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Peripheral Stem Cell Apheresis is Feasible Post ^{131}Iodine-Metaiodobenzylguanidine-Therapy in High-Risk Neuroblastoma, but Results in Delayed Platelet Reconstitution

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Abstract

Purpose: Targeted radiotherapy with ^{131}iodine-metaiodobenzylguanidine (^{131}I-MIBG) is effective for neuroblastoma (NBL), although optimal scheduling during high-risk (HR) treatment is being investigated. We aimed to evaluate the feasibility of stem cell apheresis and study hematologic reconstitution after autologous stem cell transplantation (ASCT) in patients with HR-NBL treated with upfront ^{131}I-MIBG-therapy.

Experimental Design: In two prospective multicenter cohort studies, newly diagnosed patients with HR-NBL were treated with two courses of ^{131}I-MIBG-therapy, followed by an HR-induction protocol. Hematopoietic stem and progenitor cell (e.g., CD34$^+$ cell) harvest yield, required number of apheresis sessions, and time to neutrophil (>0.5 $\times$ 10^9/L) and platelet (>20 $\times$ 10^9/L) reconstitution after ASCT were analyzed and compared with "chemotherapy-only"–treated patients. Moreover, harvested CD34$^+$ cells were functionally (viability and clonogenic capacity) and phenotypically (CD33, CD41, and CD62L) tested before cryopreservation ($n = 44$) and/or after thawing ($n = 19$).

Results: Thirty-eight patients (47%) were treated with ^{131}I-MIBG-therapy, 43 (53%) only with chemotherapy. Median cumulative ^{131}I-MIBG dose/kg was 0.81 GBq (22.1 mCi). Median CD34$^+$ cell harvest yield and apheresis days were comparable in both groups. Post ASCT, neutrophil recovery was similar (11 days vs. 10 days), whereas platelet recovery was delayed in ^{131}I-MIBG- compared with chemotherapy-only–treated patients (29 days vs. 15 days, $P = 0.037$). Testing of harvested CD34$^+$ cells revealed a reduced post-thaw viability in the ^{131}I-MIBG-group. Moreover, the viable CD34$^+$ population contained fewer cells expressing CD62L (L-selectin), a marker associated with rapid platelet recovery.

Conclusions: Harvesting of CD34$^+$ cells is feasible after ^{131}I-MIBG. Platelet recovery after ASCT was delayed in ^{131}I-MIBG-treated patients, possibly due to reinfusion of less viable and CD62L-expressing CD34$^+$ cells, but without clinical complications. We provide evidence that peripheral stem cell apheresis is feasible after upfront ^{131}I-MIBG-therapy in newly diagnosed patients with NBL. However, as the harvest of ^{131}I-MIBG-treated patients contained lower viable CD34$^+$ cell counts after thawing and platelet recovery after reinfusion was delayed, administration of ^{131}I-MIBG after apheresis is preferred.

Introduction

Neuroblastoma (NBL) is the most common extracranial solid tumor in children, accounting for 7% to 10% of all childhood malignancies (1). The majority of children presenting with NBL have "high-risk (HR) disease" with amplification of the MYCN oncogene and/or distant metastases at diagnosis, mainly involving bone marrow (BM; ref. 2). Despite the implementation of a multimodal therapy, including induction chemotherapy, surgery,

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Translational Relevance

In this study, we report on a cohort of high-risk neuroblastoma patients (HR-NBL) treated with ^{131}iodine-meta-iodobenzylguanidine (^{131}I-MIBG) before chemotherapy, that is, "upfront" ^{131}I-MIBG-therapy. We had the unique opportunity to evaluate the feasibility of hematopoietic stem cell harvesting after ^{131}I-MIBG-therapy, combined with an in-depth analysis of stem cell quality and hematologic reconstitution after autologous stem cell transplantation (ASCT). Our findings are of importance as the concept of high-dose chemotherapy and ASCT was shown to improve outcome in patients with NBL, and studies examining double transplants are being performed with promising results. Moreover, the future of ^{131}I-MIBG-therapy may expand in the coming decade by incorporation into front-line therapy, because introduction of ^{131}I-MIBG during induction will be studied in an upcoming prospective randomized trial. Thus, the optimal time to administer ^{131}I-MIBG during HR-NBL treatment is currently being investigated (www.clinicaltrials.gov: NCT03165292, NCT03126916, NCT01175356), and results of our study can assist in decision making.

autologous stem cell transplantation (ASCT), and immunotherapy, the prognosis of patients with HR-NBL is still poor. More than half of the patients with HR-NBL experience disease recurrence and long-term survival remains less than 40% (2). This poor outcome necessitates the search for new therapies.

An alternative treatment modality involves metaiodobenzylguanidine (MIBG), a norepinephrine analogue. Approximately 90% of patients with NBL have "MIBG-avid" disease, that is, MIBG will accumulate in the NBL cells (3). MIBG is therefore used as an imaging agent for diagnostic purposes, when radiolabeled with iodine-123, but is also used as a form of targeted radiotherapy when labeled with iodine-131 (^{131}I). In recurrent or refractory NBL, response rates of ^{131}I-MIBG treatment range from 20% to 40% (4–9). Dose-limiting toxicity is myelosuppression and support with ASCT at ^{131}I-MIBG doses of $\geq$12 mCi/kg is advised (6). When used as upfront therapy in newly diagnosed patients with HR-NBL, thus prior to chemotherapy, objective response rates of up to 70% have been reported (10–12). Recently, administration of ^{131}I-MIBG during induction chemotherapy and prior to myeloablative therapy (MAT) was shown to be feasible (www.clinicaltrials.gov: NCT01175356; ref. 13), and will be further studied in a prospective randomized trial (NCT03126916, ref. 1). Moreover, a combination of ^{131}I-MIBG and Topotecan will be studied as an intensification treatment strategy for patients with inadequate response after induction to proceed to MAT and ASCT (NCT03165292). Thus, optimal scheduling of ^{131}I-MIBG in the high-risk treatment plan is currently being investigated.

As ^{131}I-MIBG-therapy-related hematologic side effects have been reported, we questioned if ^{131}I-MIBG, when given upfront, would affect hematopoietic stem and progenitor cells (e.g., CD34$^+$ cells) and/or the BM microenvironment, hence impairing the ability to harvest mobilized CD34$^+$ cells. In a pilot study, that mainly focused on upfront ^{131}I-MIBG-therapy toxicity and efficacy, we observed a CD34$^+$ cell harvest failure in only 2 of 21 patients (14). The primary aim of this study was to evaluate feasibility of stem cell apheresis after upfront ^{131}I-MIBG-therapy in a larger cohort of patients with HR-NBL, and determine the effect on hematologic reconstitution after ASCT. This was combined with an in-depth analysis of the quality of the harvested CD34$^+$ cells by studying post-thaw viability, clonogenic capacity, and phenotype.

Materials and Methods

Patients and treatment

All patients included in this study were patients with HR-NBL ($\geq$1–19 years, stage 4 or MYCN-amplification) treated according to the prospective Dutch Childhood Oncology Group (DCOG), multicenter cohort protocols: pilot phase (2005–2011) and NBL-2009 (2011–October 2015). In these protocols, patients with MIBG-avid disease were treated with two courses of upfront ^{131}I-MIBG-therapy, followed by standard HR-therapy, called: "MIBG-therapy" group (Fig. 1). In the pilot phase, ^{131}I-MIBG

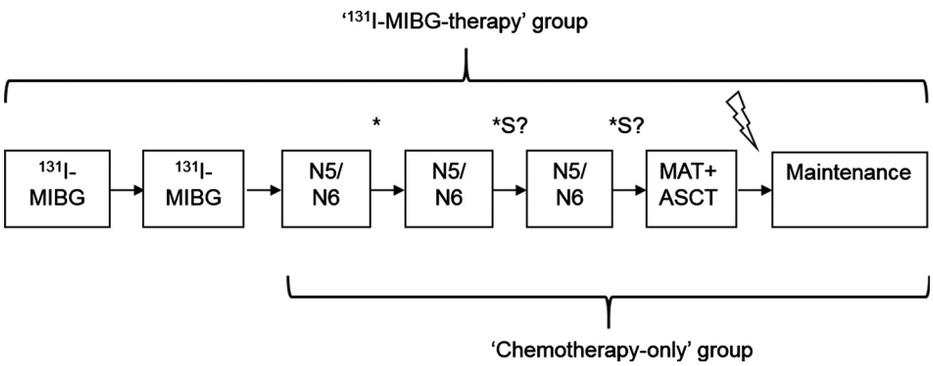

Figure 1.

Treatment overview. "MIBG-therapy" group: patients treated with upfront ^{131}I-MIBG, followed by induction chemotherapy. "Chemotherapy-only" group: patients with MIBG non-avid disease or too ill for protective nuclear isolation were excluded from receiving upfront ^{131}I-MIBG-therapy and were directly treated with induction chemotherapy (i.e., three alternating N5 and N6 courses). Hematologic requirements to start an N5 course: white blood cell count >2,000/μL, neutrophil count >0.5, platelets >50,000/μL (except patients with extensive bone marrow involvement). N5 course: 160 mg/m^2 cisplatin, 400 mg/m^2 etoposide, 3 mg/m^2 vindesine. N6 course: 2 × 1.5 mg/m^2 vincristine, 1,000 mg/m^2 dacarbazine, 7,500 mg/m^2 ifosfamide, and 60 mg/m^2 doxorubicin. MAT: 180 mg/m^2 melphalan, 40 mg/kg etoposide, and 1,500 mg/m^2 carboplatin, followed by ASCT at day 1. S?: Surgery: timing is based on response and operability.
*, Apheresis was attempted when the bone marrow was cleared from tumor cells.

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was administered as a fixed dose: first course ^{131}I-MIBG dose was 7.4 GBq (200 mCi) and second course 5.5 GBq (150 mCi; ref. 14). In the NBL-2009 study, we aimed to limit the whole-body dose to 4 Gy for the two consecutive ^{131}I-MIBG administrations. After the first administration (444 MBq/kg), the second dose was based on the total-body radiation dose calculated from the first therapeutic administration. Patients with MIBG-non-avid disease and patients that were too ill for protective nuclear isolation (e.g., superior vena cava syndrome, risk of optic nerve compression) or with uncontrollable hypertension, were excluded to receive ^{131}I-MIBG-therapy and directly treated with standard HR chemotherapy: the "chemotherapy-only" group (Fig. 1). Thus, patients were not randomly assigned to a patient group. Standard HR-therapy consisted of induction chemotherapy, surgery, MAT with ASCT and radiotherapy to the primary tumor site [identical to the Gesellschaft fur Pädiatrische Onkologie und Hämatologie (GPOH) NB2004 NBL-HR protocol, as previously described; ref. 14)]. Number of patients included in a previous cohort: Gooskens and colleagues (15): 24 patients, Kraal and colleagues (14): 32 patients. The study was conducted in accordance with the ethical guidelines of the Declaration of Helsinki. Written informed consent was obtained from patients, parents, or legal representatives.

Apheresis and hematologic reconstitution after ASCT

As is common practice in DCOG HR-NBL treatment protocols, CD34$^+$ cells were harvested after the BM was cleared from tumor. In case BM was not cleared after standard induction chemotherapy (N5/N6), patients received an additional N8 chemotherapy course. Post-chemotherapy, 10 μg/kg G-CSF was administered subcutaneously. Plerixafor was not used. When CD34$^+$ cell blood counts reached >20/μL, apheresis was performed, aimed at collecting $\geq 2 \times 10^6$ CD34$^+$ cells/kg. The sequential number of days needed for collecting sufficient CD34$^+$ cells was registered. In case of insufficient yield, a second apheresis session was attempted after the subsequent chemotherapy course.

Patients with good response to induction therapy [complete response (CR), very good partial response (VGPR) or partial response (PR)] were allowed to proceed to ASCT, with reinfusion of $\geq 2 \times 10^6$/kg CD34$^+$ cells (as measured prior to cryopreservation). Hematologic reconstitution post-ASCT was defined as a platelet count >20×10^9/L (without transfusions) and neutrophil count >0.5×10^6/L. In case of thrombocytopenia, platelet transfusions were not standard of care, only in case of severe hemorrhage platelet transfusions were given.

Cell viability

Viability and vitality testing of harvested CD34$^+$ cells and nucleated blood cells (NBC), respectively, was routinely performed prior to cryopreservation. NBC vitality testing was performed using trypan blue exclusion. Cell recovery after cryopreservation was calculated as the number of nucleated cells post-thawing divided by the number of cells prior to cryopreservation. CD34$^+$ cell viability was determined as previously described (ISHAGE guidelines; ref. 16), combined with 7-amino actinomycin D (7-AAD) staining (BD biosciences), and measured using a CANTO ll flow cytometer (BD Biosciences). Minimal two-hundred thousand CD45$^+$ events were collected. Viable CD34$^+$ cells were defined as 7-AAD negative. On a selection of 19 patients (9 ^{131}I-MIBG and 10 chemotherapy-only–treated patients), CD34$^+$ cell viability was tested post-thawing. This "subgroup" was selected based on availability of separate cryopreserved reference aliquots from the apheresis, harvest yield and dose of re-infused CD34$^+$ cells (evenly distributed between the two groups). Clinical patient characteristics of the subgroup were comparable to the other patients.

Colony-forming unit–granulocyte-macrophage (CFU-GM) assay

Progenitor capacity of collected CD34$^+$ cells was assessed using the CFU-GM assay, which was performed standard prior to cryopreservation ($n = 81$ samples of 44 patients). Additionally, one of the centers performed CFU-GM assays using post-thaw CD34$^+$ cells of the above described "subgroup" of 19 patients. Nucleated cells were plated in duplicate in 35 mm tissue culture plates (concentrations: 1.0, 0.5, and 0.25×10^5 cells/mL), in MethoCult GF 4534 (StemCell Technologies). Cultures were incubated for 12 to 14 days at 37°C (5% CO_2). CFU-GM colonies, containing at least 40 translucent cells, were scored in triplicate by microscopy (Leica). CFU-GM recovery was calculated as the number of colonies formed post-thawing divided by the number of colonies prior to cryopreservation.

Phenotypic testing of CD34$^+$ cells

Of the "subgroup" of 19 patients, post-thaw CD34$^+$ cells were characterized for surface marker expression by flow cytometry. Cells were washed, re-suspended in PBS containing 0.2% BSA and incubated (20 minutes, room temperature) with the following monoclonal-antibodies: Antibodies purchased from BD biosciences: CD45-PerCP (clone 2D1), CD34-APC (clone 8G12), CD62L-FITC (clone SK11), CD33-PE-Cy7 (clone p67.6), IgG2a-FITC, IgG1-PE, IgG1-PeCy7. Purchased from Dako: CD45-PB (clone T29/33). Purchased from Beckman Coulter: CD41-PE (clone P2). Isotype controls were used to set gating thresholds.

Statistical analysis

Groups were compared using the Chi square test for categorical variables and the independent Student t test for continuous variables. A multivariate linear regression model was used to study the association between patient characteristics, treatment and CD34$^+$ cell harvest. To account for repeated measures, a generalized linear mixed model (GLMM) was used to estimate marginal mean harvest quality (CFU-GM per CD34$^+$ cell) of the first apheresis day for each group. GLMM is a well-known statistical methodology used to study data that are correlated within subjects (17). The adjusted mean with corresponding standard error and confidence intervals were computed for each group. Percentage of CD33-, CD41-, and CD62L-expressing CD34$^+$ subsets and cell vitality/viability were compared between the two groups using the Mann–Whitney U test and t test. Survival analysis techniques were used to compare time to platelet and neutrophil reconstitution for patients treated with ^{131}I-MIBG or chemotherapy-only. The log-rank test has been used to assess the statistical significant difference between the two groups. Time to event was defined as time from infusion of CD34$^+$ cells (ASCT) until time of platelet or neutrophil reconstitution. Patients who did not engraft after the first ASCT were censored at time of second infusion. A multivariate Cox proportional hazards regression model was used to estimate the effect of risk factors on platelet and neutrophil reconstitution. Results are presented as hazard ratios (HR) with the corresponding 95% confidence interval (CI).

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Table 1. Demographic and clinical characteristics of the patients

	Overall	^{131}I-MIBG therapy	Chemotherapy-only
Total, *n* (%)	81	38 (47)	43 (53)
Gender			
Male, *n* (%)	45 (56)	25 (66)	20 (47)
Female, *n* (%)	36 (44)	13 (34)	23 (53)
Age			
At diagnosis, years (range)	3.2 (0.1–16.4)	3.3 (0.1–16.4)	3.1 (0.5–15.9)
At ASCT, years (range)	4.1 (1–17.2)	4 (1.4–17.2)	4.1 (1–11.9)
Genetic aberrations			
MYCN amplification, *n/n* measured (%)	28/74 (38)	9/36 (25)	19/38 (50)
LOH1p, *n/n* measured (%)	16/57 (28)	6/24 (25)	10/33 (30)
Metastases at diagnosis			
Bone marrow, *n* (%)	72 (89)	33 (87)	39 (91)
Curie score, median (range)	16.5 (0–30)	16.5 (1–25)	17.0 (0–30)
1st ^{131}I-MIBG dose			
GBq/kg (range)		0.42 (0.13–0.56)	
mCi/kg (range)		11.2 (3.5–15.2)	
2nd ^{131}I-MIBG dose			
GBq/kg (range)		0.37 (0.12–0.69)	
mCi/kg (range)		9.9 (3.2–18.7)	
Cumulative ^{131}I-MIBG dose			
GBq/kg (range)		0.81 (0.26–1.10)	
mCi/kg (range)		22.1 (7–29.8)	
Cumulative dose of cisplatin, mg/m^2 (range)	320 (160–640)	320 (160–480)	320 (160–640)
ASCT, *n* (%)	59 (73)	28 (74)	31 (72)
Patient characteristics before ASCT			
Months since diagnosis, median (range)	7.2 (4.3–12.1)	8.5 (6.2–12.1)	5.8 (4.3–11.4)
Curie score, median (range)	0 (0–17)	0 (0–17)	0 (0–3)
ORR, %	60	61	59
Bone marrow, *n* (%)			
Negative	52 (88)	26 (93)	26 (84)
Positive	1 (2)	1 (4)	0
NE	6 (10)	1 (4)	5 (16)

Data are expressed as median with range or number (%). LOH1p, 1p loss of heterozygosity; NE, not evaluable; ORR, objective response rate (defined as proportion of patients with complete response, very good partial response, or partial response). ^{131}I-MIBG doses are given in both GBq/kg (range) and mCi/kg (range).

Results

Patients' characteristics

Eighty-one children were included: 38/81 (47%) treated with upfront ^{131}I-MIBG-therapy and 43/81 (53%) received chemotherapy-only. The median age (range) at diagnosis was 3.3 (0.1–16.4) years (Table 1). Nearly all patients had BM metastases at diagnosis (72/81; 89%). MYCN-amplification was detected in 9/36 (25%) of ^{131}I-MIBG-treated patients, compared with 19/38 (50%) of chemotherapy-only patients ($P = 0.034$). The enclosed CONSORT figure (Fig. 2) shows the flow of the patients from enrollment to collection and reinfusion of CD34$^+$ cells.

^{131}I-MIBG-therapy

The first median ^{131}I-MIBG dose was 0.42 GBq/kg (range 0.13–0.56)/11.2 mCi/kg (3.5–15.2). For patients treated with two courses, the second median dose was 0.37 GBq/kg (range 0.12–0.69)/9.9 mCi/kg (3.2–18.7) and the total cumulative median dose was 0.81 GBq/kg (range 0.26–1.10)/22.1 mCi/kg (7–29.8; Table 1). Eight patients received only one course of ^{131}I-MIBG, with a median cumulative dose 0.41 GBq/kg (0.17–0.56).

Peripheral stem cell apheresis

Seventy-one patients underwent apheresis: 34 (89%) of the ^{131}I-MIBG-therapy group and 37 (86%) of the chemotherapy-only group. There were no significant differences in timing of apheresis between the chemotherapy-only and ^{131}I-MIBG-therapy group ($P = 0.890$, Fisher exact test). In both groups, median timing of apheresis was after the fourth chemotherapy course (Table 2). Apheresis in ^{131}I-MIBG and chemotherapy-only patient groups yielded a comparable total number of CD34$^+$ cells/kg: median of 5.4×10^6 (range 0.9–32.3) in ^{131}I-MIBG-compared to 5.6×10^6 (range 0.5–44.5) in chemotherapy-only–treated patients (Table 2). The number of apheresis days and sessions required to collect sufficient CD34$^+$ cells were also comparable between both groups: one apheresis day was sufficient to collect $\geq 2 \times 10^6$/kg CD34$^+$ cells in 59% ^{131}I-MIBG-therapy and in 65% chemotherapy-only patients, 2 days in respectively 74% and 76% (Table 3). For 4% of the patients, additional BM harvesting was performed because the number of collected CD34$^+$ cells by apheresis was not sufficient: one patient of the ^{131}I-MIBG -therapy group and two patients of the chemotherapy-only group. A multivariate regression analysis of CD34$^+$ cell harvest yield was performed, showing no association with the cumulative ^{131}I-MIBG dose (Supplementary Table S1). Instead, CD34$^+$ cell harvest yield did significantly associate with BM infiltration at diagnosis, when adjusted for age, gender, MYCN-amplification, LOH of chromosome region 1p, and cumulative dose of both ^{131}I-MIBG and Cisplatin prior to apheresis ($P = 0.004$). Taken together, total harvest yield and collection time (number of days and sessions) of apheresis were comparable between both patient groups, indicating that apheresis is feasible after upfront ^{131}I-MIBG-therapy.

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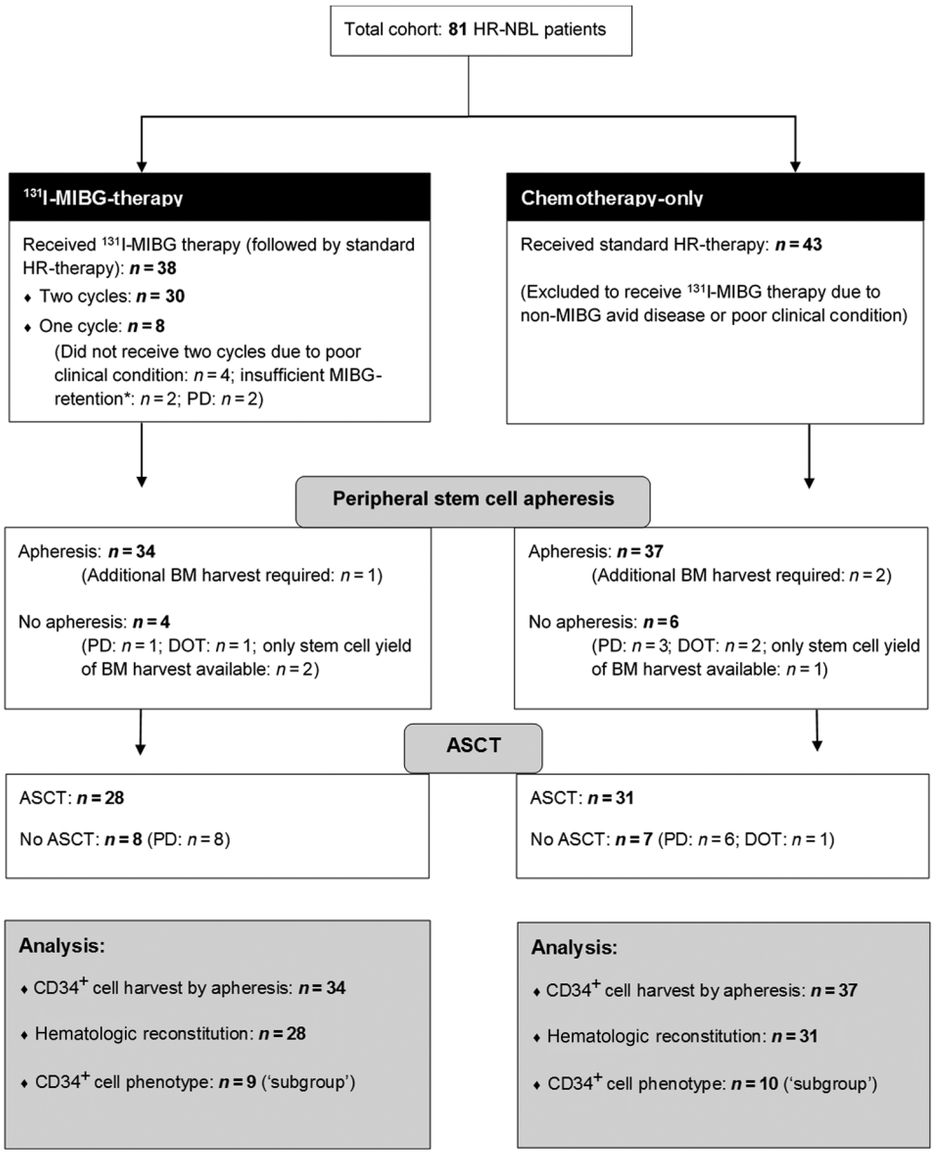

Figure 2.
CONSORT figure. Flow of the patients from enrollment to collection and reinfusion of $CD34^+$cells. *, Insufficient retention: Posttherapeutic scan (3 days after ^{131}I-MIBG-therapy) showed no, or very little, retention of MIBG. ASCT, autologous stem cell transplantation; BM, bone marrow; DOT, died of toxicity; HR, high-risk; PD, progressive disease.

Hematologic recovery after ASCT

Fifty-nine patients underwent ASCT: 28 (74%) of the ^{131}I-MIBG-therapy group and 31 (72%) of the chemotherapy-only group. Patients that did underwent stem cell harvest, but did not proceed to ASCT, had progressive disease (PD; ^{131}I-MIBG group: $n = 8$, chemotherapy-only group: $n = 6$) or died (chemotherapy-only group: $n = 1$; Fig. 2). Median dose (range) of infused $CD34^+$ cells was 3.4×10^6/kg (1.2–10.5) in ^{131}I-MIBG patients and 3.5×10^6/kg (1.2–11.6) in chemotherapy-only patients (Table 2). After ASCT, the median time (95% CI) to platelet reconstitution was 29 days (11–47) and 15 days (12–18) for ^{131}I-MIBG and chemotherapy-only group, respectively (log-rank overall 0.037; Table 2; Fig. 3). The delayed time to platelet reconstitution in ^{131}I-MIBG-treated patients was statistically but not clinically significant, as it did not result in hemorrhages or an extended length of hospital stay. Time to neutrophil reconstitution was respectively 11 days (10–12) and 10 days (refs. 9–11; log-rank overall 0.734; Table 2; Supplementary Fig. S1). A multivariate Cox's regression model was performed to estimate the effect of cumulative ^{131}I-MIBG dose, number of infused $CD34^+$ cells at ASCT and BM infiltration at diagnosis, on both platelet and neutrophil reconstitution. A significant statistical association was found between both cumulative dose of ^{131}I-MIBG (HR 0.395; 95% CI, 0.19–0.85; $P = 0.017$) and number of infused $CD34^+$ cells at ASCT (HR 1.242; 95% CI, 1.1–1.4; $P = 0.001$) with platelet reconstitution (Table 4). Concerning neutrophil reconstitution, there was a significant association with both BM infiltration at diagnosis (HR 0.377; 95% CI, 0.16–0.89; $P = 0.026$) and the number of infused $CD34^+$ cells (HR 1.282; 95% CI, 1.13–1.46; $P < 0.0001$), but not with the cumulative dose of ^{131}I-MIBG (Table 4).

In two patients (^{131}I-MIBG group) successful hematologic reconstitution was only achieved after a second stem cell infusion. A third patient (chemotherapy-only group) suffered from failure

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Table 2. Apheresis and hematologic reconstitution after ASCT

	Overall	^{131}I-MIBG therapy	Chemotherapy-only
Apheresis			
Peripheral stem cell apheresis, *n*	71	34	37
Number of chemotherapy courses before apheresis[a]	4 (1–8)	4 (1–8)	4 (2–7)
Apheresis sessions[a]	1 (1–4)	1 (1–4)	1 (1–2)
Apheresis days[a]	1 (1–8)	1 (1–8)	1 (1–8)
Harvest yield, CD34$^+$ cells $\times$ 10^6/kg[a]	5.4 (0.5–44.5)	5.4 (0.9–32.3)	5.6 (0.5–44.5)
Hematologic reconstitution			
ASCT, *n*	59	28	31
Dose of infused CD34$^+$ cells, CD34$^+$ cells $\times$ 10^6/kg[a] (range)	3.4 (1.2–11.6)	3.4 (1.2–10.5)	3.5 (1.2–11.6)
Platelet reconstitution, days[a] (95% CI)	19 (10–28)	29 (11–47)[b]	15 (12–18)
Neutrophil reconstitution, days[a] (95% CI)	11 (10–12)	11 (10–12)	10 (9–11)

Data are expressed as number (%) or

[a]As median with either range or 95% CI. Chemotherapy before apheresis: one to six courses N5/N6 (max 6 = 3 alternating courses) and three patients received one to two additional courses N8. Neutrophil reconstitution was defined as a neutrophil count >0.5 $\times$ 10^9/L, platelet reconstitution as platelet count >20 $\times$ 10^9/L without platelet transfusions.

[b]$P = 0.037$.

to engraft after two autologous stem cells infusions. Although additional allogeneic cord blood transplantation resulted in neutrophil reconstitution within 12 days, the patient died after 1 month due to septic disease and multiorgan failure, before platelet recovery was achieved.

In conclusion, treatment of patients with HR-NBL with upfront ^{131}I-MIBG results in timely myeloid but delayed platelet reconstitution after ASCT.

Functional and phenotypic testing of CD34$^+$ cells

In search of a possible explanation for the delayed platelet recovery after ASCT in ^{131}I-MIBG-treated patients, we compared the quality of the harvested cells of the two patients groups by analyzing viability. In addition, functional activity of the harvested CD34$^+$ cells was assessed using a colony-forming unit assay that determines clonogenic capacity, that is the capacity to differentiate into granulocyte/macrophage progenitors (CFU-GM). Quality assessment was routinely performed prior to cryopreservation (pre-cryo). Analysis of 81 pre-cryo apheresis samples obtained from 44 (54%) patients showed no significant difference in NBC vitality and clonogenic output (CFU-GM/CD34$^+$ cell) between the^{131}I-MIBG and chemotherapy-only group (Supplementary Fig. S2; Supplementary Table S2). Moreover, CD34$^+$ cells that were collected during the first apheresis did not differ in their clonogenic capacity compared with cells collected after multiple apheresis days (Supplementary Table S2). For a selection of 19 patients (9 ^{131}I-MIBG and 10 chemotherapy-only), CD34$^+$ cell viability and functioning was additionally tested post-thawing, on a separate apheresis aliquot. Although NBC vitality (Fig. 4A) and recovery (Fig. 4B) were comparable, we found a significant lower percentage of viable CD34$^+$ cells in post-thaw apheresis samples of ^{131}I-MIBG- compared with chemotherapy-only–treated patients, 63% and 83% respectively (Fig. 4C). Clonogenic output of CD34$^+$ cells of these 19 patients was highly variable (as commonly observed for CFU-GM), in both pre-cryo and post-thaw samples, and did not significantly

Table 3. Cumulative apheresis days and sessions needed to collect sufficient CD34$^+$ cells

	Overall *N* (Cum %)	^{131}I-MIBG therapy *N* (Cum %)	Chemotherapy-only *N* (Cum %)
Apheresis	71	34	37
Number of days			
1 day	44 (62)	20 (59)	24 (65)
2 days	9 (75)	5 (74)	4 (76)
3 days	4 (80)	3 (82)	1 (78)
4 days	7 (90)	3 (91)	4 (89)
5 days	2 (93)	1 (94)	1 (92)
6 days	NA (NA)	NA (NA)	NA (NA)
7 days	NA (NA)	NA (NA)	NA (NA)
8 days	2 (96)	1 (97)	1 (95)
Failure	3 (4)	1 (3)	2 (5)
Number of sessions			
Session 1	63 (89)	32 (94)	31 (84)
Session 2	4 (95)	NA (NA)	4 (95)
Session 3	1 (96)	1 (97)	NA (NA)

Table displaying the number of patients in whom successful apheresis ($\geq$2 $\times$ 10^6 CD34$^+$ cells/kg) was obtained. The numbers of cumulative apheresis days and sessions are analyzed. Cum % shows the cumulative percentage of patients with successful apheresis at that moment. Harvest failure: the number of collected CD34$^+$ cells by apheresis was not sufficient and additional BM harvesting was required. *N*, number; NA, not applicable.

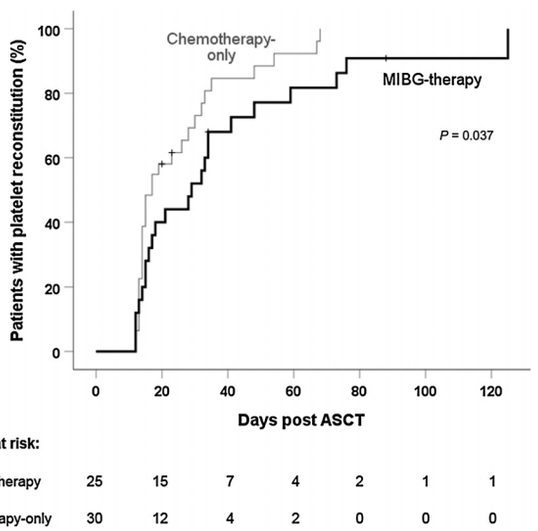

Figure 3.

Time to platelet reconstitution. Cumulative percentage of patients achieving platelet reconstitution after ASCT. ^{131}I-MIBG-therapy group: black line; chemotherapy-only group: gray line. Time to event was defined as time from ASCT until time of platelet engraftment (>20 $\times$ 10^9/L); censor (+) is defined as need for second reinfusion or death. Actual number of patients at different time points is shown below the figure = numbers at risk. *P*-value is based on log-rank test.

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Table 4. Risk factors for platelet and neutrophil reconstitution after ASCT

Platelet reconstitution	**HR (95% CI)**	***P*-value**
Bone marrow infiltration at diagnosis	1.374 (0.58–3.28)	0.474
Cumulative dose of ^{131}I-MIBG	0.395 (0.19–0.85)	0.017[a]
Number of infused CD34$^+$ cells at ASCT	1.242 (1.1–1.4)	0.001[a]
Neutrophil reconstitution	**HR (95% CI)**	***P*-value**
Bone marrow infiltration at diagnosis	0.377 (0.16–0.89)	0.026[a]
Cumulative dose of ^{131}I-MIBG	1.437 (0.68–3.03)	0.341
Number of infused CD34$^+$ cells at ASCT	1.282 (1.13–1.46)	<0.0001[a]

A multivariate Cox regression model was used to estimate the effect of BM infiltration at diagnosis, cumulative ^{131}I-MIBG dose, and number of infused CD34$^+$ cells on platelet and neutrophil reconstitution. Results are presented as hazard ratios (HR), with the corresponding 95% CI.
[a]$P < 0.05$.

differ between the two groups. Median CFU-GM potential (range) prior to cryopreservation was 30.2 $\times$ 10^4/kg (9.0–173.8) in ^{131}I-MIBG- treated patients versus 71.1 $\times$ 10^4/kg (33.0–378.1) in chemotherapy-only patients ($P = 0.203$) and CFU-GM recovery after cryopreservation was comparable (Fig. 4D).

To assess whether the delay in platelet recovery may additionally be due to exhaustion of specific progenitor cell subsets in the transplant, we next tested CD34$^+$ cells phenotypically. Viable CD34$^+$ cells in post-thaw apheresis samples were characterized by markers that indicate early myeloid (CD33) or megakaryocytic (CD41) differentiation using flow cytometry. Cell surface expression of CD33 and CD41 was not significantly different on CD34$^+$ cells of the two patient groups (Fig. 4E and F). We also compared

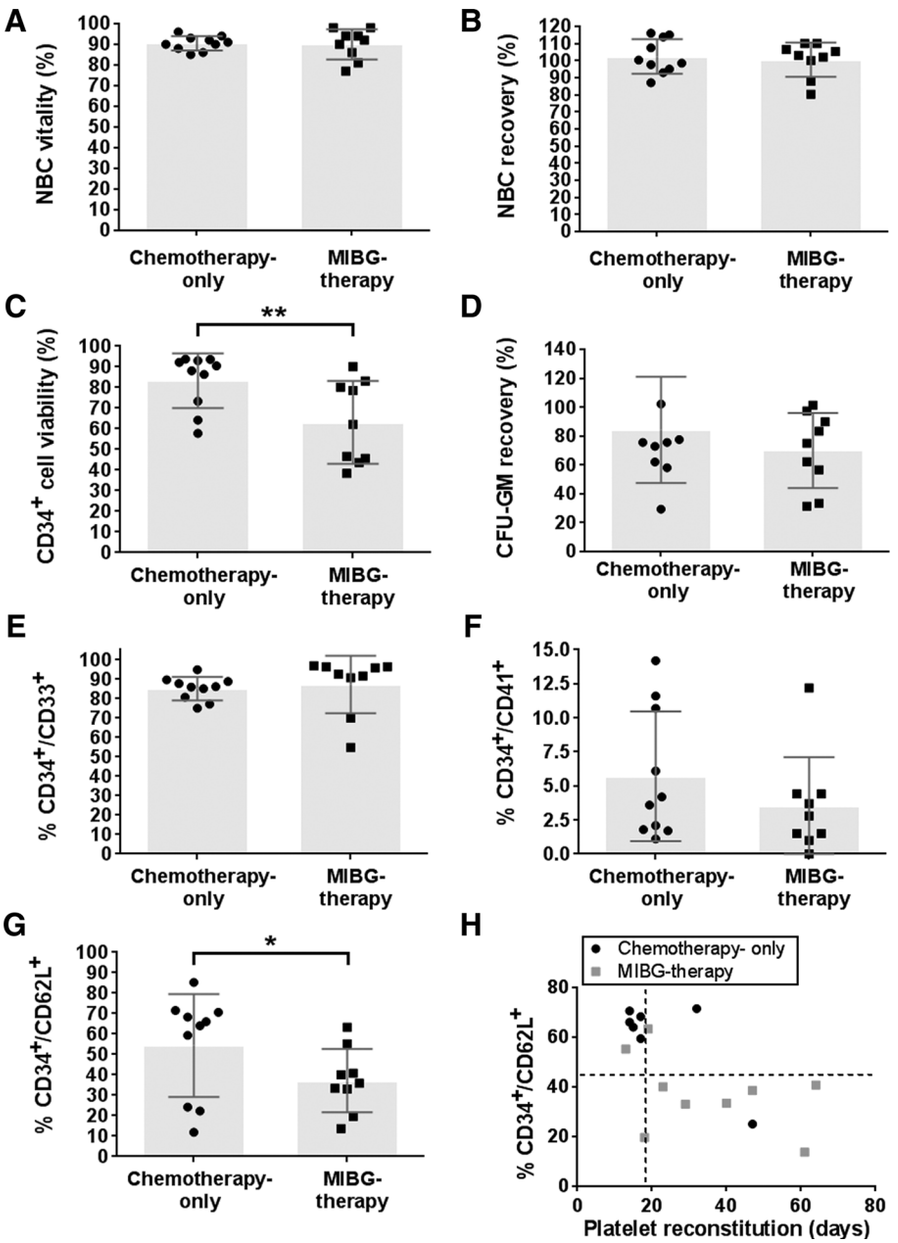

Figure 4.
Post-thaw viability, clonogenic capacity, and adhesion molecule expression of cryopreserved CD34$^+$ cells. Comparison of viability, function, and phenotype of cells in cryopreserved reference apheresis aliquots of a subgroup of 19 patients: 9 ^{131}I-MIBG- and 10 chemotherapy-only–treated patients. **A,** Post-thaw NBC vitality, determined using trypan blue. **B,** Nucleated blood cell recovery: expressed as the percentage of cells recovered after thawing in comparison to the value before cryopreservation. **C,** Percentage of viable CD34$^+$ cells after thawing, determined using 7-AAD, $P = 0.009$. **D,** Clonogenic output: CFU-GM assay. Percentage of CFU-GM recovered after thawing in comparison to the value before cryopreservation. **E,** Percentage of viable CD34$^+$ cells expressing CD33 after thawing. **F,** Percentage of viable CD34$^+$cells expressing CD41 after thawing. **G,** Percentage of viable CD34$^+$ cells expressing CD62L after thawing, $P = 0.048$. **H,** Platelet reconstitution after ASCT and percentage of CD34$^+$ cells expressing CD62L in reference apheresis aliquots of chemotherapy-only (black circle) and ^{131}I-MIBG-therapy (gray square) patient groups, moderate negative correlation: $r = -0.627$, $P = 0.009$. Platelet reconstitution was defined as a platelet count >20 $\times$ 10^9/L. Data are mean (SD). *, $P < 0.05$; **, $P < 0.01$.

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the percentage of viable CD34$^+$ cells expressing the adhesion molecule CD62L (L-selectin), which appeared to be lower in the ^{131}I-MIBG compared with the chemotherapy-only group: 37% and 54%, respectively ($P = 0.0481$; Fig. 4G). Interestingly, CD62L is proposed to be a predictive marker for platelet recovery after ASCT (18). In line, our analysis showed a moderate negative correlation ($r = -0.627$, $P = 0.009$) between the percentage of re-infused CD62L-expressing CD34$^+$ cells and the time to platelet recovery (Fig. 4H). Thus, the post-thaw viable CD34$^+$ cell count was lower in apheresis samples of ^{131}I-MIBG-treated patients and expression of CD62L, a predictive marker for platelet recovery, was reduced.

Discussion

^{131}I-MIBG is an important established treatment for relapsed or refractory NBL and its efficacy is currently investigated in front-line setting. The optimal timing of ^{131}I-MIBG-therapy during front-line treatment is not yet established. Pilot studies have demonstrated feasibility when given at the time of diagnosis (10, 14, 19) and cooperative groups in both Europe and North America currently investigate its use as part of induction or consolidation therapy (www.clinicaltrials.gov: NCT03126916; NCT01175356, NCT03165292; ref. 13). When given as front-line treatment, ^{131}I-MIBG-therapy is mostly followed by ASCT. Therefore, there is an urgent need to get insight in the impact of ^{131}I-MIBG on stem cell apheresis and on engraftment after reinfusion.

By studying our unique upfront ^{131}I-MIBG-therapy cohort, we found that stem cell apheresis is feasible post-MIBG. Treating patients with ^{131}I-MIBG early in induction did not affect the total CD34$^+$ cell harvest yield and did not extend the apheresis episode. Failure to harvest sufficient CD34$^+$ cells by apheresis occurred in only one ^{131}I-MIBG- and two chemotherapy-only–treated patients. Of interest, our findings indicate that BM tumor infiltration at diagnosis did impair the mobilization of CD34$^+$ cells, as described for other tumors (20), even though apheresis only started after clearing of initial BM disease. Concerning the timing of apheresis, there are different approaches: harvesting is performed after two induction chemotherapy courses in North America, as the Children's Oncology group previously showed that this was safe and feasible (21), whereas the consensus in Europe is still to harvest stem cells after the BM is cleared from tumor cells or post induction therapy. The cumulative median ^{131}I-MIBG dose administered to the newly diagnosed patients in our study was relatively high compared to the reported maximum tolerated dose of 12 mCi/kg for intensively pretreated patients (6), but no stem cell rescue was required. Toxicity and efficacy of upfront ^{131}I-MIBG-therapy, also for part of this cohort, has been previously described (10, 14, 19). Of note, comparisons between the two patients groups should be interpreted with caution as this study was nonrandomized and patients of the chemotherapy-only group were excluded to receive ^{131}I-MIBG-therapy because of poor clinical condition or non-MIBG avid disease.

After reinfusion of the collected CD34$^+$ cells, time to neutrophil reconstitution was similar in ^{131}I-MIBG- compared with chemotherapy-only –treated patients, but time to platelet reconstitution was prolonged. More prominent thrombocytopenia than neutropenia has been previously described for ^{131}I-MIBG in intensively pretreated NBL patients (6, 22–24). This differential toxicity towards platelets and neutrophils might, in part, be related to selective uptake of ^{131}I-MIBG by platelets or their precursors (25). The prolonged time to platelet reconstitution that we observed was not major, that is, it did not result in hemorrhages or an extended length of hospital stay. Nevertheless, the duration of thrombocytopenia after treatment with ^{131}I-MIBG and ASCT could delay additional treatment of an aggressive tumor. Hence, in light of shortening of platelet engraftment periods, we further searched for potential explanations for the ^{131}I-MIBG-related delay in recovery.

Our in-depth analysis of the quality of harvested cells from a subgroup of 19 patients revealed that post-thaw aliquots of ^{131}I-MIBG-treated patients contained lower viable CD34$^+$ cell counts. As no significant differences in harvest quality were observed in pre-cryo samples, this suggests that CD34$^+$ cells of ^{131}I-MIBG-treated patients are more sensitive to cryopreservation, which might result in reinfusion of a lower actual number of viable CD34$^+$ cells than estimated. A dose–response relationship between re-infused CD34$^+$ cells and hematologic recovery was found by us and others (26, 27). Below a threshold of 1×10^6 CD34$^+$ cells/kg, the likelihood of delayed recovery of platelets was demonstrated to increase significantly (28). We therefore attempted to achieve a minimum number of 2.0×10^6. However, these thresholds are set based on the amount at the time of collection. Based on our findings, it would be valuable to include quantification of post-thaw viable CD34$^+$ cells, which is also proposed by others as a more accurate predictor of hematologic reconstitution (29).

Delay in platelet recovery may additionally be explained by exhaustion of specific progenitor cell subsets (18, 30, 31). We showed that the percentage of CD62L-expressing viable CD34$^+$ cells was reduced in apheresis aliquots of ^{131}I-MIBG-treated patients. A correlation between the number of re-infused CD34$^+$/CD62L$^+$ cells and platelet recovery was previously described, and suggests a role for CD62L in engraftment (18, 31, 32). CD62L-mediated rolling of CD34$^+$ cells on the endothelium is suggested to be a critical step in the homing process to the BM. Although involvement of CD62L in megakaryopoiesis has also been proposed, this requires further investigation as blocking of the CD62L-ligand interaction in CFU-megakaryocyte (CFU-MK) assays did not impair clonogenic outgrowth of CD34$^+$ cells into megakaryocyte progenitors (33).

Considering that therapy for HR-NBL is intense with high doses of different chemotherapeutics, and the need to harvest 6×10^6/kg CD34$^+$ cells in current high-risk protocols for tandem transplants, we advise that determination of viable CD34$^+$ cell counts in post-thaw samples should be part of the routine quality assessment. Nevertheless, solely post-thaw CD34$^+$ cell viability does not necessarily correlate with engraftment for some patients. For example, one patient had to undergo allogeneic cord blood stem cell transplantation after engraftment failure of both autologous harvests, despite adequate post-thaw viable CD34$^+$ cell counts. Unfortunately, no functional testing could be performed because no aliquots remained after the two reinfusions. Combining post-thaw values of CD34$^+$ cell counts with functional (CFU-GM) testing is expected to further improve routine quality assurance (34). Larger prospective cohort studies should be performed to explore whether determination of CD62L status is a useful addition to CD34$^+$ cell testing.

In conclusion, we provide evidence that CD34$^+$ cell harvesting is feasible after upfront ^{131}I-MIBG-therapy in newly diagnosed patients with HR-NBL. After reinfusion, timely neutrophil but delayed platelet reconstitution occurred in ^{131}I-MIBG- compared with chemotherapy-only–treated patients. Our findings suggest

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that ^{131}I-MIBG-treated patients with prior BM tumor infiltration should be monitored more closely and the minimum acceptable number of CD34$^+$ cells/kg for reinfusion should be based on post-thaw viability counts, but the impact does not seem to be so great as to preclude the upfront use of ^{131}I-MIBG in these patients. Nevertheless, in light of our findings, ^{131}I-MIBG administration post CD34$^+$ cell collection is preferred, as will be further studied in upcoming prospective trials (www.clinicaltrials.gov: NCT03126916; NCT03165292).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Peripheral Stem Cell Apheresis is Feasible Post ^{131}Iodine-Metaiodobenzylguanidine-Therapy in High-Risk Neuroblastoma, but Results in Delayed Platelet Reconstitution

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