Chapter 1

General introduction and scope of the thesis

Neuroblastoma

Epidemiology
Neuroblastoma is the third most common childhood cancer and the most common solid extracranial tumor in children (1). In the Netherlands about 25 patients are diagnosed each year and neuroblastoma accounts for approximately 15% of all pediatric cancer deaths (2, 3). The median age at diagnosis is 19 months, and 40 percent of patients are diagnosed before the age of one year (4, 5). About 50% of patients present with bone marrow (BM) metastases at diagnosis (6).

Pathogenesis and pathology
Neuroblastoma arises from the the neural crest (7). Neural crest cells normally form the adrenal medulla and the sympathetic nervous system (1). Neuroblastoma pathology varies according to the degree of neural crest differentiation. The International Pathology Classification classifies neuroblastic tumors according to the proportion of neural-type cells and Schwann-type cells into: neuroblastoma, ganglioneuroblastoma and ganglieneuroma. Of all neuroblastic tumors neuroblastoma is the most aggressive and may be classified as differentiating, poorly differentiated and undifferentiated (the most aggressive subtype) (8, 9).

Biology and genetics
The large majority of neuroblastoma cases are sporadic, but in 1 to 2% of cases, there is an inherited predisposition (10-12). Germline mutations in anaplastic lymphoma kinase (ALK) are described to be the most important cause of familial neuroblastoma (13). Germline mutations in PHOX2B have also been associated with familial neuroblastoma and are also associated with Hirschsprung’s disease and central hypoventilation (14).

Several types of genetic aberrations have been described in neuroblastoma. Deletion of chromosome 1p36 is one of the most common chromosomal changes and is associated with a poor outcome (15, 16). Gain of chromosome 17q is observed in almost all stage 4 neuroblastoma (1, 17). Amplification of MYCN on chromosome 2p is present in 20% of neuroblastoma tumors and is associated with a poor outcome (1, 14, 18). Recently, whole genome sequencing of 87 neuroblastoma tumors revealed that chromothripsis (local shredding of chromosomes) and alterations in neuritogenisis genes frequently occur in high-risk neuroblastoma (19). Also recently an association between age at diagnosis and ATRX mutations has been described. ATRX mutations were mainly found in patients older than 12 years of age (poor prognosis) (20). Eleveld et al. performed whole genome sequencing of 23 paired diagnosis and relapse tumors and demonstrated that neuroblastoma relapse tumors show frequent RAS-MAPK pathway mutations. Eighteen of the 23 relapse tumors showed mutations predicted to activate the RAS-MAPK pathway (21).
Clinical presentation of neuroblastoma
The most common primary tumor site is the adrenal gland. However, neuroblastoma can arise anywhere throughout the sympathetic nervous system. Neuroblastoma can metastasize to lymph nodes, bone marrow, bone, orbits, liver and skin (22). The presenting symptoms depend on the location of the primary tumor and the presence and extent of metastatic disease. Approximately 40% of patients present with localized disease, ranging from a small tumor in the adrenal gland to very large and locally invasive tumors.
About half of the patients present with metastatic disease. Children with advanced disease usually have systemic symptoms. Approximately 5% of patients are infants with a phenotype of small tumors with metastases limited to skin, liver and bone marrow. The tumors in these patients can undergo spontaneous regression (stage 4s) (1, 14, 18).

Staging and risk stratification
A diagnosis of neuroblastoma requires histological confirmation with or without the evidence of bone marrow metastases with elevation of urinary catecholamines. Staging is done according to the International Neuroblastoma Staging System (INSS) (6). Stage 1, 2 and 3 represent local tumors with or without lymph node involvement. In stage 4 patients the tumor has spread to distant lymph nodes, bone, bone marrow, liver or other organs. Patients younger than 1 year of age with tumor dissemination limited to skin, liver and/or bone marrow can be staged as 4s. Stage 1-3 and 4s neuroblastoma patients have a good prognosis, whereas stage 4 patients have a poor prognosis (6). Treatment of
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neuroblastoma is based on risk stratification. Patients are classified into low, intermediate or high risk based on stage, age and genetic risk factors like MYCN and 1p status at diagnosis (figure 1).

A revised staging system to define more homogeneous risk groups has been proposed. The International Neuroblastoma Risk Group (INRG) classification system was developed to establish a consensus approach for pre-treatment risk stratification. Stage, age, histologic category, tumor differentiation, MYCN status, chromosome 11q status, and DNA ploidy were the most significant factors (23). It is likely that in the future this system will be used for risk stratification.

Treatment and prognosis

At present, the treatment of neuroblastoma by the Dutch Childhood Oncology Group (DCOG) is based on the German Society for Pediatric Oncology and Hematology (GPOH) treatment (24, 25). In general localized and stage 4S patients have an excellent prognosis (5 years EFS 86% in the German NB97 trial). In the observation group patients undergo initial surgery and are then observed for 12 months or until the end of the second year of life. Local progression or threatening symptoms are treated with N4 chemotherapy (doxorubicin, vincristine, cyclophosphamide) (figure 2).

In the medium risk protocol treatment consists of alternating N5 (cisplatinum, etoposide, vindesine) and N6 (vincristine, dacarbazine, ifosfamide, doxorubicin) courses, 4 N7 (cyclophosphamide) courses and retinoic acid for 12 months (figure 3).

Figure 2. Treatment schedule DCOG NBL2009 Observation Group
S: surgery; CR: complete remission; HRG: high risk group; MRG: medium risk group
In the Dutch high risk protocol two courses of radioactive 131I-MIBG treatment is added upfront to the German high risk protocol (26). This is then followed by alternating N5 (cisplatinum, etoposide, vindesine) and N6 courses (vincristine, dacarbazine, ifosfamide, doxorubicin) (3 each). Surgical resection is performed after the 4th or 6th cycle of chemotherapy. Induction therapy and surgery are followed by myeloablative chemotherapy (melphalan, etoposide and carboplatin) and autologous stem cell reinfusion. In the final part of treatment retinoic acid is given for 12 months (figure 4).

The Children’s Oncology Group reported that immunotherapy with ch14.18 (anti-GD2), GM-CSF and interleukin-2 was associated with a significantly better outcome compared with retinoic acid alone (27). Therefore, Dutch patients eligible for immunotherapy, receive the anti-GD2 therapy in the Children’s Hospital of Philadelphia, USA since 2012. Despite this intensive treatment high risk neuroblastoma patients have a very poor prognosis and more than half of patients will relapse. Cure after relapse is rare (28). Response to treatment is evaluated based on the International Neuroblastoma Response Criteria. Bone marrow cytology, MIBG scintigraphy, urinary catecholamine excretion and imaging (ultrasound or MRI) are used to assess the extent of the disease (6).

**Minimal residual disease**

Minimal residual disease (MRD) is the term used to describe small numbers of tumor cells remaining in blood or bone marrow during or after treatment which are below
the limits of detection using morphological assessment. MRD status is one of the most powerful predictors of survival for children with acute lymphoblastic leukemia. Several studies have shown that pediatric ALL patients with detectable MRD, at designated time points during treatment, have significantly higher relapse rates (29-33). As mentioned previously, bone marrow metastases are frequently found in neuroblastoma patients. Therefore, the detection of BM disease is of importance for stage evaluation at diagnosis and to monitor the response to treatment. According to the International Neuroblastoma Risk Group (INRG), bone marrow (BM) involvement should be assessed by two aspirates and two biopsies from bilateral sites. BM disease is determined by morphology on smears and biopsies (34). However, morphology has a limited sensitivity and is unable to detect small numbers of tumor cells in BM (35, 36). For disease monitoring during treatment of high risk patients and to sensitively assess BM involvement in localized neuroblastoma patients more sensitive techniques should be used. GD2 ImmunocytoLOGY is more sensitive than morphology. ImmunocytoLOGY is already implemented in several clinical protocols (37, 38). Real-time quantitative PCR (RQ-PCR) is another sensitive and more objective technique for the detection of tumor mRNA or DNA in blood, BM and peripheral blood stem cells (PBSC) of neuroblastoma patients.

**Minimal residual disease detection by Real-Time Quantitative PCR**

Real-time quantitative PCR (RQ-PCR) is a highly sensitive technique to detect small numbers of tumor cells in blood and BM (38, 39).

**Markers and technique**

Tumor-specific genetic alterations are ideal markers to stage malignant disease at diagnosis, to monitor the response to therapy and to detect relapse at an early stage. When recurrent genetic defects are known for a tumor type it is relatively easy to screen for the presence at diagnosis on either RNA or DNA level for MRD testing. In neuroblastoma only MYCN amplification is a recurrent gene-specific event. However, MYCN amplicons can be highly variable and not all patients have a MYCN amplification. Therefore, MRD monitoring in neuroblastoma has predominantly focused on the use of RNA markers. These RNA markers are characterized by high expression in tumor cells and low or absent expression in hematological cells. Tyrosine Hydroxylase (TH) and GD2 synthase (GD2S) have been the most widely used MRD markers. However, besides these two markers, over the past decade PHOX2B, CCND1, DCX, ELAVL4, CHRNA3, DDC and GAP43 have been described as RNA MRD markers in neuroblastoma (39-43). PHOX2B is the most specific marker (41).

Since the expression of these genes can change during treatment and at relapse and tumors are regarded to be heterogeneous, the use of a panel of markers is more reliable than using single markers (39, 44, 45). Several groups have used different panels of
markers for MRD detection and it is unknown which marker panel is optimal (39, 46-48). However, PHOX2B and TH, in combination with one or more other markers, are the most widely used marker panels at this moment. Another disadvantage of the use of these RNA markers is the expression in normal hematological cells. PHOX2B is the only specific marker and all other markers do show low expression in normal hematological cells (39, 41). Our group has applied a threshold for positivity to discriminate between tumor cell mRNA levels or normal cell mRNA levels. Other groups have used mathematical methods to determine clinically significant mRNA levels (46). Unfortunately there is no consensus yet concerning the use of which markers and techniques for defining significant mRNA levels. At the moment international studies are ongoing to compare markers and techniques. An interesting alternative for RNA markers would be the use of DNA markers. DNA is more stable than RNA and not dependent on levels of gene expression. Kryh and colleagues have described the use of MYCN amplicon junctions as tumor specific targets for MRD detection in neuroblastoma (49). Our group has described that hypermethylated RASSF1A (50) can be used as a sensitive and specific DNA MRD marker. Furthermore, several groups have performed NGS on neuroblastoma primary tumors (19, 20, 51, 52) and therefore the use of chromosomal aberrations for disease monitoring may be a good option.

**Clinical significance of MRD detection in stage 4 patients**

In childhood acute lymphoblastic leukemia (ALL) MRD levels are now used for risk-assignment in several treatment protocols (53). In neuroblastoma minimal residual disease detection may provide important prognostic information and may be used to stratify patients. Patients with a moderate or poor molecular response to treatment might benefit from more intensive treatment, whereas patients with a good molecular response can be treated with the conventional therapy. MRD guided treatment may provide better survival rates.

**Neuroblastoma mRNA levels at diagnosis**

Retrospective studies investigating the clinical significance of tumor load in BM at diagnosis show contradicting results. Our group demonstrated that the presence of BM infiltration is associated with a poor survival, but not the level of BM infiltration (54). However, only a group of 39 patients was analyzed. Trager et al. showed that high levels (above median) of TH in PB and BM at diagnosis correlated with a poor outcome (55). Recently Viprey and colleagues published the first prospective study reporting on detection of neuroblastoma mRNA in blood and BM of a large cohort of 290 patients (46). They demonstrated that high levels of PHOX2B, TH and DCX mRNA (mathematical methods to determine clinically significant mRNA levels were used) at diagnosis strongly correlated
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with a poor EFS and OS. These results suggest that at diagnosis a group of patients with a very poor outcome can be identified. For these patients current treatment is failing in the majority of patients and especially these patients might benefit from alternative treatment options.

**MRD detection in PBSCs**

It remains controversial whether reinfusion of autologous grafts contaminated with tumor cells can cause relapse after autologous stem cell transplantation. Rill et al. have shown that, by using retroviral marking, autologous grafts from neuroblastoma patients contain neuroblastoma cells and can contribute to relapse (56). However, several small studies on detection of neuroblastoma in autologous grafts show contradicting results (57-61). Handgretinger at al. even found that reinfusion of contaminated grafts, as detected by GD2-immunocytology, was associated with a better survival (61).

Kreissman et al. performed a large randomized study of immunomagnetically PBSC purging in neuroblastoma patients with high-risk disease and demonstrated that purging of neuroblastoma cells from stem cell products does not improve outcome, possibly because of incomplete purging or the presence of residual tumor (47). However, the detection of PHOX2B or TH in PBSCs from neuroblastoma patients was associated with a worse outcome. It is possible that residual BM disease is correlated with PBSC contamination and that this residual BM disease is indeed associated with outcome.

**Molecular response during induction chemotherapy**

Minimal residual disease monitoring during treatment might identify patients who clear their BM during induction chemotherapy, after completion of induction chemotherapy or patients who don’t clear their BM before stem cell transplantation. Several retrospective studies have reported that MRD detection after or during induction therapy can be predictive of outcome (54, 62-66). However, only small numbers of patients were studied and most of these studies lacked multivariate analyses to compare MRD monitoring with other clinical parameters known to be associated with survival. Recently Viprey et al. reported the first prospective study investigating the prognostic value of neuroblastoma mRNA detection after completion of induction chemotherapy in 152 patients. They demonstrated that indeed detection of neuroblastoma mRNA after induction chemotherapy is associated with a poor survival. However, in multivariate analysis this did not add to the predictive value of high levels at diagnosis. So overall, high levels of neuroblastoma mRNA at diagnosis seems the most important factor.

**Clinical significance of MRD detection in stage 1,2 and 3 patients**

Localized neuroblastoma patients (stage 1,2 and 3) have a good prognosis, but some of these patients can suffer from recurrent disease and may die because of disease
progression. GD2 immunocytology positivity in bone marrow samples of localized neuroblastoma patients has been described and is suggested to be correlated with an unfavorable outcome (67). RQ-PCR positivity in localized neuroblastoma has been described in retrospective studies (68, 69). However, large prospective studies have not been performed.

**Scope of the thesis**

Clinical data on MRD detection in neuroblastoma are promising and large prospective studies are ongoing or results have been published. The research presented in this thesis focused on improving minimal residual disease detection in neuroblastoma and to further study the clinical significance of MRD monitoring.

In the first part of the thesis we aimed to identify new MRD markers to improve MRD detection. Currently, qPCR based minimal residual disease (MRD) detection is based on neuroblastoma specific RNA markers. However, RNA MRD markers can have disadvantages, since only PHOX2B has no expression in hematological cells. Furthermore, the expression of RNA markers can vary between patients and it is unknown whether these markers are stably expressed during treatment. A tumor specific DNA MRD marker would therefore be an interesting alternative, since DNA is more stable than RNA and is not dependent on levels of gene expression. The aim of the study described in chapter 2 was to investigate whether tumor-specific DNA breakpoints (chromosomal rearrangements such as translocations, deletions, duplications and inversion), identified by whole genome sequencing (WGS) of the primary tumor, can be used as reliable patient-specific DNA MRD markers.

Cellular heterogeneity is an important aspect of many types of cancer. Epithelial to mesenchymal transition has been linked to tumor progression, metastasis and therapy resistance in several types of cancer, including neuroblastoma. Therefore, the goal of our study in chapter 3 was to identify (a) marker(s) specific for the detection of mesenchymal type neuroblastoma cells and to study the dynamics of these markers in PB, PBSCs and BM of high risk neuroblastoma patients during treatment.

Circulating DNA fragments that carry tumor specific alterations (circulating tumor DNA; ctDNA) can be found in plasma of cancer patients and several studies have shown the feasibility of using ctDNA to monitor disease in various types of cancer. In neuroblastoma detection of ctDNA has only been described at diagnosis and was not used for disease monitoring. Therefore, in chapter 4 we investigated whether ctDNA can be used to monitor treatment response in neuroblastoma patients and is of additive value compared with other techniques for disease detection.
In the second part of this thesis the clinical significance of MRD monitoring in neuroblastoma is studied. It remains controversial whether reinfusion of autologous grafts contaminated with tumor cells can cause relapse after autologous stem cell transplantation. Therefore, we investigated in chapter 5 whether infusion of a contaminated graft and/or the presence of residual BM disease influences outcome in a large retrospective cohort in a multivariate setting.

Localized neuroblastoma patients have a good prognosis, however also patients with localized disease can suffer from recurrent disease. The clinical importance of neuroblastoma mRNA detection in BM at diagnosis of localized patients remains unclear and therefore we studied the frequency and prognostic value of the detection of neuroblastoma mRNA markers in BM from neuroblastoma patients with localized disease (chapter 6).

RQ-PCR for the detection of minimal residual disease in high risk neuroblastoma patients has been demonstrated to be a predictor of survival in retrospective studies. To verify results from these retrospective studies, in 2009 the prospective MRD monitoring study was set up. This is a joint study between the DCOG and GPOH. The goal of this prospective study was to study the clinical significance of MRD monitoring in blood and BM of high risk neuroblastoma patients at different time points during treatment. In chapter 7 we describe results from an interim analysis of this study, in which we studied the prognostic value of the level of mRNA in BM at diagnosis and investigated the clinical significance of BM clearance during induction chemotherapy (after 4 courses of chemotherapy and after completion of induction chemotherapy).

Finally, in chapter 8 all the results described in this thesis are discussed.
Reference List

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