Minimal residual disease detection and monitoring in children with neuroblastoma
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Citation for published version (APA):

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Minimal residual disease detection and monitoring in children with neuroblastoma
Chapter 6

Preliminary results on minimal residual disease detection in autologous stem cell grafts in patients with high risk neuroblastoma

(manuscript in preparation)
Abstract

Introduction
The presence of minimal residual disease (MRD) detected by quantitative real-time (q)PCR in autologous stem cell grafts in children with high risk neuroblastoma seems to be associated with an unfavourable outcome, however to date only small studies have been performed. Moreover, these studies suffered from lack of specificity of the assay due to amplification of the PCR target in normal bone marrow (BM) and peripheral blood stem cells (PBSC). In this retrospective multicenter study, autografts of a large patient cohort were studied using a recently described panel of PCR targets.

Methods
In total, 21 BM, 47 PBSC and 80 CD34+ selected autografts from 148 high risk patients were retrospectively collected at 2 Dutch and 12 German centers between 1986 and 2009. In 124 patients the autograft was reinfused. qPCR was performed with six neuroblastoma specific markers: PHOX2B, TH, DDC, GAP43, CHRNA3, and DBH. The prognostic impact of MRD in autografts, the reinfusion of contaminated autografts and clinical response were assessed using Kaplan-Meier plots, log-rank tests and multivariate Cox analysis.

Results
Presence of neuroblastoma mRNA was detected in 9/21 (43%) BM grafts, in 8/47 (17%) PBSC grafts and in 6/80 (8%) CD34+ selected grafts. This was associated with poor survival (5-year overall survival (OS), 22% ± 11 versus 46% ± 5, p=0.02). When we only analyzed the patients that received autologous stem cell transplantation (n=124), this correlation lost its significance (5-year OS, 35 ± 14 versus 50% ± 5, p=0.09). In multivariate Cox analysis only BM disease at time of harvest was significantly associated with survival.

Conclusion
Our series of autologous stem cell harvests is the largest series described up till now. In this series, BM autografts were more often contaminated than PBSC or CD34+ selected grafts. Both the presence of MRD in the autografts and in BM at time of harvest were associated with poor outcome.
Neuroblastoma (NB) is the most common extra-cranial solid neoplasm in children, accounting for 10% of all childhood cancers. Approximately 50% of the patients with NB suffer from high-risk disease, with dissemination in bone marrow (BM), bone, distant lymph nodes, liver and/or other organs. Despite intensive treatment schedules with high dose chemotherapy and autologous stem cell rescue, this disseminated form of neuroblastoma has a poor prognosis.\textsuperscript{1,2}

It is thought that reinfusion of autografts contaminated with tumor cells can contribute to relapse after myeloablative therapy. Indeed, studies have convincingly shown that circulating neuroblastoma cells are highly clonogenic\textsuperscript{3} and, if reinfused, are capable of tumour formation and are implicated in relapse.\textsuperscript{4,5} Since there is less risk for tumour contamination in peripheral blood stem cells (PBSC), the use of PBSC is preferred over BM.\textsuperscript{6,7} To decrease tumour contamination in the autologous grafts, CD34+ selection can be performed on BM and PBSC collections.\textsuperscript{8,9}

Detection of tumour cells in BM or PBSC samples can rely on cytology, immunological, flow cytometric and quantitative realtime (q)PCR assays.\textsuperscript{10} The latter method is most sensitive and objective and can detect one NB cell per 10\textsuperscript{6} normal haematopoietic cells.\textsuperscript{11,12} Therefore, qPCR has been used to investigate the correlation between tumor cell contamination in autografts and survival. However, data are controversial; some studies found that reinfusion of contaminated grafts was correlated with poor survival.\textsuperscript{13,14} Others found no effect on outcome, when contaminated grafts were reinfused.\textsuperscript{15,16} Handgretinger et al. even found that reinfusion of contaminated grafts was associated with a better outcome using GD2-immunocytology to detect tumor cells.\textsuperscript{17}

The most commonly used markers in these studies were tyroxine hydroxylase (TH) and GD2 synthase (GD2S). However these markers are hampered by their expression in normal haematological cells.\textsuperscript{18-20} This could have led to false positive results and to an overestimation of graft contamination in the previous studies. Previously, we have identified the marker PHOX2B, which is neuroblastoma specific.\textsuperscript{18} In addition, we defined absolute threshold levels for positivity for markers which have low expression in normal hematological cells and showed that a panel of markers is more sensitive for detection of MRD than the use of PHOX2B alone.\textsuperscript{21} If stringent definitions for MRD positivity are used to avoid false positive results, then the incidence of tumor cell contamination in BM and PBSC autografts can be more accurately
determined and thus the correlation between reinfusion of a contaminated graft with survival as well.

The purpose of our study was to investigate the clinical relevance of detection of MRD in autologous BM and PBSC grafts and answer the following questions:
1) Is there a difference between MRD positivity in autografts of different sources, i.e. BM, PBSC and CD34+ selected material?
2) Is there a correlation between reinfusion of contaminated autografts and survival?
3) Is there a correlation between metastatic BM remission status at time of harvest and survival?
Methods

Patients and samples
In this retrospective study, 148 archived autologous stem cell grafts from 148 Dutch and German high risk patients with NB were investigated. In 30 cases more than one vial was available. In those cases the reinfused material was selected for analysis. If vials were from the same aphaeresis material (and date), the MRD positive sample was chosen for analysis. High-risk patients were defined according to the International Neuroblastoma Staging System (INSS), as those at stage 4 or stage 3 with MYCN amplified tumors, all over 1 year of age. In total, 61 grafts (21 BM, 28 PBSC and 12 CD34+ selected grafts of which 4 BM grafts and 8 PBSC grafts) from 61 Dutch patients, treated in three consecutive protocols; VECI23 (n=37); rCOJEC24 (n=8) and NB04 (n=16)25 at the Emma Children’s Hospital/AMC or Sophia Children’s Hospital/ Erasmus MC (respectively in Amsterdam and Rotterdam, the Netherlands) were collected between 1990 and 2007. In addition, 87 grafts (19 PBSC, 25 PBSC before CD34+selection and 43 CD34+ selected PBSC grafts) from 87 German patients, registered and treated in the German Neuroblastoma trials NB85 (n=2), NB90 (n=1), NB97 (n=45) and NB04 (n=39)25,26 were collected between 1986 and 2009. Both Dutch and German protocols adopted the carboplatin- melphalan association as condition regimen. According to the protocols, autologous stem cells were harvested only in patients responsive to induction chemotherapy. Data extracted from the medical record included dates of diagnosis, progression, relapse and death, MYCN gene copy number, results of BM (immuno)cytology, 123I-meta iodobenzylguanidine (123I-MIBG) scan, ultrasound, CT scan or MRI scan, and urine catecholamine metabolite levels (homovanillic acid (HVA) and 4-hydroxy-3methoxymandelic acid (HMM), formerly named vanilmandelic acid (VMA). Disease status at time of reinfusion was scored according to the International Neuroblastoma Response Criteria (INRC). Written informed consent for data collection and for the use of stored remains of autologous stem cell harvests for research purposes was obtained from parents or guardians of each participating child. The study was approved by the Medical Research Ethics Committee of the AMC.

RNA extraction, reverse transcription and real-time quantitative PCR
Total RNA extraction was performed of 1 ml to 2 ml of autologous stem cell grafts by means of the Trizol method according to manufacturer’s instructions (Invitrogen, Carlsbad, USA). cDNA was synthesized (45 min 42°C) using 1μg of total RNA, random hexamers (25μM), dNTP’s (1mM) and M-MLV reverse transcriptase (100U), total reaction volume of 20μl (all reagents from Gibco-BRL, Life Technologies, Breda, The Netherlands except for the dNTP’s: Promega, Leiden, The Netherlands). Finally, the reverse
transcriptase was inactivated by heating (3 min, 99°C) and volume diluted to 50μl.

qPCR for PHOX2B, TH, dopamine decarboxylase (DDC), cholinergic receptor3 (CHRNA3), dopamine beta hydroxylase (DBH) and growth associated protein 43 (GAP43) was performed in an ABI PRISM 7000 Sequence Detection System (PE Biosystems, Darmstadt, Germany) as described before.18,21 BM grafts were tested with PHOX2B, TH, DDC, CHRNA3 and GAP43. PBSC samples with PHOX2B, TH, DDC, CHRNA3 and DBH. CD34-selected samples were tested with all 6 markers. Reference gene beta-glucuronidase (GUS)27 was used for normalization (normalized Ct (ΔCt) = Ct GUS – Ct marker as described before.18 The number of GUS-copies was determined using GUS-plasmid DNA (Ipsogen, Marseille, France) dilutions. All qPCR experiments were carried out in triplicate and mean values were used.

Data analysis
Sample and marker positivity have been described previously.18,21 In short, clinical samples were scored positive if one of the other markers scored positive. PHOX2B was scored positive if Ct \( < 50 \) and the other markers were scored positive if \( \Delta C_t \text{sample} > 3.0 C_t \text{control} \). Thresholds for positivity in BM, PBSC and CD34+ selected cells (\( \Delta C_t \text{control} \)) were determined, respectively in 51 pediatric non-neuroblastoma BM samples, 50 non-neuroblastoma PBSC samples and 18 non-neuroblastoma CD34+ selected material, as described previously \( ^{21} \) (table 1).

Statistical analysis
Survival analysis was performed according to the Kaplan-Meier method and truncated at seven years after diagnosis. Survival curves were compared using log-rank statistics. Multivariate Cox models were used to assess the prognostic significances of the various factors. All statistics were done using SPSS 15.0.
### Table 1. Mean normalized expression levels in control BM (n=51), PBSC (n=51) and CD34+ selected material (n=18) measured by quantitative real-time PCR

<table>
<thead>
<tr>
<th>Marker</th>
<th>BM positive samples</th>
<th>Expression*</th>
<th>Treshold‡</th>
<th>PBSC positive samples</th>
<th>Expression*</th>
<th>Treshold‡</th>
<th>CD34+ positive samples</th>
<th>Expression*</th>
<th>Treshold‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHOX2b</td>
<td>0/51</td>
<td>no amplification</td>
<td>no treshold</td>
<td>0/51</td>
<td>no amplification</td>
<td>no treshold</td>
<td>0/18</td>
<td>no amplification</td>
<td>no treshold</td>
</tr>
<tr>
<td></td>
<td>15/51</td>
<td>-15.2 (±1.1)</td>
<td>-12.2</td>
<td>21/51</td>
<td>-17.0 (±0.8)</td>
<td>-14.0</td>
<td>11/18</td>
<td>-15.1 (±1.6)</td>
<td>-12.1</td>
</tr>
<tr>
<td>DDC</td>
<td>1/51</td>
<td>-16.8</td>
<td>-13.8</td>
<td>7/51</td>
<td>-18.0 (±0.7)</td>
<td>-15.0</td>
<td>7/18</td>
<td>-17.1 (±1.0)</td>
<td>-14.1</td>
</tr>
<tr>
<td>CHRNA3</td>
<td>31/51</td>
<td>-15.6 (±1.3)</td>
<td>-12.6</td>
<td>14/51</td>
<td>-17.2 (±0.9)</td>
<td>-14.2</td>
<td>10/18</td>
<td>-16.9 (±0.9)</td>
<td>-13.9</td>
</tr>
<tr>
<td>DBH</td>
<td>29/51</td>
<td>-13.5 (±1.8)</td>
<td>-10.5</td>
<td>2/51</td>
<td>-17.7 (±0.03)</td>
<td>-14.7</td>
<td>7/18</td>
<td>-15.9 (±1.4)</td>
<td>-12.9</td>
</tr>
<tr>
<td>GAP43</td>
<td>20/51</td>
<td>-14.9 (±1.6)</td>
<td>-11.9</td>
<td>47/51</td>
<td>-15.3 (±1.6)</td>
<td>-12.3</td>
<td>3/18</td>
<td>-16.0 (±1.1)</td>
<td>-13.0</td>
</tr>
</tbody>
</table>

**NOTE:**
† number of positive BM, PBSC or CD34+ samples out of total numbers tested;
* mean (± sd) normalized Ct values (ΔCt = Ct GUS – Ct Marker),
‡ threshold for positivity (ΔCt values) was defined as the ΔCT ≥ 3.0 Ct of the mean ΔCt of the normal tissue

Abbreviations:
BM, bone marrow;
PBSC, peripheral blood stem cells;
CD34+, PBSC selected for CD34
Results

Patients' characteristics
All patient were high risk patients; stage 2 or 3 with MYCNA tumors (n=21), stage 4 < 1 year with MYCNA tumors (n=2) or stage 4 > 1 year (n=125) (table 2). At diagnosis, 112 of 127 stage 4 patients had BM disease (cytology positive), which was not associated with outcome (p=0.38). The 5 year overall survival (5-year OS) was 44.3 ± 4.4%. No difference in outcome was observed between Dutch and German patients (5-year OS 46 % ± 6.6 and 42% ± 5.9, respectively p=0.87), patients treated with the different protocols for induction chemotherapy (p=0.88), and different origin of autologous grafts: BM, PBSC or CD34+selected material (p=0.45). Only stage (non-metastatic disease) was significantly associated with outcome (p=0.03). The median observation time was 81 months (range 14 – 288 months) at time of analysis (February 2010).

Detection of MRD in autologous stem cell grafts
148 autografts were analyzed with qPCR from 148 patients with high risk disease; 21 BM grafts, 47 PBSC grafts and 80 CD34+ selected grafts. MRD testing for neuroblastoma mRNA was positive in 23/148 (16%) stem cell grafts, i.e. 9/21 (43%) BM grafts, in 8/47 (17%) PBSC grafts and in 6/80 (8%) CD34+ selected grafts (figure 1). Thus BM grafts were more often contaminated with tumor mRNA than PBSC and CD34+ selected grafts (Fisher’s Exact Test, p=0.001). In positive BM grafts mRNA of several targets was detected. In contrast, in PBSC and CD34+ selected samples frequently only one PCR target was positive (figure 2a-d). This indicates that not only BM grafts were more often contaminated but also that the relative MRD levels in positive BM grafts were most likely higher than in positive PBSC and CD34+ selected grafts. In 124 of 148 patients the autologous stem cell grafts were reinfused, from which 6 of 17 (35%) BM grafts, 7 of 37 (19%) PBSC grafts and 3 of 70 (4%) CD34+ selected graft were contaminated, (figure 1).
Table 2. Characteristics of patients with high risk neuroblastoma (n=148)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (months)</strong></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>34</td>
</tr>
<tr>
<td>Range</td>
<td>7 - 267</td>
</tr>
<tr>
<td>&lt;18 months (MYCNnonA)</td>
<td>7</td>
</tr>
<tr>
<td><strong>Country</strong></td>
<td>0.87†</td>
</tr>
<tr>
<td>Netherlands</td>
<td>61</td>
</tr>
<tr>
<td>Germany</td>
<td>87</td>
</tr>
<tr>
<td><strong>INSS stage</strong></td>
<td>0.03‡</td>
</tr>
<tr>
<td>Stage 2, 3</td>
<td>21</td>
</tr>
<tr>
<td>Stage 4</td>
<td>127</td>
</tr>
<tr>
<td>BM cytology positive</td>
<td>112</td>
</tr>
<tr>
<td>BM cytology negative</td>
<td>15</td>
</tr>
<tr>
<td><strong>MYCN</strong></td>
<td>0.52</td>
</tr>
<tr>
<td>Amplification</td>
<td>66</td>
</tr>
<tr>
<td>No amplification</td>
<td>79</td>
</tr>
<tr>
<td>Not analyzed</td>
<td>3</td>
</tr>
<tr>
<td><strong>Origin of graft</strong></td>
<td>0.45¶</td>
</tr>
<tr>
<td>BM</td>
<td>21</td>
</tr>
<tr>
<td>PBSC</td>
<td>47</td>
</tr>
<tr>
<td>CD34+selected</td>
<td>80</td>
</tr>
<tr>
<td><strong>Treatment</strong></td>
<td>0.88**</td>
</tr>
<tr>
<td>VECI</td>
<td>37</td>
</tr>
<tr>
<td>rCOJEC</td>
<td>8</td>
</tr>
<tr>
<td>NB85</td>
<td>2</td>
</tr>
<tr>
<td>NB90</td>
<td>1</td>
</tr>
<tr>
<td>NB97</td>
<td>44</td>
</tr>
<tr>
<td>NB04</td>
<td>56</td>
</tr>
<tr>
<td><strong>Survival</strong></td>
<td></td>
</tr>
<tr>
<td>5-years OS (median %)</td>
<td>44</td>
</tr>
<tr>
<td>Std error</td>
<td>4.4</td>
</tr>
<tr>
<td><strong>Observation time</strong></td>
<td></td>
</tr>
<tr>
<td>Median (months)</td>
<td>81</td>
</tr>
<tr>
<td>Range</td>
<td>14-288</td>
</tr>
</tbody>
</table>

* differences in survival between sub-groups of patient characteristic were calculated with log rank tests
†, p-value for difference in survival between Dutch and German patients
‡, p-value for difference in survival between patients with stage 2,3 and patients with stage 4
§, p-value for difference in survival between patients BM cytology positive disease and patients with BM cytology negative disease
||, p-value for difference in survival between patients with MYCN amplified tumors and patients with MYCN single-copy tumors
¶, p-value for difference in survival between patients with autologous BM graft, autologous PBSC graft and autologous CD34+ selected grafts
**, p-value for difference in survival between patients treated according to different treatment protocols
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The percentage of positive grafts is shown. Black bars represent all grafts, gray bars represent the reinfused grafts.

** p=0.001 compared to PBSc and CD34+ (Fisher’s Exact Test)

Abbreviations:
BM, bone marrow grafts;  
PBSC, peripheral blood stem cells grafts,  
CD34, CD34-selected grafts
148 autologous stem cell grafts were tested with a panel of 5 or 6 qPCR markers, i.e. BM grafts for PHOX2B, TH, DDC, CHRNA3 and GAP43, PBSC grafts for PHOX2B, TH, DDC, CHRNA3 and DBH and CD34+ selected grafts for PHOX2B, TH, DDC, CHRNA3, DBH and GAP43. Each ellipse represents positive results of one marker. The number outside the ellipses represents the samples which were negative for all markers tested.

A) All grafts (n=148),
B) BM grafts (n=21),
C) PBSC grafts (n=47),
D) CD34-selected grafts (n=80).

* In figure 2a and 2d 6 markers were tested, therefore GAP43 and DBH are presented in 1 ellipse
Correlation between MRD detection in autologous grafts and overall survival

In total cohort, neuroblastoma mRNA was detected in 16% (23 of 148) of the autologous stem cell grafts. The presence of NB mRNA in autologous grafts was significantly associated with an unfavourable outcome (5-year OS, 22% ± 11 versus 46% ± 5, p=0.02), figure 3a. When we only analyzed the patients that received autologous stem cell reinfusion (n=124), the correlation between MRD positive grafts and survival lost its significance (5-year OS, 35% ± 14 versus 50% ± 5, p=0.09), figure 3b. Therefore, we hypothesized that the autologous stem cell graft just reflects remission status at time of harvest and that this metastatic remission status is correlated with survival. Since BM is one of the most favorite sites for metastasis and MRD is more sensitively measured in BM than in PB (this thesis chapter 4), BM disease would reflect remission status better than (mobilized) PB. Indeed when we analyzed the different hematological tissues separately, i.e. BM, PBSC and CD34+ cells, the presence of NB mRNA in BM graft seemed to stratify patients best and was most significantly associated with survival. Also the presence of mRNA in CD34+ selected material was associated with poor survival, while the presence of mRNA in PBSC was not, figures 4a-c.

Because the presence of neuroblastoma mRNA in autologous BM grafts was most significantly associated with survival, we also investigated the correlation between BM disease, determined by cytology, at time of harvest and outcome. At diagnosis, 112 of 127 stage 4 patients had BM positive
disease as determined with cytology. Of these 112 patients, 26 had still morphologically positive BM disease at time of harvest and this was significantly correlated with worse outcome (5-year OS 24% ± 9 versus 41% ± 6, p=0.03) (figure 5a). This correlation was even stronger when available GD2-immucytology or qPCR results were also taken into account; BM
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disease was detected in 22 extra patients (5-year OS 21% ± 7 versus 49% ± 7, p<0.001) (figure 5b). Thus a good BM response to therapy seemed to be associated with better outcome.

In addition, 3 patients that did not have BM cytology positive disease at diagnosis, also had MRD positive disease in the BM at harvest detected by
### Table 3. Results of univariate and multivariate Cox regression analyses (n=94*)

<table>
<thead>
<tr>
<th></th>
<th>p-value</th>
<th>Hazard ratio</th>
<th>95% CI of the hazard ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>qPCR of harvest (positive versus negative)</td>
<td>0.4</td>
<td>1.4</td>
<td>0.6 – 2.9</td>
</tr>
<tr>
<td>INRC response at ASCT (CR/VGPR versus PR/MR/NR)</td>
<td>0.02</td>
<td>1.9</td>
<td>1.1 – 3.2</td>
</tr>
<tr>
<td>MYCN (MYCNA versus non-MYCNA)</td>
<td>0.5</td>
<td>1.2</td>
<td>0.7 – 2.1</td>
</tr>
<tr>
<td>BM cytology at harvest (positive versus negative)</td>
<td>0.05</td>
<td>1.8</td>
<td>1.0 – 3.3</td>
</tr>
<tr>
<td>BM MRD at harvest (positive versus negative)</td>
<td>0.005</td>
<td>2.1</td>
<td>1.3 – 3.8</td>
</tr>
<tr>
<td>Therapy (VECI vs rCOJEC vs NB97 vs NB04)</td>
<td>0.5</td>
<td>1.1</td>
<td>0.8 – 1.4</td>
</tr>
<tr>
<td>Country (Germany versus Netherlands)</td>
<td>0.4</td>
<td>1.3</td>
<td>0.7 – 2.2</td>
</tr>
</tbody>
</table>

**Multivariate analysis**

<table>
<thead>
<tr>
<th></th>
<th>p-value</th>
<th>Hazard ratio</th>
<th>95% CI of the hazard ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>INRC response at SCT (CR/VGPR versus PR/MR/NR)</td>
<td>0.1</td>
<td>1.6</td>
<td>0.9 – 2.8</td>
</tr>
<tr>
<td>BM cytology at harvest (positive versus negative)</td>
<td>0.8</td>
<td>0.9</td>
<td>0.4 – 2.0</td>
</tr>
<tr>
<td>BM MRD at harvest (positive versus negative)</td>
<td>0.05</td>
<td>2.1</td>
<td>1.0 – 4.2</td>
</tr>
</tbody>
</table>

Abbreviations:
- qPCR, quantitative real-time PCR;
- INRC, International Neuroblastoma Response Criteria;
- ASCT, autologous stem cell transplantation;
- CR, complete remission;
- VGPR, very good partial response;
- MR, mixed response;
- NR, no response;
- MYCNA, MYCN amplified tumor;
- BM, bone marrow;
- MRD, minimal residual disease.

* only patients who had morphology positive BM disease at diagnosis and who received autologous stem cell transplantation were included for this analysis
** only factors significant in univariate analyses were assessed in multivariate analyses.

qPCR and all 3 patients died of disease. These 3 patients probably also had BM positive disease at diagnosis if more sensitive techniques would have been used to detect BM disease, and also in these patients BM disease was not cleared during therapy.

In line with these results, patients with CR/VGPR at time of ASCT, had a significantly better outcome than patient with PR (5-year OS 29% ± 8 versus 59% ± 6, p=0.006) (figure 5c).

In multivariate Cox analysis in which only patients who had morphology positive BM disease at diagnosis and who received autologous stem cell transplantation were included for this analysis, only BM disease at time of harvest was significantly associated with survival (p=0.05), table 3.
Discussion

In this study we have shown that autologous BM grafts are more often contaminated than PBSC and CD34+-selected grafts, but that the prognosis of patients transplanted with BM does not differ from those transplanted with PBSC or CD34+ selected grafts. In addition, we have shown that the presence of NB mRNA in autologous grafts was significantly correlated with poor outcome, whereas the correlation between contamination of reinfused grafts and prognosis did not reach significance. This suggests that the mere presence of metastatic disease at time of harvest is prognostic for survival rather than the reinfusion of tumor cells. This hypothesis is supported by our data on metastatic BM disease at time of stem cell harvest, which show that patients with BM positive disease at time of harvest have very poor outcome, irrespective of the source of the stem cells. Since BM is a more favorite site for neuroblastoma metastasis than PB and MRD is more readily detected in BM, BM disease would reflect remission status better than PB.

Since previous studies have shown that reinfused tumor cells have the potential to create a relapse,\textsuperscript{5,28} autologous stem cell transplantation protocols are designed to prevent reinfusion of tumor cells. In the protocol of GPOH, autologous stem cells are harvested only if no tumor cells are being detected in the BM using GD2 immunocytology,\textsuperscript{25} while in the COG protocol PBSC is harvested at a designated time point during treatment, regardless of the presence of BM disease.\textsuperscript{29} In addition, in the Dutch and German protocols it is recommended to reduce neuroblastoma cell contamination of autologous grafts by CD34 positive selection of the autologous PBSC graft, especially if BM disease is still present. If reinfusion of contaminated grafts is not correlated with survival, then therapy protocols could be adjusted according to these findings.

For autologous stem cell rescue, nowadays predominantly PBSC are collected, since the procedure for PBSC collection is not only less invasive, it also carries a lower risk for contamination than harvesting BM.\textsuperscript{6,7} We indeed confirmed that the frequency of contamination was higher in BM grafts than in PBSC or CD34+ selected material. Surprisingly, the frequency of contamination of PBSC material was much lower than described by others; 17\% versus 50-74\%.\textsuperscript{13-16} This can be explained by the stringent threshold levels for positivity we defined. In the previous studies, TH and GD2S were used as neuroblastoma specific PCR markers to detect neuroblastoma mRNA. These genes are known to be expressed in normal tissues, such as BM and PBSC. Consequently, some of the positive results could have been expression levels in the range of expression levels as observed in normal PBSC/CD34 cells and be false positive.
In our study, the presence of NB mRNA in autologous grafts was significantly correlated with poor survival. This correlation was most pronounced in patients with contaminated BM grafts. Since BM grafts are more often contaminated than PBSC and CD34+ selected grafts, it seems reasonable to choose for the tissue with theoretically least contamination for autologous stem cell rescue; CD34+ selected material. However, we not only show that CD34+ grafts are almost equally contaminated compared to PBSC grafts, but we also show that reinfusion of purged grafts is not correlated to better outcome than reinfusion of BM or PBSC grafts. Although we can still not exclude that reinfused tumor cells cause relapse. This observation could be explained by the hypothesis that the detection of MRD in autologous stem cell grafts reflects metastatic remission status at time of graft and that BM remission status at time of graft is correlated with survival. Indeed we show here that BM remission status at time of graft is correlated with survival. Using more sensitive techniques for MRD detection in BM, such as GD2 immunocytology and qPCR, this correlation was even more pronounced. So, the response to therapy as measured in the BM seems to be the most important prognostic factor. This is in line with studies of Fukuda et al. and Thirkov et al. which all show that early molecular BM response is correlated with better survival. Baring this in mind, we assume that the timing of harvesting does not influence survival and that grafts do not need to be selected for CD34. In line with this assumption, there are no clinical studies that have shown an advantage for purging tumour cells from stem cell product and outcome, while the disadvantage of this procedure is the loss of haematopoietic progenitors during the selection process.

However, we can not draw definitive conclusions from this study, since our study was hampered by its retrospective character. We tested autologous stem cell grafts of two patients populations; a Dutch and German cohort. Grafts from the Dutch cohort were collected during a large time span and consequently the patients were treated according to different treatment protocols. The German cohort was more homogeneous and treated mostly in two resembling consecutive protocols NB97 and NB04. However, due to retrospective character of this study we could not test all grafts of all consecutive high risk patients. In addition, centers were more willing to send vials of autologous stem cell grafts of deceased patients. Therefore, the GPOH patient cohort might be biased towards a cohort with worse survival. This might have resulted on the one hand in less significant impact of MRD positivity in the grafts, since most patients had poor outcome. But on the other hand, the results show that even in this cohort with poor outcome, the
incidence of detectable contaminated grafts, either PBSC or CD34+selected material, is very low, and might be even lower if all consecutive patients were included.

In summary, we believe that mere the presence of metastatic disease at time of harvest is prognostic for survival and thus the response to therapy rather than the reinfusion of tumor cells. However theses results need to be verified in prospective MRD studies.
References

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