to 2014.

Methods

The Netherlands Cancer Registry provided data on all patients aged <18 years diagnosed with a neuroblastoma. Trends in incidence and stage were evaluated by calculating the average annual percentage change (AAPC). Univariate and multivariable survival analyses were performed for stage 4 disease to test whether changes in treatment are associated with survival.

Results

Of the 593 newly-diagnosed neuroblastoma cases, 45% was <18 months of age at diagnosis and 52% had stage 4 disease. The age-standardized incidence rate for stage 4 disease increased at all ages from 3.2 to 5.3 per million children per year (AAPC + 2.9%, p<.01). This increase was solely for patients ≥18 months old (3.0-5.4; AAPC +3.3%, p=.01). Five-year OS of all patients increased from 44±5% to 61±4% from 1990 to2014 (p<.01) and from 19±6% to 44±6% (p<.01) for patients with stage 4 disease. Multivariable analysis revealed that high-dose chemotherapy followed by autologous stem cell rescue and anti-GD2 based immunotherapy were associated with this survival increase (HR 0.46, p<.01 and HR 0.37, p<.01, respectively).

Conclusion

Incidence of stage 4 neuroblastoma increased exclusively in patients aged ≥18 months since 1990, whereas the incidence of other stages remained stable. The 5-year OS of stage 4 patients improved, mostly due to the introduction of high-dose chemotherapy followed by stem cell rescue and immunotherapy.

INTRODUCTION

The incidence of neuroblastoma (NB) in developed countries is 11-13 per million children aged <15 years, and varies from 65 per million in children <1 year to 1 per million in children of 10-14 years.[1-3] NB is a heterogeneous tumor entity with a variable clinical course. The long term survival is good to excellent in low risk disease (5-year OS of >85% in International Neuroblastoma Staging System (INSS) stage 1, 2, 4S[4], or International Neuroblastoma Risk Group Staging System (INRGSS) stage L1, MS[5]), but poor in patients with high-risk disease (5-year OS of <50% in stage 4/M in patients ≥18 months old at diagnosis, and/or with *MYCN* amplification)[6]. Furthermore, patients with a more differentiated histology (ganglioneuroblastoma [GNB]) fare a more favorable course of disease than patients with undifferentiated histology (NB).[7,8] In the past decades, therapy for high-risk patients has been modified in several ways to increase survival. Induction chemotherapy was intensified, high-dose chemotherapy followed by autologous stem cell rescue and standard radiotherapy were introduced. Most recently, anti-GD2 immunotherapy has been added to the maintenance therapy; this monoclonal antibody is given in combination with alternating GM-CSF or IL-2 to stimulate the immune response.[9-12]

Improvements in cancer outcome are often analyzed as improvements in survival, but cancer incidence analyses should also be used to monitor changes in outcome by changes in the prevalence of (unknown) risk factors.[13] While survival provides a measure of prognosis and improvement in the treatment, trends in cancer mortality are the result of trends in both incidence and survival. The three analyses together increase the comprehension of the total progress against cancer in a given area over time.[14-16]

These epidemiological analyses were used in the evaluations of the neuroblastoma screening programs, conducted between 1985 and 2000 in Japan and parts of Germany, France, Austria, Canada and the United Kingdom. The rationale behind the screening programs was that detection at an earlier stage of disease would lead to an improved prognosis. Although the screening studies identified more young patients with low-risk neuroblastoma, this had no effect on incidence of high-risk disease or overall mortality, suggesting overdiagnosis of low risk patients.[13,16-22] This resulted in the termination of all screening programs. A disadvantage of these screening programs is that change in the incidence over time is not included. In the Netherlands, no screening programs have been performed.

The purpose of this comprehensive, population-based study was to describe the trends in incidence, treatment modalities and survival in NB patients <18 years, diagnosed between 1990 and 2014, and to study the effect of changes in treatment on the survival of patients with stage 4 NB.

METHODS

Data sources

The Netherlands Cancer Registry (NCR) is a nationwide population-based registry, established in 1989, hosted by the Netherlands Comprehensive Cancer Organization (IKNL). The NCR only registers persons with the Dutch nationality, or people who have been living in the Netherlands for at least three months before diagnosis. Trained registrars of the NCR extracted data on patient and tumor characteristics, and given treatment by retrospective medical record review. Only first line treatment modalities were registered.

The NCR registers morphology according to the International Classification of Diseased for Oncology (ICD-O-3)[23], currently the ICD-O-3.1 system[24]. Tumor stage was recorded using the TNM classification[25] until 2003 and subsequently according to the Extent of Disease[26] (EoD) classification. Localized disease (TNM/EoD) was converted to INSS stage 1/2, regional disease to stage 3 and metastatic disease to stage 4 or 4S. To validate stage and treatment modalities, hospital-based neuroblastoma databases were used to cross check these items and to identify patients with neuroblastoma stage 4S, according to the INSS staging system.[4] Information on risk stratification, *MYCN* status and other genetic prognostic factors was not available.

Patient and data selection

Clinical data from Dutch patients aged <18 years at diagnosis, and diagnosed with a NB or GNB between 1990-2014 were extracted from the NCR. Information on vital status (alive, dead, or emigration) was obtained by annual linkage with the Nationwide Population Registries Network that contains vital statistics on all Dutch residents. Last linkage was February 1 2018. Because of privacy regulations, no data on cause of death could be obtained. Nationwide disease-specific mortality data were not informative since neuroblastoma was non-consistently coded as a malignancy of the adrenal gland, the connective and soft-tissues, and the peripheral nervous system.[27]

Statistical analyses

For the NB patient population the following characteristics were described: age at diagnosis, gender, histology (NB vs. GNB), stage and location of the primary tumor. Differences in these characteristics were tested using χ^2 tests. For analysis over time, five-year periods were defined: 1990-1994, 1995-1999, 2000-2004, 2005-2009 and 2010-2014.

Overall incidence rates were calculated as the average annual number of cases per 1 million person-years, using annual midyear population sizes from Statistics Netherlands, these were provided for the age groups: 0, 1-4, 5-9, 10-14 and 15-17 years. Incidence rates were also calculated for age groups (<18 and ≥18 months), stage and stage per age

group. The population at risk <18 months was calculated as the population aged 0 years plus 1/8th of the population aged 1-4years. Similarly, the population at risk ≥18 months was calculated as the population aged 5-17 years plus 7/8th of the population aged 1-4 years. Rates were age-standardized using the age structure of the World standard population.[28] Changes in incidence over time were evaluated by calculating the average annual percentage change (AAPC). AAPC was derived from a regression line fitted to the natural logarithm of the rates, using the calendar year as regressor variable (i.e. $y = ax + b$ where $y = \ln(\text{rate})$ and x = calendar year; then $AAPC = 100 \times (e^a - 1)$) and calculated for the whole study period 1990-2014.[28]

Traditional cohort-based survival analysis using Kaplan-Meier method with log-rank test was used to calculate overall survival (OS). Survival time was calculated as the time elapsed between the date of diagnosis and the date of death of any cause or date at last follow-up (alive, censored).

For analyses in patients with stage 4 NB, treatment modalities were dichotomized to yes/no (see Table 2). Differences in frequency of applied treatment modalities by period of diagnosis were tested using χ^2 tests.

Time trends in observed 5-year OS were first evaluated by using a parametric survival model. The dichotomized treatment modalities were added to the model to investigate the effect of therapy on the hazard ratio (HR) of period of diagnosis. Age group (<18 and ≥18 months), a strong independent predictor of survival, was also entered in the multivariable models. All statistical analyses were two-sided and a p-value <.05 was considered significant. Analyses were performed with STATA/SE 14.2 (StataCorp LP, College Station, TX, 2015).

RESULTS

Patient characteristics

Between 1990 and 2014, 509 newly diagnosed patients with neuroblastoma and 84 with ganglioneuroblastoma were registered by the NCR, of which 583 (98%) were histologically confirmed. Patient and tumor characteristics are presented in Table 1. Median age at diagnosis was 21 months (range 0-16 years), male sex was slightly predominant (54%; male/female ratio = 1.2:1). Seventy percent of the patients had an adrenal or abdominal primary tumor. The majority of patients was diagnosed with stage 4 disease (52%), followed by stage 1/2 disease (28%), stage 3 (12%) and stage 4S (8%). For 8 patients no data was available on stage of disease (Table 1). In patients <18 months, stage 1/2 was the most common (41%), and stage 4 disease was observed in 26% of the patients. In patients ≥18 months, stage 4 dominated (73%; Figure 1).

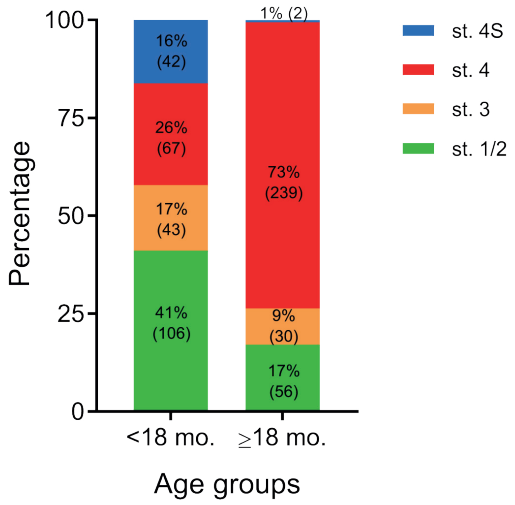

Figure 1 Stage distribution of neuroblastoma patients aged <18 and ≥18 months at diagnosis. For patients <18 months and ≥18 months of age, the percentage (number of patients between parentheses) of each stage at diagnosis is given. Blue: stage 4S; red: stage 4; orange: stage 3; green: stage 1/2. Two patients were diagnosed as stage 4S, while they were ≥18 months of age. Stage of disease was unknown in 8 patients, 7 of them aged <18 months and were not included in this graph. Abbreviations: mo.: months; st.: stage.

Table 1 Patient characteristics of patients <18 years, diagnosed with a neuroblastoma in the Netherlands between 1990 and 2014.

	1990-1994	**1995-1999**	**2000-2004**	**2005-2009**	**2010-2014**	**total**
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Age						
<18 months	45 (45)	54 (48)	56 (45)	57 (45)	53 (41)	265 (45)
≥18 months	55 (55)	59 (52)	68 (55)	69 (55)	77 (59)	328 (55)
Gender						
Male	53 (53)	61 (54)	76 (61)	66 (52)	67 (52)	323 (54)
Female	47 (47)	52 (46)	48 (39)	60 (48)	63 (48)	270 (46)
Histology						
NB	90 (90)	89 (79)	106 (85)	107 (85)	117 (90)	509 (86)
GNB	10 (10)	24 (21)	18 (15)	19 (15)	13 (10)	84 (14)
Stage						
1/2	26 (27)	37 (33)	39 (32)	34 (27)	26 (20)	162 (28)
3	14 (15)	13 (12)	15 (12)	15 (12)	16 (12)	73 (12)
4	47 (49)	52 (46)	63 (52)	67 (54)	77 (59)	306 (52)
4S	9 (9)	10 (9)	5 (4)	9 (7)	11 (8)	44 (8)
Unknown	4	1	2	1	0	8
Localization primary tumor						
Sympathetic side chain	23 (23)	32 (28)	32 (26)	33 (26)	37 (28)	157 (27)
- Cervical/thoracic	13 (13)	19 (17)	18 (15)	13 (10)	26 (20)	89 (15)
- Pelvic	5 (5)	7 (6)	6 (5)	8 (6)	4 (3)	30 (5)
- Not otherwise specified	5 (5)	6 (5)	8 (6)	12 (10)	7 (5)	38 (6)
Adrenal/abdominal	70 (70)	79 (70)	88 (71)	91 (72)	90 (69)	417 (70)
Unknown/no primary tumor	7 (7)	2 (2)	4 (3)	2 (2)	3 (2)	18 (3)

Abbreviations: NB: neuroblastoma; GNB: ganglioneuroblastoma.
Bold fonts indicate characteristics categories,italic fonts indicate subgroups

Table 2 Univariate and multivariable analyses for 5-year overall survival of patients with stage 4 neuroblastoma by age group, period of diagnosis and treatment modalities

		Univariate analysis				**Multivariable analysis, model without treatment modalities**				**Multivariable analysis, model with treatment modalities**			
	n	HR	95% CI		p	HR	95% CI		p	HR	95% CI		p
Age groups													
<18 months	68	Ref.				Ref.				Ref.			
≥18 months	238	2.12	1.42	- 3.17	<.01	2.26	1.51	- 3.38	<.01	3.11	2.04	- 4.72	<.01
Period													
1990-1994	47	Ref.				Ref.				Ref.			
1995-1999	52	0.89	0.57	- 1.40	.62	0.84	0.53	- 1.32	.44	1.03	0.65	- 1.64	.88
2000-2004	63	0.72	0.47	- 1.12	.15	0.65	0.42	- 1.01	.06	0.95	0.60	- 1.51	.83
2005-2009	67	0.54	0.34	- 0.85	.01	0.52	0.33	- 0.82	.01	0.85	0.53	- 1.38	.52
2010-2014	77	0.50	0.32	- 0.78	<.01	0.44	0.28	- 0.69	<.01	1.14	0.69	- 1.90	.60
ASCT													
no	151	Ref.								Ref.			
yes	155	0.45	0.34	- 0.60	<.01					0.46	0.32	- 0.64	<.01
Surgery													
no	82	Ref.								Ref.			
yes	224	0.58	0.43	- 0.79	<.01					0.75	0.54	- 1.04	.09
Immunotherapy													
no	262	Ref.								Ref.			
yes	44	0.38	0.23	- 0.62	<.01					0.37	0.19	- 0.72	<.01
Radiotherapy													
no	214	Ref.								Ref.			
yes	92	0.76	0.55	- 1.03	.08					1.21	0.84	- 1.74	.30

HRs were corrected for follow-up time.

Abbreviations; ASCT autologous stem cell transplantation after high-dose chemotherapy; HR Hazard Ratio; 95% CI: 95% confidence interval.

Bold fonts indicate characteristics categories.

Incidence

In the time period 1990-1994, on average 20 new patients per year were diagnosed with NB, this increased to 26 patients per year between 2010-2014 (Figure 2A). The overall incidence rate (all stages, <18 years) significantly increased by 1.6% per year from 6.4 to 9.1 per million between 1990-2014 (p=.01; Figure 2B). Stage 4 NB increased with 2.9% per year (p<.01), while the incidence of all other stages remained stable (Figure 2B). Incidence rates by age, gender, histological type and stage as well as the AAPC analyses for neuroblastoma patients aged <15 years are provided in Supplemental Table S1. No other significant changes in these rates were observed.

The age specific incidence rates for patients aged <18 and ≥18 months by stage are shown in Figure 2C and D. Incidence rates were stable for all stages in patients <18 months, while an increase in incidence of stage 4 NB was seen in patients aged ≥18 months (AAPC +3.3%, p=.01). For this age group the number of stage 4 patients almost doubled from 7 patients per year in 1990-1994 to 12 patients per year in 2010-2014. The incidence rates for the other stages in patients ≥18 months remained stable.

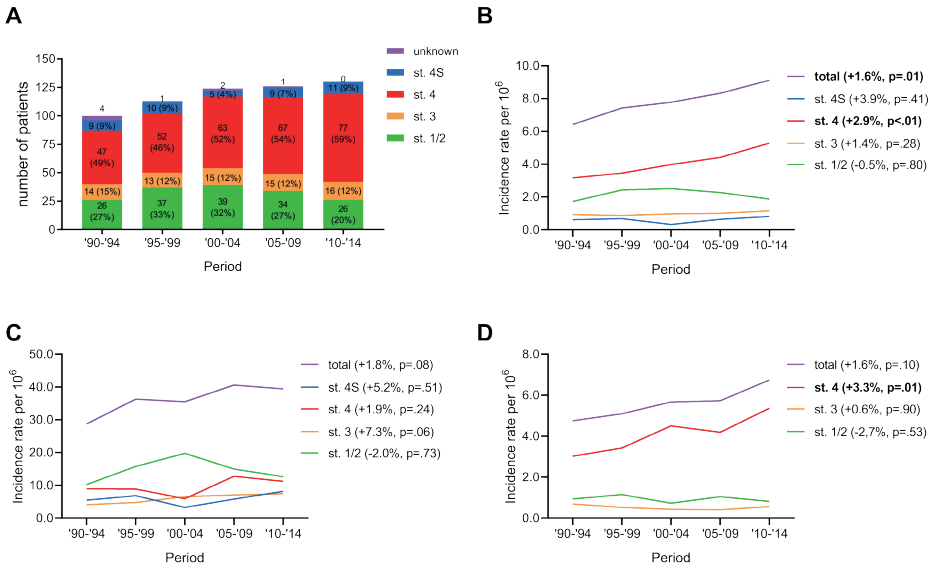

Figure 2 Time trends in neuroblastoma incidence per five year period.
Number of newly diagnosed patients (percentage in parentheses) are given by stage and diagnostic period (A); purple: unknown stage; blue: stage 4S; red: stage 4; orange: stage 3; green: stage 1/2, purple: total (in B-D).
Time trends of incidence rates according to stage were calculated per million children aged 0-17 years (B); per million children aged 0-17 months (C); and per million children aged 18 months - 17 years (D). The Average Annual Percentage Change (AAPC) is given in the legends of B-D, bold fonts indicate significant changes over time.

Therapy and survival

The 5-year survival rates varied by stage: 93±2% in stage 1/2 disease; 84±6% in stage 4S; 70±5% in stage 3 disease; 35±3% in stage 4 disease (Figure 3A). Five-year overall survival (OS) of all patients improved from 44±5% in 1990-1994 to 61±4% in 2010-2014 (p<.01; Figure 3B). Five-year OS of patients with stage 4 neuroblastoma improved significantly from 19±6% in 1990-1994 to 44±6% in 2010-2014 (p<.01; Figure 4A). For patients with the poorest outcome (stage 4 and ≥18 months old) 5-year OS significantly improved from 6±4% in 1990-1994 to 43±7% in 2010-2014(p<.01; Figure 4B). The 5- and 10-year OS rates over time for gender, age group, histologic type and stage are summarized in Supplemental Table S2.

Important changes in the treatment of patients with stage 4 disease were made between 1990 and 2014. High-dose chemotherapy with autologous stem cell transplantation was given in 21% of patients with stage 4 between 1990-1999 and in 69% between 2010-2014 (p<.01); the frequency of primary tumor surgery increased from 58% to 84% (p<.01); radiotherapy increased from 16% to 40% (p<.01); immunotherapy increased from 0% in 1990-1999 to 4% in 2005-2009 and 53% in 2010-2014 (p<.01). The number of patients receiving ^{131}I-MIBG-therapy (39%) and chemotherapy (98%) did not change between 1990 and 2014.

Multivariable survival analysis for stage 4 neuroblastoma

In univariate analysis, the risk of dying (HR) from stage 4 NB was significantly lower during the periods 2005-2009 and 2010-2014 compared with 1990-1994 (HR 0.54, p=.01 and HR 0.50, p<.01, respectively). Patients aged ≥18 months had a poorer survival probability (HR 2.12, p<.01) than patients aged <18 months (Table 2). Other prognostic factors were the treatment modalities high-dose chemotherapy with stem cell rescue, immunotherapy and surgery. The first multivariable model contained age and period of diagnosis. In this model, the two most recent periods of diagnosis were associated with better outcome (HR 0.52 and 0.44, p=.01 and p<.01, respectively). Addition of the different treatment modalities to a second multivariable model resulted in the loss of significance for the HRs of these recent periods of diagnosis (HR 0.85 and 1.14, p=.52 and p=.60, respectively; Table 2). Patients who received high-dose chemotherapy with stem cell rescue (HR 0.46, p<.01) and patients who received immunotherapy (HR 0.37, p<.01) had a significant reduction of the risk of dying. The changes in the treatment modalities were better discriminants for the changes in survival over time, than the periods of diagnosis (Table 2).

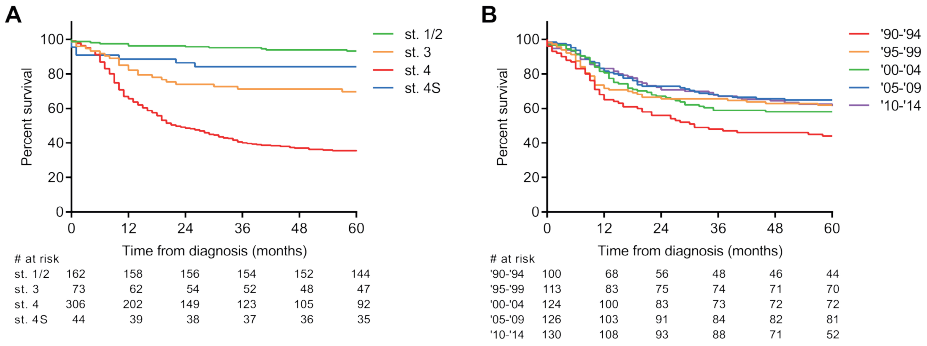

Figure 3: Five year overall survival (OS) for neuroblastoma patients.
Five year OS rates are given for different stages (A) and different time periods (B). Figure 3A) stage specific 5-yr OS was 93±2% for stage 1/2 (green), 84±6% for stage 4S (blue), 68±6% for stage 3 (orange) and 35±3% for stage 4 (red). Figure 3B) 5-yr OS for all stages combined was 44±5% in 1990-1994 (red); 62±5% in 1995-1999 (orange); 58±4% in 2000-2004 (green); 65±4% in 2005-2009 (blue) and 61±4% in 2010-2014 (purple).

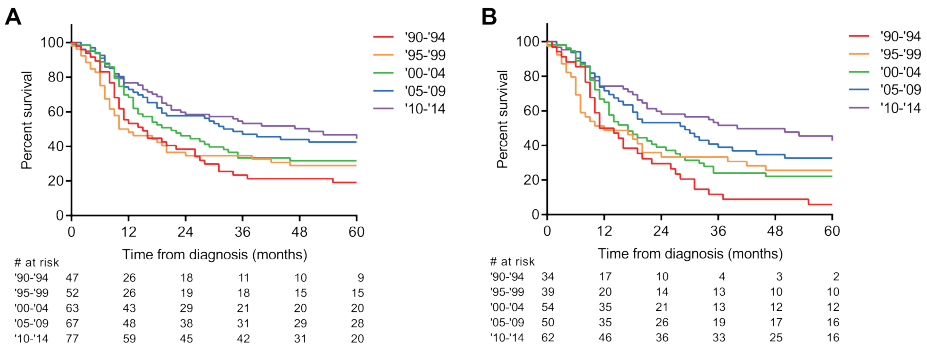

Figure 4: Time trends of five year overall survival (OS) for patients with a stage 4 neuroblastoma.
Five year OS rates are given by 5-year periods for all patients with stage 4 neuroblastoma (4A), and for patients with stage 4 neuroblastoma ≥18 months at diagnosis (4B). Figure 4A) 5-yr OS of patients with stage 4 neuroblastoma was 19 ± 6% in 1990-1994 (red); 29±6% in 1995-1999 (orange); 32±6% in 2000-2004 (green); 42±6% in 2005-2009 (blue) and 44±6% in 2010-2014 (purple). For patients ≥18 months old with stage 4 the 5-yr OS was 6±4% in 1990-1994 (red); 26±7% in 1995-1999 (orange); 22±6% in 2000-2004 (green); 33±7% in 2005-2009 (blue) and 43±7% in 2010-2014 (purple).

DISCUSSION

This is the first report on incidence and survival of children and adolescents with NB in the Netherlands. Over a 25-year period, we observed a significant increase in incidence of stage 4 disease in patients ≥18 months, while the incidence of other stages and ages remained stable. Five-year OS improved for all ages and stages, the most distinct for patients aged ≥18 months with stage 4 NB, where an improvement of 37 percentage points was seen.

The age-standardized incidence rate of around 10.5 cases per million children in 2010-2014 observed in this study is similar to other high income countries as Canada, USA and neighboring European countries (WSR 0-14 years 10.1-15.0).[29,30] The overall increase in NB incidence of 1.6% per year is in line with the increase in NB incidence in older children (1-4 year) of 1.7% per year in Europe (1978-1997), and of 1.6% per year in Canada (1992-2010).[2,3] However, in Denmark NB incidence has been stable between 1981-2000 for all stages and age categories,[31] whereas in England, a slight decrease in incidence of 0.2% for all stages and age categories was seen between 1993 and 2000.[16] In Germany, analyses of both tumor stage and age were performed. They found a small (7% per 10 year) increase in overall incidence, but this was attributed to an increase in stage 1-3 and stage 4S and a decrease in stage 4, which is contradicting our data.[32] Etiological factors for NB are largely unknown other than 'it is a developmental tumor of the sympathetic nervous system'. Genetic predisposition is rare (estimated at 1-2%)[33], and no environmental factors have been consistently associated with neuroblastoma.[34] Improved prenatal ultrasounds only contribute to an increase in patients <18 months at diagnosis. In fact, this has also been shown in NB screening studies based on urinary catecholamine measurements in infants. [17,20,35] Higher registration rates caused by immigration for medical reasons can be ruled out because the Netherlands has a long standing population-wide cancer registry, covering at least 95% of all newly diagnosed malignancies in Dutch inhabitants.[36]

The increase in overall incidence is caused by an increase in the incidence of stage 4 NB in patients ≥18 months old. In this group, the number of newly diagnosed patients almost doubled. The increase cannot be assigned to higher sensitivity of molecular markers (amplification of *MYCN* or loss of heterozygosity of chromosome 1p), since these influence risk stratification and not stage of disease. Improved sensitivity of diagnostics and upstaging of patients with lower stage disease can play a small role, but seems to be negligible because only a minimal (non-significant) decrease in lower stage disease was observed, while there was a significant increase in overall incidence and in stage 4 incidence. This leaves the cause of the increased incidence for this subgroup unclear.

The improved survival for patients with stage 4 disease, is associated with changes in therapy. Multivariable analysis showed that high-dose chemotherapy followed by autologous stem cell rescue and immunotherapy (HR 0.46, p<.01 & HR 0.37, p<.01) were the treatment modalities that more adequately predicted the survival improvement than the periods of diagnosis. Berthold *et al.* and Pinto *et al.*[9,37] reported previously of a

survival benefit for high-dose chemotherapy in high-risk NB, compared to maintenance therapy. Immunotherapy was introduced in 2009, and in this cohort only 44 of the 306 patients with stage 4 disease received immunotherapy. Despite this very small number, we observed a significant effect on OS in both the univariate(HR 0.38, p<.01), and multivariable analysis (HR 0.37, p<.01). This cohort seems to confirm earlier studies demonstrating a benefit for maintenance therapy with immunotherapy.[12,38] In addition, we expect roles for the intensified induction chemotherapy and the improved supportive care over time, but the current data set did not allow these analyses.

The longstanding population-based Netherlands Cancer Registry follows international standards and coding practices, and has, also through its participation in international projects (Eurocare, ACCIS, CI 5) many quality checks. The NCR is one of the few registries that also registers stage and initial treatment. A limitation of this study is the lack of data on prognostic markers such as *MYCN* amplification and on cause of death. However, because the pediatric population in this study is not suspected for other serious underlying diseases or competing causes of death, the observed survival, as reported here, is representative for the NB-specific survival.[39] Another limitation is the relative small size of the Dutch population, resulting in a smaller cohort than the German, European or American SEER databases.[1,32,39]

CONCLUSIONS

Our population-based study comprehensively analyzed incidence, incidence changes over time, survival and treatment of NB during a 25-year period in the Netherlands. We observed an increase of 1.6% per year in total incidence and more particularly for patients with stage 4 disease who were ≥18 months of age. Survival for this group improved from 6±4% in 1990-1994 to 43±7% in 2010-2014. The improved survival of stage 4 patients is predominantly associated with the introduction of high-dose chemotherapy with autologous stem cell rescue and immunotherapy.

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SUPPLEMENTAL MATERIAL

Supplemental Table S1 Incidence for children, aged <15 years, diagnosed with neuroblastoma in the Netherlands between 1990 and 2014

							AAPC				
Incidence Males & Females		**1990-94**	**1995-99**	**2000-04**	**2005-09**	**2010-14**	**1990-2014**	**SE**	**95% CI low**	**95% CI high**	**p-value**
	Average number of new cases/ year	20	23	25	25	25					
	Incidence rate (per 10^6)	7.2	7.8	8.3	8.5	8.8	0.9	0.5	-0.2	2.0	0.10
	Age-standardized incidence rate (per 10^6)[a]	7.9	8.8	9.3	9.8	10.5	1.3	0.5	0.2	2.4	0.02
Age (years)											
0	Average number of new cases/ year	7	8	9	9	8					
	Incidence rate (per 10^6)	35.8	42.2	44.4	47.6	47.1	1.4	0.9	-0.5	3.2	0.14
1-4	Average number of new cases/ year	10	12	13	12	14					
	Incidence rate (per 10^6)	13.5	15.3	16.1	15.7	18.6	1.2	0.8	-0.5	2.9	0.17
≥ 5	Average number of new cases/ year	3	3	3	4	3					
	Incidence rate (per 10^6)	1.4	1.3	1.4	2.1	1.7	3.2	3.1	-3.3	9.6	0.32
Age (months)											
< 18	Average number of new cases/ year	9	11	11	12	11					
	Incidence rate (per 10^6)	30.8	37.0	36.8	41.3	39.4	1.4	0.9	-0.5	3.4	0.15
≥ 18	Average number of new cases/ year	11	12	14	14	15					
	Incidence rate (per 10^6)	4.8	5.1	5.7	5.7	6.7	1.4	1.0	-0.5	3.4	0.15
Incidence Males		**1990-94**	**1995-99**	**2000-04**	**2005-09**	**2010-14**	**1990-2014**	**SE**	**95% CI low**	**95% CI high**	**p-value**
	Average number of new cases/ year	11	12	15	13	13					
	Age-standardized incidence rate (per 10^6)[a]	8.5	9.5	11.5	9.9	10.7	1.0	0.7	-0.4	2.4	0.16
Age (months)											
< 18	Average number of new cases/ year	5	6	7	6	6					
	Incidence rate (per 10^6)	30.9	37.4	42.5	41.7	43.7	5.2	4.7	-4.6	14.7	0.29
≥ 18	Average number of new cases/ year	6	7	9	7	7					

Supplemental Table S1 Conrinued

							AAPC				
	Incidence rate (per 10^6)	5.2	5.6	7.0	6.0	6.3	0.6	1.4	-2.3	3.5	0.69
Incidence Females		**1990-94**	**1995-99**	**2000-04**	**2005-09**	**2010-14**	AAPC **1990-2014**	**SE**	**95% CI low**	**95% CI high**	**p-value**
	Average number of new cases/ year	9	10	10	12	12					
	Age-standardized incidence rate (per 10^6)[a]	7.5	8.3	7.4	9.5	10.5	1.3	1.0	-0.8	3.3	0.21
Age (months)											
< 18	Average number of new cases/ year	4	5	5	6	5					
	Incidence rate (per 10^6)	30.8	36.6	30.9	40.9	34.9	-0.2	1.3	-2.9	2.4	0.87
≥ 18	Average number of new cases/ year	5	5	5	6	8					
	Incidence rate (per 10^6)	4.5	4.6	4.3	5.4	7.2	2.5	1.6	-0.8	5.7	0.13
Incidence neuroblastoma		**1990-94**	**1995-99**	**2000-04**	**2005-09**	**2010-14**	AAPC **1990-2014**	**SE**	**95% CI low**	**95% CI high**	**p-value**
	Average number of new cases/ year	18	18	21	21	23					
	Age-standardized incidence rate (per 10^6)[a]	7.1	7.0	7.9	8.5	9.5	1.5	0.6	0.3	2.7	0.01
Age (months)											
< 18	Average number of new cases/ year	8	9	10	11	10					
	Incidence rate (per 10^6)	28.1	31.6	32.9	37.8	37.2	1.9	1.1	-0.4	4.0	0.10
≥ 18	Average number of new cases/ year	10	9	11	11	13					
	Incidence rate (per 10^6)	4.3	3.8	4.6	4.6	5.8	1.6	1.0	-0.4	3.6	0.11
Incidence ganglioneuroblastoma		1990-94	1995-99	2000-04	2005-09	2010-14	1990-2014	SE	95% CI low	95% CI high	p-value
	Average number of new cases/ year	2	5	4	4	3					
	Age-standardized incidence rate (per 10^6)[a]	0.8	1.8	1.4	1.4	1.1	2.4	3.1	-4.0	8.7	0.45
Age (months)											

Supplemental Table S1 Conrinued

							AAPC				
< 18	Average number of new cases/ year	1	2	1	1	1					
	Incidence rate (per 10^6)	2.8	5.4	3.9	3.5	2.2	-9.5	8.1	-26.8	6.8	0.23
≥ 18	Average number of new cases/ year	1	3	2	3	2					
	Incidence rate (per 10^6)	0.5	1.3	1.0	1.1	0.9	3.3	4.5	-6.1	12.6	0.48
							AAPC				
Incidence by stage[b]		**1990-94**	**1995-99**	**2000-04**	**2005-09**	**2010-14**	**1990-2014**	**SE**	**95% CI low**	**95% CI high**	**p-value**
	Average number of new cases/ year	19	22	24	25	25					
	Age-standardized incidence rate (per 10^6)[a]	7.5	8.7	9.1	9.7	10.5	1.5	0.5	0.4	2.6	0.01
Stage											
1/2	Average number of new cases/ year	5	7	8	7	5					
	Age-standardized incidence rate (per 10^6)[a]	2.0	2.9	2.9	2.7	2.2	-0.5	1.8	-4.2	3.3	0.80
3	Average number of new cases/ year	3	3	3	3	3					
	Age-standardized incidence rate (per 10^6)[a]	1.1	1.0	1.1	1.2	1.4	1.5	1.3	-1.3	4.2	0.28
4	Average number of new cases/ year	9	10	13	13	15					
	Age-standardized incidence rate (per 10^6)[a]	3.7	4.1	4.7	5.2	6.0	2.7	0.7	1.2	4.2	<0.01
4S	Average number of new cases/ year	2	2	1	2	2					
	Age-standardized incidence rate (per 10^6)[a]	0.7	0.8	0.4	0.8	0.9	4.0	4.7	-5.8	13.7	0.41

[a] age standardization according to the World standard rate (0-14 years).

[b] calculated for patients with known stages (the unknown 8 were excluded).

In case of zero patients in a year, an incidence rate of 0.01 was assumed.

Abbreviations: AAPC: average annual percentage change; SE: standard error; 95% CI: 95% confidence interval. Bold fonts indicate characteristics categories.

Supplemental Table S2 Five-year and 10-year overall survival for neuroblastoma patients diagnosed and treated in the Netherlands over time.

	1990-2014					1990-1994					1995-1999					2000-2004					2005-2009					2010-2014				
	n at risk	5-yr OS	SE	10-yr OS	SE	n at risk	5-yr OS	SE	10-yr OS	SE	n at risk	5-yr OS	SE	10-yr OS	SE	n at risk	5-yr OS	SE	10-yr OS	SE	n at risk	5-yr OS	SE	10-yr OS	SE	n at risk	5-yr OS	SE	p* 5-yr	p* 10-yr
Total	593	59%	2%	56%	2%	100	44%	5%	44%	5%	113	62%	5%	55%	5%	124	58%	4%	55%	5%	125	65%	4%	58%	5%	130	61%	4%		
Gender																														
Male	323	57%	3%	54%	3%	53	51%	7%	51%	7%	61	52%	6%	46%	7%	76	57%	6%	54%	6%	66	66%	6%	60%	6%	67	58%	6%	0.07	0.11
Female	270	60%	3%	58%	3%	47	36%	7%	36%	7%	52	73%	6%	66%	7%	48	60%	7%	56%	8%	60	63%	6%	60%	7%	63	65%	6%	0.03	0.04
Age (mo)																														
<18	265	81%	2%	81%	2%	45	64%	7%	64%	7%	54	81%	5%	81%	5%	56	89%	4%	89%	4%	57	84%	5%	84%	5%	53	81%	5%	0.06	0.02
≥18	328	41%	3%	36%	3%	55	27%	6%	27%	6%	59	44%	6%	37%	6%	68	32%	6%	31%	6%	69	49%	6%	41%	6%	77	48%	6%	0.01	0.06
Histology																														
NB	509	54%	2%	51%	2%	90	43%	5%	43%	5%	89	53%	5%	49%	5%	106	52%	5%	51%	5%	107	60%	5%	55%	5%	117	60%	5%	0.01	0.05
GNB	84	87%	4%	84%	4%																									
Stage**																														
1/2	162	93%	2%	92%	2%	26	77%	8%	77%	8%	37	97%	3%	95%	4%	39	95%	4%	95%	4%	34	100%	0%	100%	0%	26	92%	5%	0.04	<0.01
3	73	70%	5%	70%	5%																									
4	306	35%	3%	30%	3%	47	19%	6%	19%	6%	52	29%	6%	23%	6%	63	32%	6%	30%	6%	67	43%	6%	33%	6%	77	45%	6%	<0.01	0.01
4S	44	84%	6%	84%	6%																									

* p for trend in observed 5 and 10-year OS by using parametric survival model adjusted for follow-up time.

** Stage is missing for 8 patients.

The analysis per period was not performed when patient number at start of the interval was below 20. Bold p-values indicate statistical significance. For the period 2010-2014, only 5-yr OS could be calculated.

Abbreviations: n: number; SE: standard error; OS: overall survival; NB: neuroblastoma; GNB: ganglioneuroblastoma. Bold fonts indicate characteristics categories.

CHAPTER

Anti-GD2 based immunotherapy prevents late events in high-risk neuroblastoma patients over 18 months at diagnosis

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ABSTRACT

Background

Anti-GD2 based immunotherapy has improved overall (OS) and event free survival (EFS) for high-risk neuroblastoma (HR-NBL) patients. Here, we evaluate the long-term efficacy of anti-GD2 immunotherapy in combination with isotretinoin, GM-CSF and IL-2.

Methods

Dutch HR-NBL patients treated with immunotherapy according to the COG-ANBL0032 protocol (n=47) were included and compared to historical controls (n=37) treated with single-agent isotretinoin maintenance therapy. Survival time was calculated from start of the maintenance therapy.

Results

The study and control group were similar concerning baseline characteristics. In the complete cohort, 5 year OS was 64±7% and 49±8% for the immunotherapy group and the control group, respectively (p=0.16). Five year EFS was 57±7% and 41±8%, respectively (p=0.16). In the subgroup of patients ≥18 months, 5-yr OS was 63±8% and 39±9, respectively (p=0.04) and EFS 54±8% and 29±8%, respectively (p=0.05). Landmark analysis for EFS with landmark point at 6 months after start of maintenance suggests a larger effect on the prevention of late than early events.

Conclusions

This study is the first to confirm the results of the COG-ANBL0032 study, regardless of the different induction regimen. Anti-GD2 immunotherapy prevents late events, most significantly in patients older than 18 months of age at diagnosis.

INTRODUCTION

Neuroblastoma is a pediatric tumor with an incidence of 11 per million children <15 years of age each year.[1] It accounts for 7% of newly diagnosed pediatric malignancies, and for 10-12% of pediatric cancer mortality.[2,3] It is a heterogeneous tumor with good survival (>85%) for low-risk patients with minimal treatment, but with poor survival (<50%) for high-risk patients, despite intensive multimodality treatment.[4,5] The main challenges in treating high-risk patients are improving complete response (CR) rates and reducing the number of recurrences after CR to initial treatment: 50-60% of patients will develop recurrent disease, which is associated with 5-yr survival rates below 20%.[6,7] To improve survival, therapy for high-risk neuroblastoma has been intensified in the last decades. Most recently, anti-GD2-based immunotherapy was added to the maintenance phase of treatment.[4,8] Ganglioside-2 (GD2) is a tumor-associated antigen, expressed on 95% of neuroblastoma cells.[9] In normal tissues, expression is limited to the central and peripheral nervous system. Other tumors expressing GD2 include melanoma, glioblastoma multiforme, medulloblastoma, small cell lung carcinoma, breast cancer and some sarcomas. [10-13]

Between 2001 and 2009, the American Children's Oncology Group (COG) conducted a randomized controlled trial (ANBL0032) comparing the ch14.18 antibody (dinutuximab) in combination with isotretinoin and alternating GM-CSF and IL-2 to single-agent isotretinoin in the maintenance phase of treatment.[3,14] This improved event free survival (EFS) by 20% and 11%, and overall survival (OS) by 11% and 16% after 2 and 5 years, respectively.[3,14] The authors concluded dinutuximab to be more powerful in preventing early recurrences than late recurrences.

Here, we conducted a retrospective analysis in a Dutch cohort treated according to the COG-ANBL0032 protocol. We present data on long-term survival suggesting a preventive effect on late events, and show that this is the most pronounced in patients ≥18 months of age at diagnosis.

METHODS

Patients characteristics

High-risk neuroblastoma patients in the Dutch Childhood Oncology Group (DCOG) database, diagnosed between 1999 and 2015, were identified based on completion of induction therapy including consolidation therapy with high-dose chemotherapy and autologous stem cell rescue. Exclusion criteria were similar to the ANBL0032 study: 1) less than partial response (PR) after induction therapy; 2) interval between start of induction therapy and ASCT of more than 12 months; 3) progressive disease before start of the maintenance treatment; 4) induction therapy according to other protocols than the DCOG NBL2004[15] or POG9640[16] protocols. Patients who received immunotherapy

according to the ANBL0032 protocol were diagnosed between 2009 and 2015 and were compared to historical controls diagnosed between 1999 and 2014. Five patients in the control group were diagnosed between 2010 and 2014, when immunotherapy was available, for all these cases it was parents' choice not to receive the immunotherapy. The historical control group was treated with an identical induction regimen and high dose chemotherapy as consolidation. The maintenance treatment was single-agent isotretinoin 160 mg/m^2/day for subsequent 14 days followed by 14 day rest for a total of 6-9 cycles.

Clinical and pathological characteristics were collected for all patients at diagnosis: age, INSS stage, *MYCN* status and LOH1p status. All available tumor samples were centrally reviewed using the International Neuroblastoma Pathology Classification (INPC)[17] by an expert pathologist (RdK). Response after induction treatment was retrieved from patient charts and reconstructed from the available clinical, pathological, radiological and biochemical data and centrally discussed by MT, LD, GT and MvN following the 1993 International Neuroblastoma Response Criteria (INRC)[18]. Since all patients were diagnosed before 2017 and assessments from charts were not yet according to the 2017 INRC criteria[19]. Collection of patient data and use of tumor material was approved by the Medical Research Ethics Committee of the University Medical Center Utrecht, Utrecht, The Netherlands (reference number: WAG/nb/18/021561). In addition, all patients who received immunotherapy signed a written consent for the ANBL0032 study.

Statistical analysis

Data were analyzed using SPSS Statistics 26 (IBM Corp. Armonk, NY, 2019). Fisher's exact test was used to analyze clinical and pathologic characteristics. Estimated OS and EFS were estimated according to Kaplan-Meier methodology[20] and are reported ±SE. Survival was calculated from start of maintenance treatment to event or last follow-up. Events were defined as recurrence, progression, or death. To investigate the effect of immunotherapy on survival outcomes a Cox regression model was estimated.[21] To deal with the violation of the proportional hazard assumption for treatment, two separate models were estimated.[22,23] The first was estimated from start of the maintenance treatment until the end, 6 months later. The second model was estimated from the landmark point (6 months after start of maintenance) until 5 years after start of maintenance therapy. Multivariable Cox Regression estimation was used to estimate the effect of induction protocol on survival. $P<0.05$ was considered significant for all tests.

RESULTS

Patients characteristics

A total of 103 patients was identified who were diagnosed with high-risk neuroblastoma between 1999 and 2015 and had completed induction therapy and consolidation therapy with high-dose chemotherapy followed by autologous stem cell transplantation (ASCT). Nineteen patients were excluded from analysis because they received chemotherapy according to other protocols than the DCOG NBL2004 or the POG9640 protocols (n=10), interval of more than 12 months between start of induction and ASCT (n=3), less than PR at response evaluation after induction (n=2), progressive disease prior to start of maintenance treatment (n=3), and missing data (n=1). The remaining 84 patients were included, 47 patients in the immunotherapy group and 37 in the control group.

Table 1 Patient characteristics.

	Control N (%)	**IT N (%)**	**Total N (%)**	**Sig (p)**
Age				**0.76**
<18 months	6 (16)	6 (13)	12 (14)	
≥18 months	31 (84)	41 (87)	72 (86)	
INSS stage				NA
3	3 (8)	3(6)	6(7)	
4	34 (92)	44 (94)	78 (93)	
Histology				**0.43**
Undifferentiated	6 (19)	11 (28)	17 (24)	
Poorly differentiated	18 (58)	24 (62)	42 (60)	
Differentiating	6 (19)	4 (10)	10 (14)	
GNB nodular	1 (3)	0 (0)	1 (1)	
Unknown	6	8	14	
MYCN				**NA**
Not-amplified	19 (61)	27 (61)	46 (61)	
Amplified	12 (39)	17 (39)	29 (39)	
Unknown	6	3	9	
Response prior to ASCT				**0.40**
CR	21 (57)	22 (47)	43 (51)	
VGPR	2 (5)	7 (15)	9 (11)	
PR	14 (38)	18 (38)	32 (38)	
Induction protocol				**0.35**
DCOG NBL2004	23 (62)	34 (72)	57 (68)	
POG9640	14 (38)	13 (28)	27 (32)	

Abbreviations: IT: immunotherapy, sig: significance, NA: not applicable, GNB: ganglioneuroblastoma, ASCT: autologous stem cell transplantation, CR: complete response, (VG)PR: (very good) partial response

Median follow-up of surviving patients was 7.5 years (range 4.3-18.4 years). This was 6.7 years (range 4.3-9.8 years) for the immunotherapy group and 11.6 years (range 7.5-18.4 years) for the control group. At baseline, the immunotherapy and control group were similar for clinical and pathological characteristics (Table 1). In the immunotherapy group, 39 patients (83%) completed all 6 cycles of maintenance therapy. Immunotherapy was discontinued in six patients (13%) because of progressive disease, and in two patients (4%) due to anaphylaxis. In the control group, 32 patients (86%) completed at least six cycles of isotretinoin, five patients (14%) stopped early due to progressive disease.

Outcome

At 2 years, EFS was equal to 66±7% in the immunotherapy group, and to 51±8% in the control group (p=0.23), a 15% difference. At 5 years, EFS was equal to 57±7%, and 41±8% (p=0.16; Δ16%) for the immunotherapy and control group, respectively (Figure 1A). At 2 years, OS was equal to 79±6% and 65±8% (p=0.18; Δ14%), respectively. At 5 years, OS was equal to 64±7% and 49±8% (p=0.16; Δ15%), respectively (Figure 1B).

Overall survival in patients ≥ 18 months at diagnosis significantly improved

Patients older than 18 months at diagnosis are at higher risk of relapse compared to younger patients.[4,5] We performed separate analyses aimed at investigating the effect of immunotherapy in this patient group. EFS at 5 year was equal to 54±8%, for the immunotherapy group and 29±8% for the control group (p=0.05; Figure 2A), a difference of 25%. OS at 5 years was equal to 63±8% for the immunotherapy group and 39±9% for the control group, an improvement of 24% (p=0.04; Figure 2B). Landmark analysis was performed at the end of treatment, six months after start of maintenance treatment. Univariate Cox regression estimated on the first six months – during maintenance therapy – showed no difference in survival between the immunotherapy and control group (HR 1.30, 95% CI: 0.44-3.89; Figure 2C). From the landmark point until 5 years after start of maintenance treatment, a protective effect of the immunotherapy (HR 0.34, 95% CI 0.16-0.75; Figure 2D) was found. Addition of induction protocol to the regression model did not influence the effect of immunotherapy on survival (HR 0.36, 95% CI 0.16-0.79), and induction protocol was not associated with survival (HR 1.28, 95% CI 0.58-2.83).

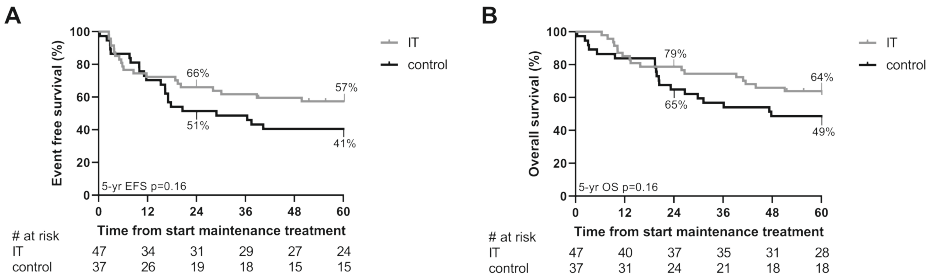

Figure 1 Estimated event free and overall survival for the complete cohort
Figure 1A/B) Estimated event free survival (EFS; A) and overall survival (OS; B) in months from start of the maintenance treatment, treated with immunotherapy (IT) or single-agent isotretinoin (control). Estimated survival and p-values are calculated by Kaplan-Meier method. Numbers at risk are given for 12 months intervals below the graphs.

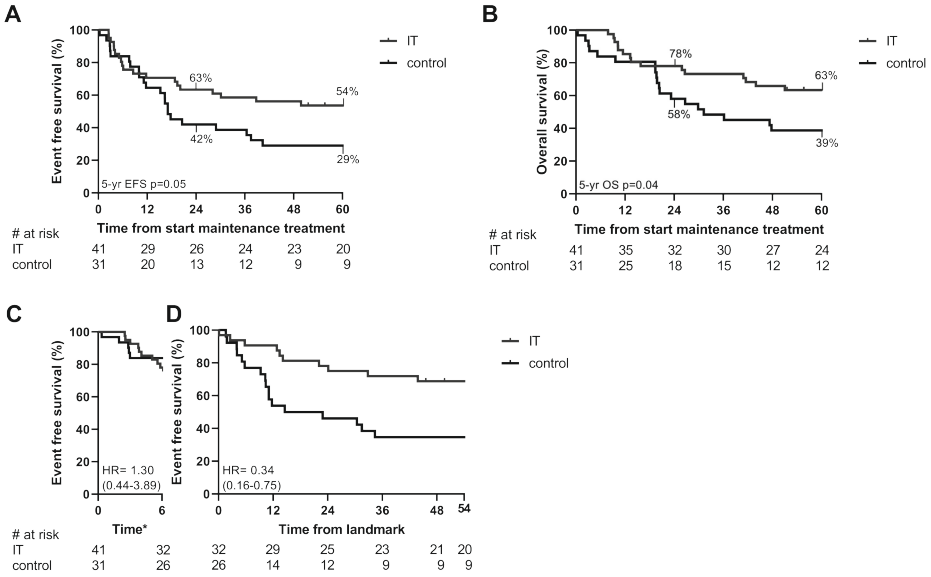

Figure 2 Estimated event free and overall survival of patients ≥18 months
Figure 2A/B) Estimated event free survival (EFS; A) and overall survival (OS; B) in months from start of the maintenance treatment for patients ≥18 months of age at diagnosis, treated with immunotherapy (IT) or single-agent isotretinoin (control). Numbers at risk are given for 12 months intervals below the graphs.
Figure 2C/D) EFS, with a landmark at the end of treatment, 6 months after start of maintenance treatment. 2C shows EFS from start of maintenance treatment to landmark (6 months after start of treatment) 2D shows EFS from landmark until 5-yr after start of maintenance treatment (54 months from landmark). Estimated survival and p-values are calculated by Kaplan-Meier method. Numbers at risk are given for 12 months intervals below the graphs.
*Time from start maintenance therapy.

DISCUSSION

This is the first confirmation of the ANBL0032 immunotherapy protocol in a cohort outside the COG. The patients in our cohort received different induction therapy compared to the original study. The data presented here not only suggests an improved EFS and OS for high-risk neuroblastoma patients, but a long-term protective effect of immunotherapy against late events, particularly in patients ≥ 18 months of age at diagnosis.

After the ANBL0032 study, immunotherapy-based maintenance treatment became standard treatment for high-risk neuroblastoma patients. Different studies have reported on the efficacy of anti-GD2 based immunotherapies (Table 2 and Figure 3). Patients in our cohort and in the COG[3,14,24] were treated according to the ANBL0032 protocol, with dinutuximab (ch14.18)[25] and alternating IL-2 and GM-CSF. In contrast, patients in the GPOH (Gesellschaft fur Padiatrische Onkologie und Hamatologie)[26,27] and SIOPEN (Société International d'Oncologie Pédiatrique European Neuroblastoma)[28,29] cohorts received dinutuximab-beta (ch14.18/CHO)[30] with/without IL-2 and no GM-CSF. Both anti-GD2 antibodies are chimeric human-mice antibodies. Dinutuximab is produced in SP2/0 cells while dinutuximab-beta is produced in CHO cells. All patients, the GPOH excepted, received concomitant isotretinoin. Despite differences in anti-GD2 antibody and concomitant drugs, the studies report an improved 5 year EFS of 11-16% and an improved OS of 14-17%. In our cohort, POG9640 or DCOG NBL2004 induction did not influence survival. In line, the pattern of improved survival for IT patients was similar for all studies, despite different induction and high-dose chemotherapy regimens (Table 2 and Figure 3).

IL-2 was given as an immunostimulant in multiple studies. Recent studies showed that IL-2 increases toxicity without improving survival and it has been deleted from all immunotherapy protocols.[29,31] The effect of the concomitant administration of isotretinoin, and GM-CSF remains unclear. All studies suggest a beneficial effect on (long-)term survival. This effect seems independent of the administered anti-GD2 antibody, the induction therapy regimen, if high-dose chemotherapy is administered and if immunostimulatory drugs are administered with the anti-GD antibody.

Table 2 Literature cohorts of patients receiving immunotherapy

author	**Yu**	**Simon**	**Ladenstein**	**Ozkaynak**	**Ladenstein**	**Tas**
year	2010/2021	2004/2011	2020	2018	2018	Current study
study group	COG	GPOH	SIOPEN	COG	SIOPEN	DCOG
comparison to control group	Randomized	historical controls	historical controls	only IT	only IT	historical controls
induction and minimal response						
induction treatment	COG A3973	GPOH NB97	Rapid Cojec	not reported	rapid COJEC	POG9640/DCOG NBL2004
minimal response	VGPR	NR	PR	PR	PR	PR
immunotherapy group						
n	113	166	378	105	206/200[a]	47
HD chemotherapy	CEM	CEM/N7 courses	BuMel	CEM	CEM/BuMel	CEM
radiotherapy	all patients	MIBG avid masses	all patients	all patients	all patients	all patients
antibody	ch14.18	ch14.18/CHO	ch14.18/CHO	ch14.18	ch14.18/CHO	ch14.18
IL2	iv	no	sc/no[a]	iv	sc/no[a]	iv
GM-CSF	sc or iv	no	no	sc or iv	no	sc
RA	yes	no	yes	yes	yes	yes
Non-immunotherapy group						
n	113	69	466	0	0	37
HD chemotherapy	CEM	CEM/N7 courses	CEM/BuMel	NA	NA	CEM
radiotherapy	all patients	MIBG avid masses	all patients	NA	NA	MIBG avid masses
RA	yes	no	yes	NA	NA	yes
Reported EFS and OS						
EFS	2, 5yr	2, 3, 5, 9yr	5yr	1, 2, 3, 4, 5yr	3, 5yr	2, 5yr
OS	2, 5yr	2, 3, 5, 9yr	5yr	1, 2, 3, 4yr	3, 5yr	2, 5yr

Abbreviations: IT: immunotherapy, COG: Children's Oncology Group, GPOH: Gesellschaft fur Padiatrische Onkologie und Hamatologie, SIOPEN: Société International d'Oncologie Pédiatrique European Neuroblastoma, DCOG: Dutch Childhood Oncology Group, VGPR: very good partial remission, NR: no response, PR: partial remission, HD: high-dose, CEM: carboplatin/etoposide/melphalan; BuMel: busulphan/melphalan, MIBG: Iodine-123 metaiodobenzylguanidine, IL2: interleukin 2, sc: subcutaneously, iv: intravenously, GM-CSF: granulocyte macrophage colony stimulating factor, RA: retinoic acid, EFS: event free survival, yr: year, OS: overall survival. [a] randomization was performed with/without IL2.
Discrepancies within a study between the immunotherapy and control groups are indicated by italic font.

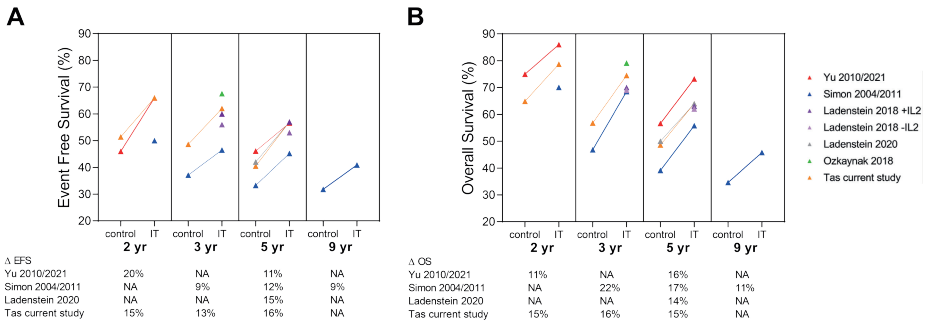

Figure 3 Event free and overall survival according to the literature
Event free survival (EFS; A) and overall survival (OS; B) of published cohorts at different times of follow-up. The X-axis shows the time of follow-up. Within each follow-up moment, the left side shows the EFS or OS of patients treated without anti-GD2 immunotherapy (control) and the right side of patients treated with immunotherapy (IT). When an article contained both patients treated with and without immunotherapy the two groups are connected by a line. Below the graph the difference (Δ) in EFS or OS is given for these articles.
Abbreviations: IT: immunotherapy, yr: year, EFS: event free survival, OS: overall survival, NA: not available.

Interestingly, landmark analysis showed that immunotherapy had a protective effect on EFS after – but not during – maintenance therapy. This suggests that immunotherapy is more powerful in preventing late than early events. This is in line with the studies of the GPOH, who observed a more pronounced effect on long-term survival.[26,27] In contrast, the COG observed a more pronounced effect on short-term survival.[3,14] In our cohort, 68% of patients were treated with the DCOG NBL2004 protocol, which is based on the GPOH NB2004 protocol. Therefore, we wonder if the induction regimens influence this difference in prevention of late and early events. We do, however, not have the power to draw conclusions on this subject.

CONCLUSION

In conclusion, immunotherapy improved outcome by approximately 15% in high-risk neuroblastoma patients, with the greatest clinical benefit for patients ≥18 months. All studies show a sustainable effect. The effect seems most pronounced in prevention of late events, although early and some late events still occur.

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CHAPTER

Discussion and future perspectives

Neuroblastoma is a developmental tumor of neural crest cells. It is a disease of young children and has a broad range of clinical presentations. To better understand neuroblastoma heterogeneity, it is important to realize that neuroblastoma is a developmental tumor of the neural crest. This implies that different spatiotemporal defects during neural crest development result in different tumor subtypes. In addition, the different clinical entities require different treatment strategies. It is therefore safe to say that there remain many challenges and questions in both understanding neuroblastoma biology and treatment.

This thesis focuses on two major topics: **the first part** has a central theme focused on understanding the developmental etiology and biology of neuroblastoma. Can different developmental cells of origin explain the different clinical subtypes of neuroblastoma? Is there a pattern in spontaneous regression? Do all tumorigenic lesions regress completely? Are there features that could explain the indolent course of disease, and can we recognize these patients earlier during treatment?

The second part focuses on progress in the treatment of high-risk neuroblastoma. What are the trends in incidence and survival of Dutch neuroblastoma patients in the past 25 years? Did anti-GD2 immunotherapy affect the survival of Dutch patients?

PART I: EMBRYONIC ORIGIN AND SUBGROUPS OF DISSEMINATED NEUROBLASTOMA

Different cells of origin result in different tumor subtypes

Considering neuroblastoma biology, roughly two major clinical groups can be distinguished with distinctly separated genetic characteristics: the aggressive high-risk tumors and the favorable low-risk tumors. Based on the current knowledge of neural crest development and clinical characteristics, we propose that different *cells of origin* result in different clinical entities. In early embryonic development – as described in **Chapter 2** – the neural crest cells are divided into two major populations: the sympathoadrenal progenitor and the Schwann cell precursor. The sympathoadrenal progenitor will mainly form sympathetic ganglia, while the Schwann cell precursors will mainly form adrenal chromaffin cells. Current knowledge has shown that the Schwann cell precursor and chromaffin progenitors are a source for neuroblastoma tumors arising from the adrenal gland. However, low stage tumors are more often located at the sympathetic side chain.[1] We hypothesize that low-risk tumors arise from sympathoadrenal progenitors and early sympathetic ganglion cells. This hypothesis, which argues that different clinical entities are the result of defects in different developmental stages and *cells of origin* of the neural crest, is the best explanation why these tumor types are developed in different age groups, behave as biologically separate tumor entities with different genetic characteristics.

Metastatic disease with an excellent outcome

In **Chapter 3**, the clinical aspects of regression of stage MS tumors are described. This entity is characterized by infants with a specific pattern of disseminated disease, a high rate of spontaneous regression and an excellent outcome, often without treatment. The standard of care is therefore 'watchfull waiting'. Treatment is only indicated in patients with tumor progression or life threatening symptoms. In these cases treatment is aimed to reduce the rapid tumor growth and accelerated the expected process of spontaneous regression. This study evaluated the outcome, risk factors for fatal outcome and the rate of regression of tumors with and without treatment. An important conclusion of the study was that fast progression of liver metastases was only fatal in patients younger than 4 weeks old. This was due to organ compression (lungs, kidney, large blood vessels) by the enlarged liver due to massive tumor involvement. In addition, the study analyzed biochemical and radiological regression rates. Normalization of catecholamine excretion and liver size was reached in most patients and occurred after a median of two months. Regression of the primary tumor occurred in 69% of patients after a median of 13 months. Regression rates were similar for treated patients and untreated patients. Likewise, a similar proportion of patients reached complete regression. This raises the question what the effect of chemotherapy is on the regression rate or if this is an autonomous process, regardless of treatment administered.

From this study we advise to start early with treatment in children younger than two months, but to try to avert treatment in patients older than three to four months, as no clear benefit of treatment could be established in these patients.

Patients without regression or progression

In **Chapter 4**, we describe a small cohort of patients with metastatic disease, who follow a course of disease distinct from the known clinical patterns. The patients presented with true stage M disease with bone metastases, and had a refractory response to induction chemotherapy. In this historic group, treatment was discontinued and the patients were expected to suffer from tumor progression and to succumb of disease. However, the survival of this cohort was 100% after a median of 17 years of follow-up (range 7-27 years). Analyzing the cases, it appeared to be a group with specific features. Meningeal disease was overrepresented, patients were relatively asymptomatic, and tumor morphology was largely classified as ganglioneuroblastoma. All patients showed a slow metabolic regression, but tumor volume tended to expand over time. From this clinical course, we concluded that, like neuroblastoma stage MS, this clinical subtype is not truly malignant. Interestingly, meningeal metastases were identified in 5/7 patients. As in neuroblastoma stage MS, we questioned if the metastases were true metastases with a preferential homing at the meninges, or multifocal spread of primary tumor nodules. If the latter is true, this could mean that the tumorigenic hit occurred very early in embryonic development, before separation of the cranial and trunk neural crest. From

a clinical point of view, it is important to recognize patients as described in this cohort early, to prevent overtreatment. We advised to perform consecutive tumor biopsies to establish if tumor differentiation has occurred. If tumors have differentiated, treatment can be discontinued.

Germline rearrangement of a pluripotency marker

In **Chapter 5** we describe a patient with a germline aberration affecting *ZFP42*, which we investigated in depth. This patient developed two neoplasms during childhood: a neuroblastoma at the age of 15 months and an atypical cartilaginous tumor at the age of 16 years. ChIP-seq analysis of the germline aberration revealed that a 154kbp region with H3K27ac and H3K4me1 marks, indicative for active transcription, was positioned near the promoter of *ZFP42*. The active transcription was confirmed by expression of *ZFP42* detected by RT-PCR on the cartilage tumor. ZFP42 is a zinc finger protein known as a pluripotency marker for embryonic stem cells and induced pluripotent stem cells.[2,3] ZFP42 functions as a repressor of differentiation by inhibiting the effect of retinoic acid on differentiation.[3] Both neural crest cells and the precursors of chondrocytes (cartilage producing cells) depend on retinoic acid for differentiation. In addition, retinoic acid is used in the current treatment protocols of high-risk neuroblastoma. We could not proof that the alteration was a driver mutation of one or both of the tumors. However, recent research show that about 8% of childhood cancer is related to germline alterations, and that this could be an underestimation.[4,5] It is possible that this genetic alteration and the development of two tumors in one patient at early age is all connected within a developmental syndrome.

PART II: PROGRESS IN THE TREATMENT OF (HIGH-RISK) NEUROBLASTOMA

Increasing incidence and survival of neuroblastoma patients

In **Chapter 6**, the incidence and survival of all Dutch neuroblastoma patients diagnosed between 1990 and 2014 is analyzed. An increased incidence of 1.6% per year for all stages of neuroblastoma was observed. Further analysis showed that this was mostly due to an increase in subgroup of children >18 months with stage 4/M disease. Biological and environmental risk factors for neuroblastoma remain unknown or unlikely and there is no clear explanation for the increased incidence in this high-risk subgroup.

Five year overall survival improved for all ages and stages. The largest improvement was made for patients ≥18 months with stage 4/M neuroblastoma. In this patient group, the 5-yr overall survival improved from 6±4 to 43±7%. Multivariable analysis showed that high-dose chemotherapy followed by stem cell rescue and immunotherapy

were the treatment modalities associated with the improved survival over time. This corresponds with earlier research performed by Berthold et al. and Pinto et al. on the effect of the high-dose chemotherapy.[6,7] Despite the major increase in survival of stage 4 patients older than 18 months at diagnosis, still 57% of patients die of neuroblastoma or treatment. This urges the need to further adjust therapy to ultimately improve outcome of high-risk neuroblastoma patients. The major question is what strategy should be taken to obtain this.

Increased long-term survival for Dutch patients treated with ch14.18 anti-GD2 antibodies

In 2010, Yu et al. showed the efficacy of anti-GD2 based maintenance immunotherapy in high-risk neuroblastoma in a randomized controlled trial. Anti-GD2 antibody (dinutuximab) in combination with IL-2, GM-CSF and isotretinoin improved the 2-year event free survival and overall survival by 20% and 11%, respectively, compared to isotretinoin alone. After publication of these results anti-GD2 immunotherapy was incorporated in all international high-risk neuroblastoma protocols. However, shortly after publication, the treatment combination was not approved by the European Medicines Agency (EMA). Between 2009 and 2016, all eligible Dutch patients received this treatment in the Children's Hospital of Philadelphia. This was a unique cohort, treated with the DCOG/GPOH or POG based induction treatment in the Netherlands, and immunotherapy with the COG protocol. In **Chapter 7,** we investigated the effect of anti-GD2 antibody based immunotherapy for Dutch patients. An improved overall and event free survival of approximately 15% was observed after both 2 and 5 years. For patients older than 18 months at diagnosis, 5-yr overall survival improved even by 24%. Landmark analysis revealed that immunotherapy was more effective in preventing late relapses than early relapses. This Dutch cohort study is the first and only confirmation of the efficacy of this immunotherapy combination outside the COG study. It corroborates the Dutch Children's Oncology Group (SKION) early decision to include anti-GD2 based immunotherapy in the standard treatment for high-risk neuroblastoma as early as 2009. For anti-GD2 immunotherapy it is important to investigate strategies to reduce toxicity. Also new strategies to use the GD2 epitope are in development to further improve survival.

Future directions

Tumor biology and evolution

The field of developmental biology and tumor biology has been moving fast forward by using single cell analyses, characterization of super-enhancer profiles and genome wide epigenetic chromatin analyses.[8-13] This has opened avenues for research into tumor heterogeneity and tumor evolution. In 2017, Van Groningen et al.[14] and Boeva et al.[15] discovered two cell states, an adrenergic and a mesenchymal, in neuroblastoma tumors and cell lines. Tumor cells were able to switch between these states *in vitro*. In 2017,

Furlan et al.[10] identified, using genetic cell lineage tracing techniques, that Schwann cell precursors are the major ancestor of the adrenal chromaffin cells. They identified a 'bridge' cell state between the Schwann cell precursor and the chromaffin state. Based on these findings, many research groups are currently investigating if the Schwann cell precursors resemble mesenchymal neuroblastoma and if the more differentiated chromaffin cells resemble the adrenergic neuroblastoma cell state. In addition, the switch between the cell states is investigated in depth to better understand chemotherapy resistance, tumor heterogeneity as well as identifying factors influencing heterogeneity and tumor evolution and progression. Ultimately, better understanding of the malignant processes of individual tumors will enhance tumor specific treatment strategies, in order to minimize toxicity and maximize response.

Strategies for further improvement of survival in high-risk neuroblastoma

The mission statement of the Princess Máxima Center, "to cure every child with cancer with an excellent quality of life" resonates in the need for neuroblastoma research and improvement of outcome. Survival rates of high risk neuroblastoma patients have increased in the past 25 year, but do not exceed 50%. New strategies are needed to further increase the cure rate. Several possible routes and strategies lie in front of us to make choices from:

1. Induction treatment can be intensified by adding targeted treatment in biomarker positive patients. Patients with ALK mutations will potentially benefit from adding an ALK-inhibitor during induction. This is currently being investigated in the COG phase III high-risk treatment protocol (NCT03126916) and will be included in the SIOPEN high-risk study (NCT04221035). Introduction of anti-GD2 antibodies during induction is another opportunity. Phase II studies in both relapsed/refractory and newly diagnosed patients demonstrated that the combination of anti-GD2 and chemotherapy was tolerable and seemed effective.[16,17]
2. Consolidation treatment can be intensified by adding a second high-dose treatment. In the United States of America, tandem transplant has been studied, in which two courses of high-dose chemotherapy followed by autologous stem cell transplantations were performed.[18] This was feasible, and improved 3-yr EFS by 14% compared to single transplant. In Europe, the SIOPEN focusses on identifying ultra-high risk patients based on insufficient response after induction chemotherapy. These patients undergo a double transplant via the VERITAS study (NCT03165292) to investigate the best intensification of treatment. Patients are randomized between double transplants according to two different regimens ^{131}I-MIBG + BuMel or Tiothepa + BuMel.
3. Immunotherapy has revolutionized cancer treatment. However, high-risk neuroblastoma tumors are immunogenic cold tumors, with low expression of MHC I antigens, with a low mutational burden and few neoantigens.[5,19] For better results with immunotherapies in neuroblastomas these tumors need to be transformed to immunogenic hot tumors. CAR-T cell strategies are promising for solid tumors and are in development for neuroblastoma.[20] A first phase 1 study showed that CAR-T cells

against GD2 are well tolerated and have no on-target off-tumor toxicity. This study did, however, not show objective clinical responses.[21] A second phase 1/2 study is currently being conducted (NCT03373097). Preliminary results of this study show at least partial response in 14 of 22 patients (64%) (data not published). In addition, strategies are being developed to incorporate the anti-GD2 antibody in combination with other immunotherapies such as checkpoint inhibitors, to optimize the immune response.[16,22]

4. For relapsed and refractory patients many national and international programs are active, which profile tumors on CGH-arrays, and WES, NGS and RNA sequencing platforms. These programs are aimed to identify actionable events to refer the patients subsequently to the best befitted precision medicine studies. This provides the patients the best chance on tumor response, and this way the drugs are being tested in the population for whom they have been developed.

The rarity of neuroblastoma, the different clinical/genetic subgroups and the scarcity of drugs being developed for pediatric use, hampers the fast implementation of new treatment (strategies) in neuroblastoma and other pediatric malignancies. The European Innovative Therapies for Children with Cancer (ITCC) consortium has launched the Neuroblastoma New Drug Development Strategy (NDDS) to accelerate the development of new drugs for neuroblastoma.[23] The NDDS prioritizes targets based on (pre-)clinical data and mechanisms of action and promotes collaborations between academia, pharmaceutical companies, regulatory bodies and patient advocates. The NDDS urges the need to start as early as possible with testing of combinations based on mechanisms of action in early phase clinical trials.

CONCLUSION

Neuroblastoma is a developmental tumor of the sympathetic nervous system. Despite this being well established, the precise *cell of origin* remains unknown. Recent developments have brought insight in the *cell of origin*. This thesis underlines that preclinical research for *cell of origin* can highly benefit from clinical observations and studying exceptional patient entities.

The treatment of high risk patients has greatly benefitted from recent advances, such as high dose chemotherapy and immunotherapy. In the future, patient specific additional treatment based on in-depth tumor analysis can increase the outcome of individual patients. Fundamental understanding of tumorigenesis and tumor evolution is needed to develop more targeted treatment to improve survival and quality of life for patients with neuroblastoma.

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CHAPTER

English summary &
Nederlandse samenvatting

ENGLISH SUMMARY

Neuroblastoma is a common pediatric malignancy of the developing autonomic nervous system. It affects mostly young children in the first years of life. In the Netherlands, 25-30 new patients are diagnosed each year. The clinical course is variable, ranging from spontaneous regression to highly aggressive chemoresistant tumors. Although different subgroups can be recognized based on clinical and biological features, the cause of variability is poorly understood. This thesis has a focus on two main topics: 1) As is true for many pediatric malignancies, the cell of origin remains elusive in neuroblastoma. We aimed to understand the connection between the normal neural crest development of the autonomous nervous system and the clinical appearance of different neuroblastoma subgroups. 2) We studied the progress in neuroblastoma treatment, especially for high risk patients.

Part I: embryonic origin and subgroups of disseminated neuroblastoma

Neuroblastoma is a developmental tumor of the neural crest. During embryonic development, neural crest cells proliferate and migrate towards their target tissues and undergo differentiation into among others sympathetic ganglia and adrenal chromaffin cells, the most common organs affected by neuroblastoma. During development, (precursors of) neuroblastoma tumors arise. However, the precise cell-of-origin of neuroblastoma remains unknown, and it is unclear if all neuroblastoma subtypes arise from the same precursor cell. In the first part of the thesis we searched for similarities between normal development and neuroblastoma genesis in specific subgroups of patients. The first subgroup we describe – in **Chapter 3** – are patients with stage MS ('metastatic special') neuroblastoma. This is a specific subgroup of patients aged younger than 18 months at diagnosis, with metastatic spread limited to liver, bone marrow (less than 10% invasion) and skin. This subgroup is special, because spontaneous tumor regression is expected, despite the metastatic spread at diagnosis. However, metastatic nodules in the liver can be fast proliferating causing life threatening enlargement of the liver. In a cohort of 32 patients, we observed that fast growth of metastatic tumor nodules was only fatal in patients younger than 4 weeks old at diagnosis, these patients were at risk of multi-organ failure due to pressure of the enlarged liver on the lungs, internal organs and blood vessels. Next we investigated (spontaneous) regression of the stage MS tumors. Strikingly, regression velocity and complete regression rates were similar for treated patients and untreated patients. This raised the question what the effect of chemotherapy is on the regression rate or if this is an autonomous process, regardless of treatment administered. We wonder if the tumor spread with subsequent spontaneous regression in patients with MS neuroblastoma is truly malignant and metastatic, or if it multifocal disease, originating from a very early developmental defect of non-malignant cells.

The second subgroup we describe – in **Chapter 4** – are 7 patients with stage M disease, who had a course of disease distinct from the known clinical patterns. They had a refractory response to induction chemotherapy and treatment was stopped. Disease progression with fatal outcome was expected. However, fast tumor progression did not occur. Tumor volumes tended to expand slowly over time, but, unexpectedly, the patients showed a slow metabolic regression. We hypothesized that, like neuroblastoma stage MS, this clinical subtype is not truly malignant, but an aberration of normal development. In one of these patients we identified a germline alteration involving *ZFP42*, studied in depth in **Chapter 5.** The patient described developed two malignancies during childhood, both with an indolent course of disease. The germline aberration seemed to activate *ZFP42*, which is normally repressed in the first few days after conception. ZFP42 is a pluripotency marker and a repressor of retinoic acid induced differentiation. The role of ZFP42 in very early embryonic development could again suggest that in this patient, a very early tumorigenic hit led to a subsequent indolent clinical course.

In **Chapter 2,** we reviewed recent literature on embryonic neural crest development in light of the different neuroblastoma subtypes. For long, chromaffin cells and sympathetic neurons were thought to be the pedigree of a shared sympathoadrenal progenitor. However, recently developed single cell tracing and RNA techniques have shown that the majority of adrenal chromaffin cells do not arise from the sympathoadrenal progenitor, but from the Schwann cell precursor. In contrast, the majority of sympathetic neurons arise from the sympathoadrenal progenitor. This model with not one – the sympathoadrenal progenitor – but two progenitor cell types – the sympathoadrenal progenitor and the Schwann cell precursor – changed the view on neural crest development and is changing the view on neuroblastoma tumorigenesis. In neuroblastoma, two major subtypes can be recognized: 1) the aggressive high-risk tumors, which present in older patients, with mainly adrenal primary tumors, metastatic spread at diagnosis, and segmental chromosomal aberrations; 2) the favorable low-risk tumors, which present in young patients, with mainly thoracic primary tumors, localized at diagnosis or have tumor spread according to the stage MS pattern. From this marked difference in progenitor cells, and clinical subtypes, we hypothesized that low-risk tumors are a deviation of normal development arising from sympathoadrenal progenitors and early sympathetic ganglion cells, while high-risk tumors are truly malignant tumors arising from Schwann cell precursors and early chromaffin cells. We believe this model can explain why these tumor types are developed in different age groups, behave as biologically separate tumor entities with different genetic characteristics.

Part II: progress in the treatment of (high-risk) neuroblastoma

A major challenge in treating neuroblastoma is to further improve survival of high-risk patients. Survival rates are currently estimated around 50%. Incidence and outcome of all NBL patients between 1990-2014 was studied in **Chapter 6**. We showed that five year overall survival for patients ≥18 months at diagnosis with stage 4/M neuroblastoma

improved from 6±4 to 43±7% between 1990 and 2014. This improvement could largely be explained by the introduction of high-dose chemotherapy and immunotherapy. In this period, incidence increased with 1.6% per year for all stages and with 3.3% per year for high-risk patients. No clear explanation for the increased incidence could be identified.

To investigate the efficacy of immunotherapy in depth, we compared – in **Chapter 7** – patients treated with immunotherapy to historical controls. Both patient groups received the same induction and consolidation regimen, but maintenance was either according to the ANBL0032 or single-agent isotretinoin. A 15% difference in overall and event free survival in favor of the immunotherapy group was observed. For patients older than 18 months at diagnosis, 5-yr overall survival improved even by 24%. Landmark analysis revealed that immunotherapy was more effective in preventing late events than early events. In the future, new anti-GD2 strategies need to be investigated in order to reduce toxicity. Also new strategies and combination using the GD2 epitope are in development to further improve survival of high-risk neuroblastoma patients.

This thesis contributed to the current knowledge on the origin of neuroblastoma during embryonic development, and on the correlation of tumorigenesis and clinical subgroups. In addition, it corroborated the effect of the ANBL0032 immunotherapy study in a cohort treated with non-COG induction protocols, and on an epidemiological level. Future research using single cell analyses could further increase the knowledge on the sympathoadrenal progenitor and Schwann cell precursor as precursors of low-risk and high-risk tumors, as well as the adrenergic and a mesenchymal cell state, found in neuroblastoma tumors and cell lines. With better understanding of these mechanisms, chemotherapy resistance, tumor heterogeneity and tumor evolution/progression could be clarified. Ultimately, better understanding of the malignant processes of individual tumors will enhance tumor specific treatment strategies, minimizing toxicity and maximizing response.

NEDERLANDSE SAMENVATTING

Neuroblastoom is een veelvoorkomende vorm van kinderkanker. Neuroblastomen ontstaan tijdens de ontwikkeling uit neurale lijst cellen van het sympathische zenuwstelsel. Het sympathische zenuwstelsel is onderdeel van het autonome zenuwstelsel. Het autonome zenuwstelsel regelt de onbewuste lichaamsprocessen zoals spijsvertering, ademhaling, hartslag enzovoort. Het bestaat uit de chromaffinecellen in de bijnier en zenuwcellen in de zogenaamde grensstrengen aan weerszijden van de wervelkolom vanwaar autonome zenuwvezels lopen naar darmen, longen, hart, etc.

Het neuroblastoom is een ziekte die zich vooral in de eerste levensjaren presenteert. In Nederland worden elk jaar bij 25-30 patiënten een neuroblastoom gediagnosticeerd. Het klinisch beloop is zeer variabel, met een spreiding van spontane tumorregressie tot zeer agressieve tumoren die resistent zijn voor chemotherapie. Hoewel ten tijde van de diagnose al verschillende patiëntgroepen kunnen worden herkend op basis van klinische en biologische karakteristieken, blijft de oorzaak van de variabiliteit onduidelijk. Dit proefschrift focust zich op twee hoofdonderwerpen: 1) De precieze voorlopercel van neuroblastoomcellen is, net als bij de meeste vormen van kinderkanker, nog niet vastgesteld. Wij hebben daarom de normale neurale lijst ontwikkeling van het autonome zenuwstelsel en verschillende neuroblastoom-subgroepen naast elkaar gelegd, op zoek naar verbanden. 2) De behandeling van neuroblastomen wordt steeds verder ontwikkeld. Wij hebben onderzocht welke veranderingen in de therapie het meeste effect hebben gehad, met extra aandacht voor de behandeling van patiënten met een hoog-risico neuroblastoom.

Deel 1: embryonale oorsprong en subgroepen binnen gemetastaseerd neuroblastoom

Neuroblastoom is een ontwikkelingstumor van de neurale lijst. Tijdens de embryonale ontwikkeling vermenigvuldigen en verplaatsen de neurale lijst cellen zich richting hun eindorganen, waar zij uitrijpen in onder andere sympathische zenuwcellen (neuronen) van de sympathische grensstreng en chromaffinecellen (cellen die de stresshormonen adrenaline en noradrenaline maken) van de bijnier. De bijnier en grensstreng zijn de organen waar de meeste neuroblastomen gevonden worden. Het neuroblastoom is een ontwikkelingstumor, dat wil zeggen dat deze ontstaat tijdens de embryonale ontwikkeling van het autonome zenuwstelsel. Het is echter onbekend vanuit welk stadium van ontwikkeling of welke voorlopercellen het neuroblastoom ontstaat. Ook is het onduidelijk of alle neuroblastoomsubgroepen van dezelfde voorloper afstammen. In het eerste deel van dit proefschrift onderzoeken we de gelijkenissen tussen de normale ontwikkeling en het ontstaan van neuroblastomen in specifieke patiëntgroepen. De eerste patiëntgroep die we beschrijven – in **Hoofdstuk 3** zijn patiënten met stadium MS (‘metastatisch speciaal’) neuroblastoom. Dit is een specifieke groep van kinderen met een leeftijd onder de 18 maanden en uitgezaaide ziekte die zich beperkt tot lever,

huid en beenmerg (<10% invasie). Het bijzondere aan deze subgroep is dat spontane regressie van de tumor en uitzaaiingen vaak wordt waargenomen. Hoewel er regressie verwacht wordt, zijn er wel levensbedreigende complicaties mogelijk door in eerste instantie snelle groei van de tumorbollen, met name in de lever die hierdoor erg groot kan worden. In een cohort van 32 patiënten zagen wij dat de snelle progressieve groei van levermetastasen alleen fataal was voor kinderen jonger dan 4 weken. Deze kinderen lopen risico op multi-orgaanfalen door druk van de vergrote lever op de longen, interne organen en bloedvaten. Vervolgens hebben we onderzocht hoe de (spontane) regressie van stadium MS tumoren verloopt. Opvallend was dat de snelheid waarmee de tumoren afnamen gelijk was tussen de behandelde en onbehandelde patiënten. Het is daardoor onbekend wat het effect van chemotherapie is op de regressiesnelheid of dat dit een autonoom proces is, waarop (chemo)therapie geen invloed heeft. Van het intrigerende beeld van uitgezaaide ziekte die spontaan verdwijnt, vragen wij ons af of dit daadwerkelijke uitgezaaide ziekte is, of dat dit multipele goedaardige tumoren zijn, afkomstig van een zeer vroeg ontwikkelingsdefect van niet kwaadaardige cellen die op meerdere plaatsen in het lichaam terecht komen tijdens de ontwikkeling.

De tweede patiëntgroep die we beschrijven in **Hoofdstuk 4** zijn 7 patiënten met stadium M neuroblastoom. Deze patiënten hadden een ziektebeloop dat niet past bij het bij stadium M passende klinische patronen van hoog-risico patiënten. Hun tumoren waren ongevoelig voor de inductie chemotherapie en de behandeling werd – volgens destijds geldende therapie stratificatie – afgebroken. Ziekteprogressie met fatale afloop werd verwacht, maar de verwachte snelle tumorprogressie bleef uit. Het volume van de primaire tumoren nam wel langzaam toe, maar tegelijkertijd werd er een langzame metabole regressie gezien. Onze hypothese is dat deze tumoren, net als stadium MS tumoren, niet daadwerkelijk maligne en uitgezaaid zijn, maar een afwijking van de normale ontwikkeling. In één van deze patiënten ontdekten wij een kiembaanafwijking die betrekking had op het *ZFP42* gen. Deze afwijking en dit gen hebben wij nauwkeuriger onderzocht in **Hoofdstuk 5**. De beschreven patiënt ontwikkelde naast het neuroblastoom ook een kraakbeentumor op de kinderleeftijd, beide met een langzaam en mild beloop. De kiembaanafwijking lijkt *ZFP42* te activeren, een gen dat normaal gesproken enkele dagen na de conceptie wordt onderdrukt. *ZFP42* wordt daarom gebruikt om pluripotente cellen te herkennen. Het onderdrukt de differentiatie die door vitamine A-zuur ingezet wordt. Dat *ZFP42* zo belangrijk is in de hele vroege embryonale ontwikkeling, kan suggereren dat er in deze patiënte de eerste stap in de tumorontwikkeling ook vroegembryonaal heeft plaatsgevonden, met vervolgens een mild ziektebeloop.

In **Hoofdstuk 2** hebben we de recente literatuur over de embryonale ontwikkeling van de neurale lijst en het autonome zenuwstelsel bekeken met het oog op verschillende neuroblastoom subtypen. Lange tijd werd aangenomen dat chromaffinecellen en sympathische zenuwcellen afstammen van een gezamenlijke sympathoadrenale voorloper. Verschillende internationale onderzoeksgroepen hebben analyses van foetaal weefsel verricht met behulp van RNA-sequentie van iedere losse cel en de expressie (activiteit) van alle genen af te lezen en om losse cellen gedurende

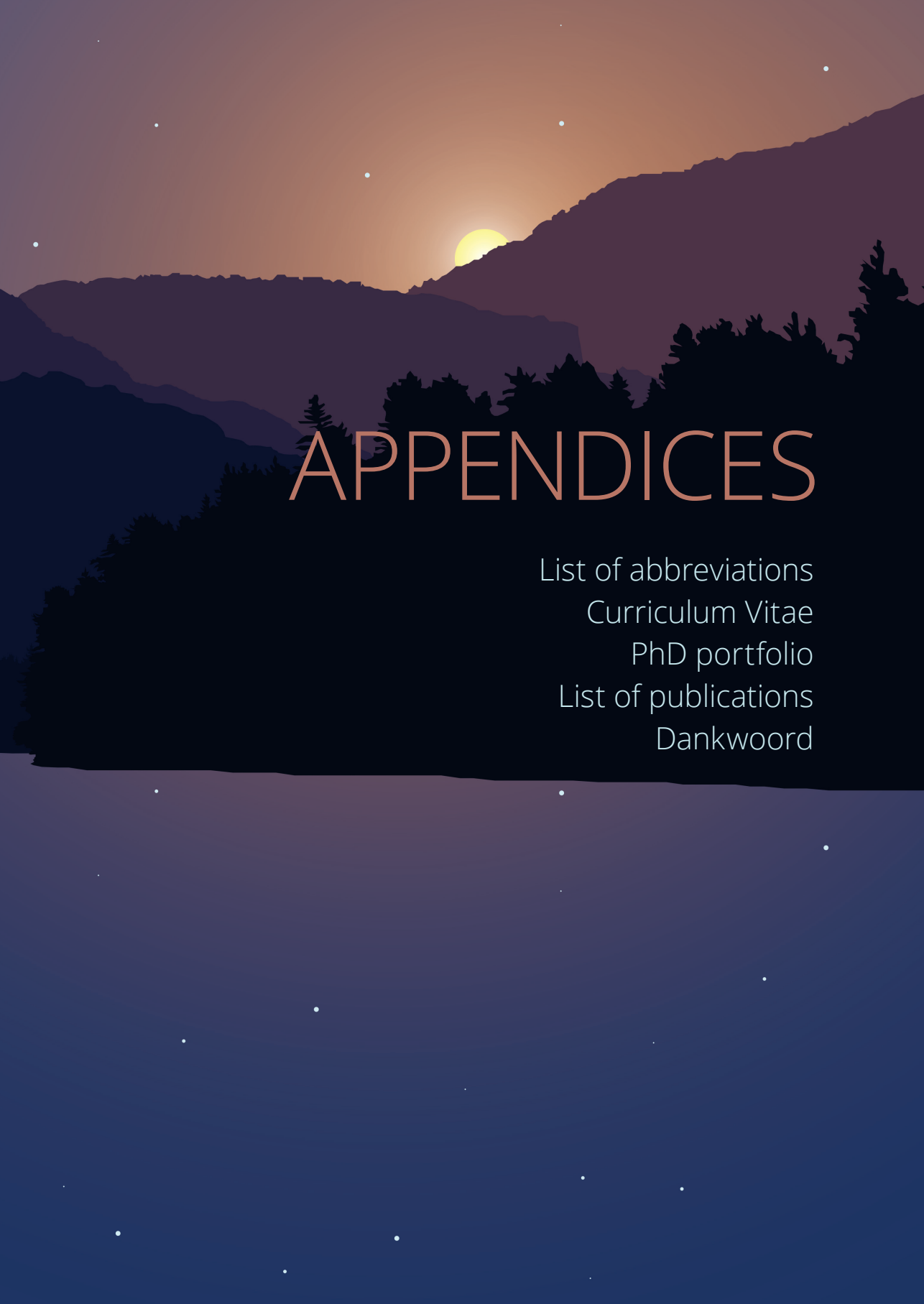
embryonale ontwikkeling te volgen (zogenaamde 'single cell RNA sequencing' en 'single cell tracing'). Hiermee is ontdekt dat het merendeel van de chromaffinecellen in de bijnier niet afstammen van de sympathoadrenale voorloper, maar van de Schwann cel voorloper. Het merendeel van de zenuwcellen in de sympathische grensstreng stamt echter wel af van de sympathoadrenale voorloper, zoals reeds werd aangenomen. Dit ontwikkelingsmodel met niet één – de sympathoadrenale voorloper – maar twee – de sympathoadrenale voorloper en de Schwann cel voorloper – voorlopercellen heeft de visie van de ontwikkeling van het autonome zenuwstelsel veranderd en verandert het beeld van het ontstaan van neuroblastomen. Binnen neuroblastomen kunnen grofweg twee subgroepen onderscheiden worden: 1) de agressieve hoog-risico tumoren, die zich presenteren in oudere kinderen, met bijniertumoren, en uitgezaaide ziekte bij diagnose. Deze tumoren hebben vaak breuken in de chromosomen en genafwijkingen zoals het bekende amplificatie van MYCN; 2) de milde laag-risico tumoren, die zich presenteren in jonge kinderen, vaak met tumoren in de borstholte en bij wie er geen uitzaaiingen zijn ten tijden van diagnose, of uitzaaiingen volgens het stadium MS patroon. Deze tumoren bevatten meestal geen breuken in de chromosomen, en alleen een toename of afname van hele chromosomen. Met dit opvallende verschil in voorlopercellen en klinische subgroepen, vragen wij ons af of laag-risico tumoren wel echt kwaadaardige tumoren zijn of dat ze merendeels laag-maligne of goedaardige varianten zijn van de normale neurale lijst ontwikkeling, ontstaan uit sympathoadrenale voorlopercellen of vroege sympathische neuronen. Hiernaast bestaan dan de hoog-risico kwaadaardige tumors, die zich ontwikkelen uit Schwann cell voorlopers en vroege bijniercellen. Een model met twee verschillende voorlopers zou kunnen verklaren waarom deze tumortypen zich ontwikkelen in verschillende leeftijdsgroepen, waarom ze zich biologisch anders gedragen en waarom ze verschillende genetische profielen hebben.

Deel 2: vooruitgang in de behandeling van (hoog-risico) neuroblastoom

Een belangrijke uitdaging in de behandeling van patiënten met een neuroblastoom is om de genezingskans van hoog-risico neuroblastoom te verbeteren. Op dit moment geneest ongeveer 50% van de patiënten. In **Hoofdstuk 6** hebben we de incidentie en overleving bestudeerd van alle patiënten met een neuroblastoom die in Nederland gediagnosticeerd zijn tussen 1990 en 2014. In deze periode nam de 5-jaar overleving van patiënten ouder dan 18 maanden met een stadium M tumor (hoog-risico patiënten) toe van 6 naar 43%. Deze stijging is met name toe te schrijven aan de introductie van hoge-dosis chemotherapie en immunotherapie. Tegelijkertijd werd er een stijging gezien van de incidentie van 1,6% per jaar voor alle leeftijdscategorieën en een stijging van 3,3% per jaar voor patiënten met het ongunstigste profiel (stadium M en ouder dan 18 maanden bij diagnose). Voor de stijging in incidentie kon geen eenduidige verklaring gevonden worden. Om de effectiviteit van immuuntherapie beter te bekijken, hebben we – in **Hoofdstuk 7** patiënten die immuuntherapie kregen vergeleken met historische controle patiënten. Beide groepen ontvingen dezelfde inductie- en consolidatietherapie, maar de

onderhoudsbehandeling bestond óf uit immuuntherapie volgens het ANBL0032 protocol óf uit monotherapie met isotretinoïne (de historische standaard therapie). Patiënten die immuuntherapie ontvingen bleken een 15% hogere genezingskans te hebben. Voor de patiënten ouder dan 18 maanden was dit verschil zelfs 24%. Aanvullende analyse toonde dat immuuntherapie krachtiger was in het voorkómen van recidieven of ziekteprogressie na het afronden van de therapie dan gedurende therapie. In de toekomst zal er gekeken moeten worden naar verbeterde strategieën om anti-GD2-antilichamen toe te passen, om de bijwerkingen te verminderen. Ook kunnen nieuwe strategieën gericht zijn op het verbeteren van de effectiviteit van anti-GD2 voor patiënten met een hoog-risico neuroblastoom.

Dit proefschrift draagt bij aan de kennis over het ontstaan van neuroblastomen tijdens de embryonale ontwikkeling en over de verbanden tussen tumorontwikkeling en klinische subgroepen. Daarnaast hebben we de effectiviteit van anti-GD2 immuuntherapie volgens het ANBL0032 bevestigd op epidemiologisch niveau en in een cohort dat andere inductiechemotherapie heeft gehad dan de originele studie. Het opent ook de deur voor toekomstig onderzoek naar, bijvoorbeeld de rol van de sympathoadrenale voorloper en de Schwann cell voorloper als grondleggers van laag- en hoog-risico tumoren. Wanneer deze mechanismen beter begrepen worden, zal ook de resistentie tegen chemotherapie, tumorheterogeniteit, tumorevolutie en –progressie beter onderzocht en begrepen worden. Uiteindelijk zal met meer kennis van kwaadaardige processen in samenhang met de embryonale oorsprong van tumor kunnen leiden tot een effectievere, persoonlijkere, en minder toxische behandeling voor patiënten met een neuroblastoom.



APPENDICES

List of abbreviations
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LIST OF ABBREVIATIONS

3MT	3-methoxytyramine
$^{123/131}$I	Iodine-123/131
^{18}FDG	Fluor-18 fluorodeoxyglucose
AAPC	Average annual percentage change
ASCT	Autologous stem cell transplantation
COG	Children's Oncology Group
CNS	Central nervous system
CNV	Copy number variation
CR	Complete response
CRC	Core regulatory circuit
CT scan	Computed tomography scan
DCOG	Dutch Childhood Oncology Group
DRG	Dorsal root ganglia
EFS	Event free survival
EMA	European Medicines Agency
EMT	Epithelial to mesenchymal transition
EoD classification	Extent of Disease classification
FFPE	Formalin-fixed paraffin-embedded
FISH	Fluorescent in situ hybridization
GD2	Ganglioside 2
GN	Ganglioneuroma
GNB	Ganglioneuroblastoma
GNBi	Ganglioneuroblastoma intermixed
GNBn	Ganglioneuroblastoma nodular
GPOH	Gesellschaft für Pädiatrische Onkologie und Hämatologie
GWAS	Genome wide association study
HR	Hazard ratio
HR-NBL	High-risk neuroblastoma
HVA	Homovanillic acid
ICD-O system	International Classification of Diseased for Oncology system
IDRF	Image defined risk factors
IKNL	Netherlands Comprehensive Cancer Organization
INPC	International Neuroblastoma Pathology Classification
INRC	International Neuroblastoma Response Criteria
INRG(SS)	International Neuroblastoma Research Group (Staging System)
INSS	International Neuroblastoma Staging System
IT	Immunotherapy
ITCC consortium	Innovative Therapies for Children with Cancer consortium

MIBG	Metaiodobenzylguanidine
MRI	Magnetic resonance imaging
MS	Metastatic special
NB	Neuroblastoma
NC	Neural crest
NCA	Numerical chromosomal aberrations
NCC	Neural crest cell
NCR	Netherlands Cancer Registry
NDDS	Neuroblastoma New Drug Development Strategy
OS	Overall survival
PCP	Planar cell polarity
PCR	Polymerase chain reaction
PD	Progressive disease
PET scan	Positron emission tomography scan
PR	Partial response
RA	Retinoic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SA progenitor	Sympathoadrenal progenitor
SCA	Segmental chromosomal aberrations
SCP	Schwann cell precursor
SIOPEN	Société International d'Oncologie Pédiatrique European Neuroblastoma
SKION	Stichting Kinderoncologie Nederland
SNP	Single nucleotide polymorphism
SPECT	Single-photon emission computed tomography
VGPR	Very good partial response
VMA	Vanillylmandelic acid
WES	Whole exome sequencing
WGS	Whole genome sequencing
WSR	World standardized incidence rate

CURRICULUM VITAE

Michelle Tas was born in Capelle aan den IJssel in The Netherlands on June 25th 1991. She was raised in Rotterdam until the age of 14, when her family moved to Middelburg. In 2009, she graduated cum laude from the Stedelijke Scholengemeenschap Nehalennia, Middelburg. After her graduation she started medical school at the Erasmus University, Rotterdam. In the first year, she was selected to join the Honours Class, in which she was introduced to medical research. She combined her medical study with working at the Hematology ward of the Erasmus MC, where her interest for working with critically ill patients was aroused. During the minor Pediatric Oncology in 2011, she started performing research under the supervision of Max van Noesel and after the minor she continued as a research student, assisting the research of Kathelijne Kraal.

In the academic year 2013/2014 she was a fulltime board member of the medical students association Rotterdam (MFVR). In this year she collaborated with the educational office of the Erasmus MC to improve the Medical Education Program.

During the last 5 months of the Master in Medicine, she worked as an intern at the clinical ward of the Princess Máxima Center for Pediatric Oncology. During this internship she became determined she wanted to be a pediatrician. After finishing medical school in 2016, she started her PhD project at the Princess Máxima Center for Pediatric Oncology under the supervision of prof. dr. M.M. van Noesel, prof. dr. J.J. Molenaar and dr. G.A.M. Tytgat. During this PhD project she supervised two medical students and she combined her work as PhD student with organizing different retreats. She joined the PriMá PhD group and started a buddy system to help start first year PhD students. As of July 2020 she works as a resident in pediatrics in the Jeroen Bosch Ziekenhuis in ‘s Hertogenbosch.

PHD PORTFOLIO

Name:	Michelle Tas
PhD period:	November 2016 – May 2020
Research School:	Clinical Translational Oncology (Utrecht University, Graduate school of life sciences)
Department	Pediatric Oncology (Princess Máxima Center for Pediatric Oncology)
Promotors	Prof. dr. M.M. van Noesel Prof dr. J.J. Molenaar
Co-promotor	Dr. G.A.M. Tytgat

1. PhD training

	Year	ECTS
General courses		
Introductory Biostatistics for Researchers, GSLS/UMC Utrecht	2017	4.5
Academic Writing in English, GSLS	2017	2.0
BROK, UMC Utrecht	2018	1.0
Supervision of Master's Students, GSLS	2018	0.6
Adobe InDesign Essentials, GSLS	2018	0.6
Specific courses		
Research in the Princess Máxima Center for Pediatric Oncology, CTO	2016	0.3
Clinical Trial and Development, CTO	2017	1.5
Clinical Translational Oncology Introductory Course, GSLS	2018	1.0
Epigenetic Regulation in Health and Disease, GMC	2018	0.8
Advanced Molecular Pathology, CTO	2019	1.5
Translational immune-oncology: cancer & immune therapies from bench to bedside, CTO	2019	1.5
Seminars and Workshops		
PhD retreat Clinical Translational Oncology	2017-2019	0.9
PhD Day GSLS	2017-2019	0.9
Research Meetings Princess Máxima Center	2016-2020	4.5
Research Seminars Princess Máxima Center	2019-2020	1.5
Conferences		
49th Annual Congress of the International Society of Paediatric Oncology (SIOP), Washington DC (oral presentation)	2017	1.2
1st SIOPEN/GPOH Neuroblastoma Research Symposium &SIOPEN Annual General Meeting, Berlin	2017	0.9

Advances in Neuroblastoma Research Association (ANR) Conference, San Francisco (rapid fire pitch, poster presentation)	2018	1.2
Princess Máxima Center, international scientific symposium	2018	0.6
51th Annual Congress of the International Society of Paediatric Oncology (SIOP), Lyon (poster presentations)	2019	1.2
Advances in Neuroblastoma Research Association (ANR) 2021 Conference, Amsterdam	2021	1.0

2. Teaching activities

Supervision master student (Erasmus University, Medicine) – 6 months fulltime	2018	2.0
Supervision master student (Utrecht University, Medicine) – 3 months fulltime	2019-2020	1.0

3. Other activities

Organization Research Retreat Molenaar/Tytgat research groups	2018	1.0
Research Retreat Committee, Princess Máxima Center	2019	0.5
Organization PhD retreat Clinical Translational Oncology	2019	1.0
PriMá PhD group, member	2019-2020	0.5
PriMá PhD group, PhD Buddy System	2019-2020	1.5

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This thesis

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Other projects

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DANKWOORD

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mijn western blot skills kunnen perfectioneren t/m het gieten van eigen gelen aan toe. En wat kon ik α-tubuline prachtig in beeld krijgen... Wat heb ik een respect voor jullie engelengeduld met alle labexperimenten en hoe die keer op te perfectioneren.

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Lieve **Evelyn**, wat ben jij een topper. Je weet elk onderzoek met je kritische feedback te verbeteren. Iedere groep verdient een Evelyn te hebben. Je hebt daarbij ook nog eens een hele lieve persoonlijkheid. Ik wens je heel veel geluk in je verdere carrière en met je gezinnetje. Samen gaan we de review nog tot een succesvol einde brengen en daarna genieten van ons PhD-pensioen en vooral leuke dingen samen doen.

Ardine, waar wij in twee verschillende uithoeken van het Máxima zaten heeft het lot ons aan elkaar weten te koppelen. Jij met jouw epidemiologische kennis, ik met mijn neuroblastoom kennis. Samen hebben we een mooi overzicht kunnen maken van de incidentie en genezing van neuroblastoompatiënten in de afgelopen 25 jaar. Daarnaast ben ik blij dat ik jou persoonlijk heb leren kennen en kijk ik met veel plezier terug op het SIOP congres in Lyon.

Natanja en Janna, jullie waren mij net voorgegaan met de start van jullie PhD-trajecten. Dank voor jullie opvang in het Máxima, en het wegwijs maken in de PhD-wereld en alle dingen die daarbij komen kijken. Dat waren er meer dan ik me van tevoren had bedacht. Als ik jullie niet had gehad, was ik waarschijnlijk nog steeds niet bij een onderzoeksschool ingeschreven. Janna jij natuurlijk ook nog heel erg bedankt voor het in elkaar zetten van ons IKEA-krukje. Daarnaast heb ik natuurlijk enorm genoten van alle gezellige momenten, de congressen, lunches, etentjes en had ik zonder jullie door het leven moeten gaan zonder kennis te hebben gemaakt met de pink fluffy unicorn. We wonen inmiddels wat verder uit elkaar, maar onze wegen zijn gelukkig niet gescheiden.

Lianne, vrolijke krullenbol. Wat was het heerlijk om jou als collega naast me te hebben, samen alles ontdekken, samen in dezelfde valkuilen lopen, samen doorhebben dat promoveren véél langzamer gaat dan we allebei hopen. Enige nadeel blijft natuurlijk dat jij in 020 woont, en je ook erg Amsterdammer voelt, maar wie weet verandert dat in de toekomst...

Lieve **Karin**, wat bof ik ook dat ik jou heb mogen ontmoeten. Jij bent echt een ongelooflijk harde werker. Ik heb oneindige waardering voor jouw sterke doorzettingsvermogen en je verbindende kracht binnen de groep. Laten we snel weer een biertje (of wijntje) op een zonnig terras pakken!

Lieke en Nathalie, wat ben ik blij met de fantastische vriendschap die we hebben ontwikkeld. Jullie verfrissende, maar ook kritische kijk heeft dit proefschrift verbeterd. Ik vind het heerlijk om met jullie te discussiëren over alles van onze onderzoeken, van statistiek tot logistiek. Jullie eindeloze vertrouwen en positieve kijk op dit proefschrift leek soms irreëel, maar jullie hebben toch gelijk gekregen. Nu aan mij de taak om jullie te steunen bij jullie proefschriften, die er zeker ook gaan komen. Dank jullie voor alle leuke momenten die we binnen, maar zeker ook buiten het Máxima hebben beleefd. Gelukkig gaan we nog vele nieuwe momenten tegemoet.

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Lieve **Simone en Nick**, jullie zijn een prachtig stel! Jullie snappen wel helemaal hoe het er in het ziekenhuis en de onderzoekswereld aan toe gaat. Simone, we zitten redelijk in eenzelfde schuitje en toch – of juist daardoor – kunnen we altijd steun bij elkaar vinden. Ik ben je ook heel dankbaar hoe we de laatste tijd een mooi team blijken te zijn. Ik ben onwijs blij met je en kan me geen beter zusje wensen.

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