Minimal residual disease detection and monitoring in children with neuroblastoma
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Minimal residual disease detection and monitoring in children with neuroblastoma
Chapter 1

Introduction

Introduction

Neuroblastoma (NB) is the most common extra-cranial solid neoplasm in children with a wide range of clinical behavior ranging from spontaneous regression in patients with metastatic disease (4s), to dismal prognosis despite intensive treatment. At present, treatment allocation is based on pre-treatment risk stratification. However, using the current parameters in the risk stratification schemes, it is not possible to predict which patients will succumb and which will survive, especially in patients with an expected poor prognosis. Therefore additional parameters are needed to improve risk stratification and treatment allocation. In this introduction we will give an overview of our current knowledge of neuroblastic tumors and we will discuss if the use of minimal residual disease (MRD) detection and monitoring can be used as an additional parameter in risk stratification.
Chapter 1 Introduction

Neuroblastoma

Epidemiology

Neuroblastoma is the third most common pediatric cancer, accounting for about 8% of childhood malignancies and for approximately 15% of cancer deaths in children. The incidence of neuroblastoma per year is 10.5 per million children less than 15 years of age. The incidence peaks at age 0 to 4 years, with a median age of 23 months. Forty percent of patients who present with clinical symptoms at diagnosis are under 1 year of age, and less than 5% with clinical symptoms are over the age of 10 years. In the Netherlands there are about 25 new cases diagnosed each year.

Pathology of neuroblastoma

Neuroblastic tumors are derived from neuroectodermal cells that originate from the neural crest during fetal development. These cells are normally destined to form the adrenal medulla and sympathetic nervous system. Failure of these cells to respond to differentiation signals is the first step towards malignant transformation. In the histological classification of neuroblastic tumors the balance between neural type cells (neuroblasts and ganglion cells) and Schwann type cells is being taken into account. Neuroblastoma is the most aggressive of this family of tumors and in turn may be classified as differentiating, poorly differentiated and the most aggressive subtype, undifferentiated. Undifferentiated neuroblastoma (UN) is composed almost entirely of neuroblasts which appear as small round blue cells. Poorly differentiated neuroblastoma (PDN) is composed of neuroblasts and with <5% of cells showing differentiation. Differentiating neuroblastoma (DN) shows >5% cells showing differentiation toward ganglion cells. Ganglioneuroblastoma (GNB) contains neuroblasts with a more mature appearance that are clustered in small foci surrounded by Schwannian stroma. Ganglioneuroma (GN) is predominantly composed of Schwann cells with mature ganglion cells.

Biology of neuroblastoma

Allelic gains and losses

Numerous biologic factors have been shown to predict clinical behavior of neuroblastoma. In a systematic review of Riley and coworkers, six genetic aberrations were found to be prognostic tumor markers: ploidy, MYCN amplification, chromosome 1p loss, chromosome 17q gain, chromosome 14q loss, and loss of chromosome 11q. Of these genetic aberrations MYNC
Minimal residual disease detection and monitoring in children with neuroblastoma has the strongest prognostic impact and is used in most risk stratification protocols (figure 1 and 2). MYCN amplification is found in 30% to 40% of stage 3 and 4 neuroblastomas and in only 5% of localized or stage 4s neuroblastomas.\textsuperscript{10,11} Loss of tumor suppressor regions is reported in neuroblastomas for many chromosomal regions. The most frequently affected regions are chromosome 1p (30–40%), 4p (20%), 1q (25%), and 14q (25%).\textsuperscript{10,11} In almost all tumors with MYCN amplification chromosome 1p is lost, but loss of 1p also occurs in MYCN single-copy cases.\textsuperscript{12} 11q loss is inversely correlated with MYCN amplification.\textsuperscript{13} Therefore 1p and 11q might be used to identify high-risk patients in MYCN single-copy tumors. Prospective evaluation of 1p and 11q status is ongoing in several cooperative group trials, respectively DCOG/GPOH and COG, and is especially aimed at risk stratification of intermediate-risk patients.

**Molecular factors**

A number of biological pathways regulating cancer seems to be disrupted or affected in neuroblastoma, however no target genes have been identified with the exception of MYCN.\textsuperscript{14} Biological pathways which have been and are extensively being studied are tumor differentiation, apoptosis, drug resistance, angiogenesis, and metastasis. To unravel the molecular regulation of neuronal differentiation, research is focused on neurotrophin signaling in which Trk receptors and their ligands have been extensively investigated. High TrkA expression seems to be correlated with good outcome,\textsuperscript{15} while TrkB is correlated with aggressive neuroblastomas and poor outcome.\textsuperscript{16} Research on the disruption of normal apoptotic pathways includes mainly the following genes: BCL2 family, survivin, and caspase-8.\textsuperscript{17,18} The latter is mainly affected by inactivation due to epigenetic silencing. CpG-island hypermethylation of gene promoters is a frequent mechanism for functional inactivation of genes. In neuroblastoma, this mode of inactivation has been demonstrated not only for caspase-8 but also for the four TRAIL apoptosis receptors, the caspase-8 inhibitor FLIP, the RASSF1A tumor suppressor, and many others.\textsuperscript{19-21} For the other biological pathways the following genes are investigated: multidrug resistance gene 1 and the gene for multidrug resistance-related protein (drug resistance),\textsuperscript{22-24} vascular endothelial growth factor and basic fibroblast growth factor (angiogenesis),\textsuperscript{25,26} metalloproteinases, activating matrix-degrading proteolytic enzymes, and molecules regulating tumor cell adhesion and migration (metastasis).\textsuperscript{27,28} Insight into the molecular regulation of the biological pathways mentioned above will hopefully lead to the identification of novel drug targets.
Clinical presentation

Neuroblastoma can arise anywhere throughout the sympathetic nervous system. The adrenal gland is the most common primary site followed by abdominal, thoracic, cervical and pelvic sympathetic ganglia. Neuroblastoma may metastasize to the lymph nodes, bone marrow, bone, liver and skin. Bone metastases tend to appear in the orbit and therefore periorbital ecchimoses is a classical sign of disseminated neuroblastoma. Around 40% of the patients present with localized disease, with a clinical presentation ranging from small intra-adrenal mass found by ultrasonography, to very large and locally invasive tumors. Paraspinal located tumors tend to invade the neuronal foramina and can cause compression of the spinal cord. These cases are oncological emergencies and require acute treatment. About half of the patients present with evidence of metastatic disease, ranging from loco regional spread in lymph nodes to distant metastasis. Children with metastasis mostly have extensive tumor burden and are frequently very ill. About 5% of the patients present with a striking phenotype of 4S disease: these are infants with tumors that metastasize to skin, liver and bone marrow (< 10% invasion). 4S Tumors tend to disappear spontaneously without treatment.5,6,29

Diagnosis and staging

To positively diagnose a neuroblastoma either a positive histological analysis is required or evidence of neuroblastoma cells in the bone marrow with catecholamine metabolites in the urine. Tumors are staged according to the International Neuroblastoma Staging System (INSS) which was revised in 1993.30 Stage 1,2 and 3 represent loco regional tumors with or without positive lymph nodes. Stage 4 tumors are those with distant metastasis. Especially bone marrow (BM) involvement is very common in children with metastatic disease detected by standard morphologic examination. The specific subgroup of stage 4S tumors is reserved for patients less than 1 year of age with dissemination limited to skin liver and/or bone marrow (<10% involvement). More recently the International Neuroblastoma Risk Group (INRG) classification system was developed to establish a consensus approach for pre-treatment risk stratification, since the INSS is a postsurgical staging system.31 The INRG staging system (INRGSs) is based on clinical criteria and image-defined risk factors (IDRFs). In de INRGSSs, locoregional tumors are staged L1 or L2 based on absence or presence of one or more of 20 IDRFs, respectively. Metastatic tumors are defined as stage M, except for stage MS, in which metastases are confined to skin, liver, and/or bone marrow in children younger than 18 months of age.
**Risk stratification**

Up till now, risk classification of neuroblastoma is not uniform in different collaborative groups. However, all risk classification systems use age, stage, and MYCN copy number as risk factors. Histopathological grading is used in different variants, in Europe all groups include neuroblastoma and ganglioneuroblastoma in the treatment protocols for malignant neuroblastoma. Ganglioneuroma are classified as a benign disease and not included.

The Dutch Childhood Oncology Group (DCOG) Neuroblastoma Disease Committee has launched a new treatment protocol in 2009 for which the stratification is shown in **figure 1**. This risk stratification scheme is identical to that of the German Pediatric Oncology and Hematology group (GPOH).

In line with the INRGSS, the INRG Task Force also developed and proposed a new risk stratification system.\(^{32}\) In this new system INRG stage, age, histologic category, grade of tumor differentiation, the status of the MYCN oncogene, chromosome 11q status, and DNA ploidy are taken into account, see **figure 2**.
### Figure 2. Stratification of neuroblastoma tumors according to the INRG classification system

<table>
<thead>
<tr>
<th>INRG Stage</th>
<th>Age (months)</th>
<th>Histologic Category</th>
<th>Grade of Tumor Differentiation</th>
<th>MYCN</th>
<th>11q Aberration</th>
<th>Ploidy</th>
<th>Pretreatment Risk group</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1/L2</td>
<td></td>
<td>GN maturing; GNB intermixed</td>
<td></td>
<td>A</td>
<td></td>
<td></td>
<td>Very low</td>
</tr>
<tr>
<td>L1</td>
<td></td>
<td>Any, except</td>
<td>GN maturing or GNB intermixed</td>
<td>NA</td>
<td>Amp</td>
<td></td>
<td>B Very low</td>
</tr>
<tr>
<td>L2</td>
<td>&lt;18</td>
<td>Any, except</td>
<td>GN maturing or GNB intermixed</td>
<td>No</td>
<td></td>
<td></td>
<td>D Low</td>
</tr>
<tr>
<td>L2</td>
<td>≥18</td>
<td>Poorly differentiated</td>
<td></td>
<td>NA</td>
<td></td>
<td></td>
<td>H Intermediate</td>
</tr>
<tr>
<td>M</td>
<td>&lt;18</td>
<td>Hyperdiploid</td>
<td></td>
<td>NA</td>
<td>Amp</td>
<td></td>
<td>F Low</td>
</tr>
<tr>
<td>M</td>
<td>12 to &lt;18</td>
<td>Diploid</td>
<td></td>
<td>NA</td>
<td>Amp</td>
<td></td>
<td>J Intermediate</td>
</tr>
<tr>
<td>M</td>
<td>≥18</td>
<td>P High</td>
<td></td>
<td>Amp</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MS</td>
<td>&lt;18</td>
<td>No</td>
<td></td>
<td>NA</td>
<td>Yes</td>
<td></td>
<td>C Very low</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Poorly differentiated or undifferentiated</td>
<td>Amp</td>
<td></td>
<td></td>
<td>Q High</td>
</tr>
</tbody>
</table>

Diploid (DNA index 1.0); hyperdiploid (DNA index 1.0 and includes near-triploid and near-tetraploid tumors); very low risk (5-year EFS 85%); low risk (5-year EFS 75% to 85%); intermediate risk (5-year EFS 50% to 75%); high risk (5-year EFS < 50%). GN, ganglioneuroma; GNB, ganglioneuroblastoma; Amp, amplified; NA, not amplified; L1, localized tumor confined to one body compartment and with absence of image-defined risk factors (IDRFs); L2, locoregional tumor with presence of one or more IDRFs; M, distant metastatic disease (except stage MS); MS, metastatic disease confined to skin, liver and/or bone marrow in children 18 months of age (for staging details see text and Monclair et al14); EFS, event free survival. Figure adapted from Cohn et al.JCO 2009.32
Current treatment and prognosis
The DCOG treatment regimen is based on the GPOH treatment strategy. The schedule for high-risk patients is shown in figure 3. The medium-risk protocol is the same as the high-risk protocol without upfront MIBG therapy and without high-dose chemotherapy with stem cell transplantation. In low-risk patients a wait and see strategy is followed. However, when necessary (tumor progression, organ dysfunction) mild chemotherapy is given.

The DCOG has chosen the GPOH treatment as best current protocol, and added 2 courses of radiolabeled MIBG upfront to the high-risk protocol. In the GPOH protocol there is a randomisation between addition of 2 courses of Topotecan upfront, or just start with the regular protocol. In this way, the addition of the 2 courses of $^{131}$I-MIBG therapy to the high-risk protocol will be comparable to both the standard and investigational arm of the GPOH high-risk schedule.

MIBG is a neurotransmitter-like substance that can be radioactive labelled with $^{131}$I for treatment or $^{123}$I for diagnostics. The radiopharmacon shows uptake in 95% of the neuroblastoma.
# Chapter 1 Introduction

<table>
<thead>
<tr>
<th>Response</th>
<th>Primary tumor</th>
<th>Metastatic site *</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR</td>
<td>No tumor</td>
<td>No tumor; catecholamines normal</td>
</tr>
<tr>
<td>VGPR</td>
<td>Decreased by 90%-99%</td>
<td>No tumor; catecholamines normal</td>
</tr>
<tr>
<td>PR</td>
<td>Decreased by &gt; 50%</td>
<td>All measurable sites decreased by &gt; 50%. Bones and bone marrow; number of positive sites decreased by &gt; 50%; no more than 1 positive bone marrow site allowed*</td>
</tr>
<tr>
<td>MR</td>
<td>No new lesions; &gt;50% reduction of any measurable lesion (primary or metastasis with &lt; 50% reduction in any other; &lt;25% increase in any existing lesion.</td>
<td></td>
</tr>
<tr>
<td>NR</td>
<td>No new lesions; &lt;50% reduction but &lt;25% increase in any existing lesion.</td>
<td></td>
</tr>
<tr>
<td>PD</td>
<td>Any new lesion; increase of any measurable lesion &gt; 25%; previous negative marrow positive for tumor</td>
<td></td>
</tr>
</tbody>
</table>

Table adapted from Brodeur et al. 1993.30

* one positive marrow aspirate or biopsy allowed for PR if this represents a decrease from the number of positive sites a diagnosis.

Abbreviations: CR: complete remission; VGPR: very good partial response; PR: partial response; MR: mixed response; NR: no response; PD: progressive disease.

After MIBG therapy, 3 cycles N5 chemotherapy (cisplatinum, etoposide, vindesine) and 3 cycles N6 chemotherapy (vincristine, dacarbazine, ifosfamide, doxorubicin) are given. Then resection is performed if substantial tumor remains after induction therapy. This resection should be attempted after the 4th or 6th chemotherapy cycle. After the primary induction therapy and surgery a high-dose-chemotherapy with melphalan, etoposide and carboplatin is given, followed by autologous stem cell reinfusion. Finally retinoic acid is given in 6 cycles followed by a short break of 3 months and 3 more cycles. The prognosis for patients with high risk tumors is very poor with a 5 year overall survival around 30% with the current protocol33,34

**Response evaluation**

Currently, response evaluation is based on the International Neuroblastoma Response Criteria (INRC), in which results on BM cytology, MIBG scintigraphy, urinary catecholamines excretion and imaging (echo/ MRI) are combined to assess the extent of disease. The definitions of the INRC are listed in **table 1**. Most protocols evaluate response after 3 to 4 months of intensive induction therapy. The final response to treatment is (mostly) determined at the end of chemotherapy, after induction chemotherapy, surgery and ASCT.
Minimal residual disease

As stated above, the high risk group, 40% of all patients with NB, contains all patients with metastatic disease over 12 months of age (INSS stage 4) and all patients with MYCN amplification. One of the most important hallmarks of high-risk disease is dissemination to the bone marrow (BM). Therefore, detection of BM metastasis is crucial for clinical staging and thereby risk assessment at diagnosis. Furthermore, to monitor response to therapy, BM is tested during therapy. The current golden standard to measure BM infiltration is by cytological investigation of bilateral BM aspirates (morphological investigations) and histological assessment of bilateral bone biopsies. According to the International Neuroblastoma Staging System, BM infiltration is assessed by microscopic examination of both aspirate smears and bone biopsies. BM trephine biopsies, taken from both iliac crests, are reviewed after H&E staining. A biopsy is considered positive when neuroblasts are detected in at least 1 of 10 sections of any biopsy. For cytology, BM aspirates are taken from both iliac crests (one or two samples for each crest, i.e., anterior and/or posterior) and slides immediately prepared. BM cytology is considered positive when neuroblast aggregates are detected in at least one of six slides. Conventional morphological techniques are mostly unable to detect tumor cell infiltration below the level of 0.1% and are therefore not regarded sensitive enough to monitor minimal residual disease (MRD). Antibodies detecting GD2 are used to detect NB with immunocytology and have been shown to be specific in combination with morphological analysis of the cell. Immunocytology is a more sensitive technique to detect MRD than morphology alone, and can detect 1 tumor cell in $10^5$ normal haematopoietic cells. Immunocytology has been standardized and is already applied in several clinical protocols. Since other cells, such as macrophages, can also take up the GD2 antigen, the light-microscopic evaluation of immunocytochemical results requires a detailed cytomorphological study of immunopositive–and negative cells. Therefore, immunocytology is not only very time consuming, it is also dependant on the skills of individual observers. Another sensitive and more objective technique that has been developed to detect MRD is real-time quantitative PCR (RQ-PCR). This technique has a considerable higher sensitivity than morphology, and has been applied to detect tumor cells not only in BM, but also in peripheral blood (PB) and peripheral blood stem cells (PBSC). Provided that adequate controls are included, the identification of tumor cells by NB specific markers introduces a greater level of objectivity than morphological and (immuno)cytological assays.
Markers for molecular testing

To be able to use molecular testing to discriminate tumor cells (NB cells) from non-tumor cells (the surrounding hematological cells), the NB cell must have acquired distinguishable differences in DNA or mRNA expression. At DNA level, neuroblastomas are genetically quite heterogeneous, so there is no universally applicable DNA marker available yet. Therefore, molecular diagnostic assays for neuroblastomas focuses on RNA markers. Optimal detection for mRNAs requires the identification of a target mRNA that is expressed in the target tumor cells but not in hematological cells. The identification of suitable target mRNAs has been one of the primary challenges for successful application of RT-PCR to detect MRD in neuroblastoma. For the last two decades the first enzyme in the catecholamine synthesis pathway tyrosine hydroxylase (TH) has been used by several groups. Cathecholamines are produced by 98% of all neuroblastomas, thus TH is highly expressed in almost all neuroblastoma tumors. Importantly, TH is hardly expressed in hematological cells and therefore a very sensitive and specific marker. However, recently it has been described that TH is also expressed in a subset of hematological cells within the different compartments; BM, PB and PBSC. This illegitimate background expression limits the sensitivity of the assay. Nevertheless, several authors claim a sensitivity of $10^6$ using TH as an MRD marker to detect neuroblastoma.

Another widely applied target is GD2 synthase (GD2S). Ganglioside 2 (GD2) is present on the cell membrane of neuroblastomas and used as immunological marker for the detection of neuroblastoma. GD2S is required for synthesis of GD2. However, normal cells in the BM also express GD2S, especially mesenchymal stromal cells express high levels of this enzyme. Because of this relatively high expression, especially in bone marrow, GD2S cannot be used to discriminate between stage 1-3 and stage 4 disease. In addition, NB cells do not always express GD2 at the cell surface and NB cells can loose expression during therapy (become GD2 negative), this might indicate that in these cells GD2S is down regulated.

Because TH and GD2S, the currently most widely applied markers, are not totally specific, the search for other more specific targets for detection of minimal residual disease continued. Many new targets have been described, amongst others: PGP9.5, GAGE54,55, DDC56, DCX42, ELAVL443, ST8Siall57 and CyclinD1. However, even for these markers expression in normal hematological cells has been described. Therefore, efforts have been taken to discover new NB markers by systematical approaches.
Clinical relevance of molecular MRD detection

Although it is possible to detect MRD with RQ-PCR at a very low level, its clinical relevance still needs to be established. In patients presenting with non-metastatic disease, MRD detection is focused on comparison of RQ-PCR with conventional methods at diagnosis to clarify if presence of MRD in BM at diagnosis is correlated with survival. Patients with positive BM might benefit from upstaging and from more intensive treatment. In patients with metastatic disease, level of tumor load in BM at diagnosis might be correlated with survival. Furthermore, by monitoring MRD at diagnosis and during treatment response to treatment can be evaluated. PCR guided stratification might identify responders, which can be cured with current, conventional therapy and might identify non-responders who might benefit from other (new) therapies. In this way, PCR guided therapy might result in better survival rates. In addition, since obtaining a PB sample is less invasive than a BM sample, the prognostic value of the presence of circulating tumor cells (CTC) in the PB at diagnosis and the clearing of CTC during treatment are also subject of research. Lastly, the presence or absence of MRD detection is investigated in autologous stem cell grafts, to elucidate the significance of reinfusion of a MRD positive graft. Several studies have already been performed, in which MRD in BM, PB and PBSC has been investigated. However, all these studies have been performed with markers that are also expressed in normal hematological cells, GD2S or TH; this might have influenced the results in these studies. Below, relevant literature on MRD studies at different time points during treatment is summarized.

MRD detection in BM at diagnosis

RQ-PCR based MRD testing of BM at diagnosis in medium and low risk groups, might identify patients that have no sign of tumor invasion as tested with conventional methods, but test positive with RQ-PCR. The frequency as well as the clinical significance of low level of BM infiltration still needs to be established. Neuroblastoma cells have been detected in BM of patients with localized disease (stage 1-3) using RT-PCR and immunocytology. These children are likely to have disseminated disease that is not detected by conventional cytology and they might therefore benefit from more intensive therapy. Shono et al. reported that in 4 of 14 patients with localized disease a positive BM by TH mRNA PCR was detected; 2 of 4 patients with BM disease detected by PCR, died of recurrent disease in BM. Corrias et al. found 13% (19 of 145) of low stage patients to be BM GD2-positive using GD2 immunocytology, and this was significantly correlated with relapse.

In patients with stage 4 disease, the frequency of BM positive disease detected by RQ-PCR is reported to be higher compared to conventional methods.
RT-PCR detects NB in diagnostic BM samples in 95-100%\textsuperscript{59} compared with a reported frequency of 90% by conventional cytology.\textsuperscript{61} In addition, it has been hypothesized that the extent of marrow disease at diagnosis could be an indicator of poor prognosis in patients with metastatic disease. Trager et al.\textsuperscript{49} found in a cohort of uniformly treated high risk patients (n=24) that the concentrations of TH mRNAs in BM at diagnosis had a prognostic significance. Patients with transcript concentrations below the median in BM had a significantly better outcome than the group with transcript concentrations above the median (5 years survival 91% versus 33%, p=0.009). However, they chose a mathematically calculated cut-off level, which did not correspond to disease severity nor implied a clinical decision limit. Many RQ-PCR values of patients with PCR results below the median were just above cut-off, and would probably have been negative with more strict definitions for TH MRD positivity. In addition, there was no relation of transcript level to survival in patients with high transcript levels in their BM. Apparently, it is more relevant whether the bone marrow is infiltrated or not, rather than the actual level of infiltration.

**Detection of MRD in peripheral blood**

Several groups also investigated the correlation between presence of circulating tumor cells (CTCs) in peripheral blood at diagnosis and survival. Although the tumor load in PB has been shown to be one log (10 fold) lower than in BM,\textsuperscript{62} the presence or clearance of CTCs during treatment, could be a prognostic factor. Burchill et al.\textsuperscript{63} found that TH mRNA could be detected in PB in 67% (33 of 49) of stage 4 patients > 1 year. The presence of TH mRNA in peripheral blood at diagnosis in these patients was a significant predictive factor for overall survival (hazard ratio= 2.4, p=0.014). Trager et al.\textsuperscript{49} also reported the presence of TH mRNA in PB in 16 of 18 stage 4 patients. However, using a more specific marker for PB, DDC, only 10 of 18 stage 4 patients were defined as positive. Furthermore, they showed that DDC and TH mRNA transcripts levels above the median were correlated with poor survival (80% versus 34%, p=0.053 for both markers). Parareda et al.\textsuperscript{64} analyzed PB of 13 high risk stage 4 patients and found 7 patients to be positive for TH mRNA. In contrast to Trager et al., they did not find a difference in survival between patients with or without circulating tumor cells present in the PB; 4 of 7 TH positive patients died compared to 2 of 6 TH negative patients, p=ns. In conclusion, the presence of circulating tumor cells in PB and MRD in the BM might be correlated with poor survival, but large prospective studies of uniformly treated patients are needed to confirm these findings.
**MRD monitoring to study early clearance of BM**

Detection of minimal residual disease (MRD) in BM during therapy can be used to monitor response to therapy and subsequently to identify different patterns of response kinetics (‘early’ versus ‘late’ responders). The hypothesis is that patients that respond early to current therapy (early responders) are more likely to survive. If so, the response to therapy could then be used to identify those patients that benefit from the current treatment strategies or to identify patients that would benefit from different or more treatment. In Acute Lymphoblastic Leukemia (ALL), MRD results of BM samples drawn during the first months of therapy are currently used to stratify patients. Until now, only two studies investigated the impact of early clearance of BM MRD on outcome using RT-PCR.\(^65,66\) Fukuda et al. found that 29% (6/21) of patients with metastatic BM disease had TH negative BM within 4 months after start of therapy and none of these patients died.\(^65\) Tchirkov et al. compared survival in high risk stage 4 patients with less than 1000 TH transcripts in the BM after 3 cycles of induction chemotherapy to patients with more than 1000 TH transcripts. They found that 50% (11/22) of patients with metastatic BM disease had less than 1000 TH transcripts per 10\(^6\) copies of the reference gene GAPDH, which was significantly correlated with survival 85% versus 0%, respectively.\(^66\) Both studies did not perform multivariate analysis to compare the prognostic value of RQ-PCR response with other clinical response parameters.

**MRD detection before high dose chemotherapy**

Patients with no or partial response to induction chemotherapy as measured with conventional methods before high dose chemotherapy, have poor survival.\(^67\) More sensitive detection of MRD in BM might identify more non-responders. Several studies have shown that patients with MRD in (either PB or) BM at the end of induction, regardless of high dose chemotherapy and vitamin A therapy, have poor survival due to relapse or progression of their disease. The largest study performed until now is performed by Cheung et al. who investigated MRD status using GD2S mRNA in 45 high risk patients. They found that 32 of 45 (71%) patients tested positive for GD2S after induction chemotherapy. These patients had a significantly worse outcome than patients that were GD2S negative after induction chemotherapy. For patients in complete remission (CR)/ very good partial response (VGPR), GD2S negative patients (n=9) had a 5 years overall survival of 88% versus 50% for GD2S positive patients (n=27). For patients in partial response (PR), 3 of 4 GD2S negative patients were still alive after 5 years, compared to none of the 5 GD2S positive patients.
Tumor cell contamination of autologous stem harvests
The clinical significance of reinforcing neuroblastoma cells during transplantation procedures has been questioned, although gene marker studies demonstrating the clonogenic properties of tumour cells isolated from peripheral blood and the identification of reinjected tumour cells at the site of metastases suggest that they may play a role in the development of secondary disease and relapse. Since there is less risk for tumour contamination in PBSC, the use of peripheral blood stem cells (PBSC) is preferred over BM. To decrease the tumour content in the autologous harvests, BM and PBSC harvests can be purged using CD34+ selection. Several studies have been performed to investigate the correlation between contamination of autologous stem cell harvest (ASCH) and survival using RQ-PCR, however data are controversial. Tchirkov et al. found that a level of > 500 copies TH mRNA per 10^6 GAPDH copies in autologous PBSC was associated with a decreased survival. 10 of 21 patients had > 500 TH mRNA copies and their 5 years OS was 25 % compared to 75% in patients with <500 TH mRNA copies in their PBSC. In line with these results, Burchill et al. reported that in their cohort 9 of 18 PBSC were positive for TH mRNA and 7 of these 9 patients died of progressive disease compared to 4 of 9 with no detectable disease in their graft. Due to small numbers this was not significant. The same trend was also found by Avigad et al. who detected TH mRNA in 26 out of 45 (58%) patients. Patients harboring high TH expression had a reduced progression-free survival (23%) versus those with low/negative TH expression (43%). However, due to small patient numbers this difference in PFS was not significant. In contrast, Corrias et al. tested 27 PBSC from stage 4 patients with RQ-PCR for TH of which only 6 were positive. The survival of patients after reinjection of TH positive or negative harvests did not differ (5-y-OS 50% versus 48%). Handgretinger et al. measured MRD using GD2 immunocytoLOGY in 24 CD34+ selected harvests and found that patients with positive harvests were even long term survivors. They hypothesized that the presence of a critical number of graft-contaminating neuroblastoma cells can elicit a protective anti-tumor immune response after autologous stem cell transplantation. In conclusion, data are controversial and larger preferably prospective studies are needed to investigate the true effect of reinjected tumor cells on survival.

Molecular response to immunotherapy
Various treatment strategies have been developed to target MRD that might be present after induction chemotherapy, such as myeloablative therapy with autologous stem cell transplantation and differentiation therapy with
13-cis-retinoic acid. A new promising treatment strategy to target MRD is immunotherapy using GD2 antibody alone or in combination with cytokines such as interleukin-2 and granulocyte-macrophage colony-stimulating factor (GM-CSF). In a recent COG trial, immunotherapy was administered every 1 to 2 months, and in some patients, for up to 2 years. Because the BM is a common site for recurrence of the disease, molecular response in BM is thought to reflect patients that benefit from this treatment. Cheung et al. performed two studies to investigate the molecular response on GD2 immunotherapy using GD2S mRNA as MRD marker. In the first study, they examined BM samples of 45 stage 4 patients. Before antibody treatment 32 of 45 patients had BM positive for GD2S mRNA. Post antibody treatment (one cycle of radio immunotherapy with stem cell rescue and one cycle of unlabeled immunotherapy) 20 patients (63%) became GD2S negative. However it was not described whether this was related to survival. In the second study, Cheung et al. showed that the GD2S PCR became negative following antibody plus GM-CSF therapy in 10 of 13 (77%) patients with CR/VGPR, 9 of 20 (45%) patients with primary refractory, 2 of 8 (25%) patients with secondary refractory, 0 of 9 (0%) patients with progressive disease. Molecular responders were significantly less likely to relapse than non-responders (75% survival compared to 0%). GD2S mRNA can be a useful surrogate marker for evaluating adjuvant (anti-GD2) treatment efficacy in neuroblastoma with prognostic potential. However, it has to be determined whether other markers might be superior.

Follow up after therapy

High risk patients that are in complete remission after completing therapy are still at risk to develop recurrent disease. There seems to be a plateau after 5 years after diagnosis, resulting in a survival of around 35%, but long term follow up shows that patients still die from recurrent disease. Several studies have looked at early detection of relapse both in BM and PB. Cheung et al. showed that detection of GD2S mRNA in BM samples 24 months after diagnosis correlated strongly with overall survival. Parareda et al. found that patients with TH positive PB at 6 months after end of therapy (n=20) had a worse prognosis than patient who were negative (n=5), 40% versus 100% 5 years EFS, identifying early relapse in PB. In line with these results, Burchill et al. showed that TH mRNA was detected in PB around 4 months (range 1 –11 months) before clinical relapse. In prospective studies, follow up in PB will have to be done at regular intervals to investigate the value of PB positivity after the end of treatment, in detecting early relapse.
However, it also has to be investigated whether early detection of relapse and thus early start of treatment with low tumor load will contribute to better survival in these patients.

**Conclusion**

Clinical data on MRD detection in PB, BM and PBSC are very promising, however several issues still need to be addressed. First of all, there is a need for more specific RNA markers and better guidelines for MRD positivity or negativity for the existing markers. In addition, it should be investigated whether a panel of markers would increase the sensitivity, since marker genes are heterogeneously expressed between the primary tumors in patients and within one tumor itself. Furthermore, it is unknown if PCR targets are stably expressed during treatment and if so how to deal with MRD quantification. In this respect a DNA MRD marker would be more suitable for MRD quantification. Lastly, the clinical relevance of MRD detection during and after induction chemotherapy and the correlation of contaminated autologous stem cell harvests with survival, should be confirmed in preferably large prospective studies, in which efforts should be taken to detect MRD using international guidelines.
**Scope of the thesis**

The aim of the work presented in this thesis is to improve MRD detection in neuroblastoma and to study the clinical impact of MRD detection in neuroblastoma treatment.

We first put effort in identifying new, more specific, MRD markers for neuroblastoma by comparing mRNA expression profiles of neuroblastoma tumors and cell lines to normal tissues. The most promising candidate MRD-marker, PHOX2B, was selected for further testing in parallel to the currently most widely used MRD markers, TH and GD2synthase (chapter 2).

We further described the technology, by which candidate markers were selected and the panel that we selected (chapter 3). To determine the specificity of the markers, the expression in control tissues needed to be established. So in this chapter we describe further the method used to establish thresholds for positivity by RQ-PCR testing in control tissues, such as bone marrow (BM) and peripheral blood (PB), and their cell subsets. Finally, the additional value of the panel of markers over one marker was determined.

Because it is not known if PCR markers are stably expressed, we studied the stability of the panel of RQ-PCR markers (chapter 4). We compared expression in primary tumors at diagnosis to 1) their distant metastasis (liver, lymph node and BM), 2) treated tumors and BM and 3) relapse tumors.

If target genes are not stably expressed, quantification of MRD using these markers will be difficult. This information might be essential for a reliable selection of MRD-PCR targets with a minimum chance of false-negative results as well as for accurate quantification of MRD level.

The clinical impact of MRD detection is investigated in chapter 5 and 6. Chapter 5 addresses which time-points of MRD detection are most informative for outcome. And chapter 6 deals with the detection of MRD in autologous stem cell harvests and the association with outcome.

Since the expression of RNA markers varies greatly between tumors and are not always stably expressed during treatment, we studied the possibility of MRD detection using a DNA marker in chapter 7. DNA is more stable than RNA and is not dependant on levels of gene expression. Thus we hypothesized that a DNA marker would be more suitable for accurate quantification of MRD in bone marrow of patients with NB. However, a general neuroblastoma specific DNA aberration has not been found yet.
Common epigenetic changes, such as aberrant DNA methylation, have been described in neuroblastoma. Therefore we used methylated RASSF1a as a DNA MRD and determined whether this marker was comparable in sensitivity and specificity as the commonly used RNA markers and more suitable for MRD quantification.

Finally in chapter 8 the results are summarized and discussed with respect to the clinical value of MRD detection by PCR. In addition the (study-) design of the ongoing prospective study on MRD detection (joint effort DCOG-GPOH) is discussed in which the results of this thesis were taken into account.
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