

# Higher CD34<sup>+</sup> and CD3<sup>+</sup> Cell Doses in the Graft Promote Long-Term Survival, and Have No Impact on the Incidence of Severe Acute or Chronic Graft-versus-Host Disease after In Vivo T Cell-Depleted Unrelated Donor Hematopoietic Stem Cell Transplantation in Children

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The aim of our study was to compare the results of unrelated donor (UD) peripheral blood stem cell transplantation versus UD bone marrow transplantation and to analyze the impact of infused CD34<sup>+</sup> and CD3<sup>+</sup> cell doses on survival and incidence of severe graft-versus-host disease (GVHD) in 187 children who underwent UD hematopoietic cell transplantation with the use of in vivo T cell depletion (antithymocyte globulin or CAMPATH-1H). HLA typing was performed at the “high-resolution” level. Patients receiving  $\geq 10 \times 10^6$  CD34<sup>+</sup> cells/kg and  $\geq 4 \times 10^8$  CD3<sup>+</sup> cells/kg had better overall and disease-free survival. Multivariate analysis has shown that both infused CD34<sup>+</sup> cell dose  $< 10 \times 10^6$ /kg and CD3<sup>+</sup> cell dose  $< 4 \times 10^8$ /kg were independent risk factors for mortality (relative risk [RR] 1.8 and 1.71,  $P = .009$  and  $.016$ , respectively). Regarding disease-free survival, multivariate analysis has revealed another independent risk factor for poor outcome apart from the 2 earlier-mentioned cell doses, which was the use of donors mismatched at 2 HLA antigens or 3 HLA allele/antigens (RR 2.5,  $P = .004$ ). In age groups 0-10 years and 10-20 years, CD34<sup>+</sup> cell doses higher than the age-adjusted median dose clearly favored survival. Higher infused doses of CD34<sup>+</sup> and CD3<sup>+</sup> cells did not result in an increased rate of severe GVHD. The use of mismatched donors was the only independent risk factor for the incidence of severe acute GVHD (RR 2.2,  $P = .046$ ). The report demonstrates for the first time in a pediatric cohort, that higher doses of transplanted CD34<sup>+</sup> and CD3<sup>+</sup> cells lead to an improved survival without an increased risk of severe GVHD. The study findings may be limited to the population of patients receiving in vivo T cell depletion, which is now broadly used in unrelated donor setting in Europe.

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**KEY WORDS:** Hematopoietic stem cell transplantation, Unrelated donor, Graft-versus-host disease, Children, CD34<sup>+</sup> cell dose, CD3<sup>+</sup> cell dose, Graft content

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## INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) has become a standard therapy for children with a variety of both malignant and nonmalignant diseases [1]. In Poland, only approximately 20% of children have an HLA matched sibling donor (MSD) available. The remaining 80% of patients have to be transplanted from alternative donors. For children with rare HLA haplotypes, it remains extremely difficult and time consuming to find a fully matched unrelated donor (MUD). It is generally acceptable to use unrelated donors (UD) mismatched at 1 HLA allele or antigen. Such UD are categorized as MUD, together with fully matched ones (10/10-allele matched).

According to the acute lymphoblastic leukemia (ALL) international BFM SCT 2008 (Vienna, Austria) transplant protocol, UD HSCT from donors mismatched at more than 1 HLA antigen or allele, requires extensive in vitro T cell depletion. There are, however, two published studies confirming low mortality of children undergoing HSCT from 7 to 8/10 HLA allele-matched UD with the use of antithymocyte globulin (ATG), but not T cell depletion in vitro [2,3].

There is an unresolved question whether unmanipulated UD-peripheral blood stem cell transplantation (PBSCT) leads to a worse overall outcome, and a higher incidence of severe graft-versus-host disease (GVHD) in both adults and children, in comparison with well-established UD bone marrow transplantation (BMT). It is well known that PBSC grafts contain approximately 1 log more mature T cells, which may result in a higher rate of severe (often fatal) acute or/and chronic GVHD (aGVHD, cGVHD) posttransplant.

The aim of our study was to compare the results of UD PBSCT versus UD BMT and analyze the impact of CD34<sup>+</sup> and CD3<sup>+</sup> cell doses in the grafts in a large pediatric patient population. PBSC grafts with larger amounts of CD34<sup>+</sup> and CD3<sup>+</sup> cells may lead to a prompt engraftment and improved outcome because of a more pronounced graft-versus-malignancy effect [4,5]. However, studies on both adults and children show discrepant results with regard to overall survival (OS) and occurrence of aGVHD and cGVHD after UD PBSCT [6-12]. Based on the fact that the majority of volunteer donors in Germany and Poland prefer (sometimes exclusively) PBSC donation, the number of UD PBSCT in our center has increased steadily over the last decade. To our knowledge, there is no published study with a higher number of UD PBSCTs performed in children and adolescents. Furthermore, data on the impact of CD34<sup>+</sup> and CD3<sup>+</sup> cell doses in the UD graft on the OS and disease-free survival (DFS) and aGVHD/cGVHD is scarce for adults [13-17] and virtually nonexistent for children. In this multivariate detailed analysis of different factors influencing the outcome and the incidence of severe GVHD post-UD HSCT, we demonstrate for the first time in a large pediatric cohort, that higher doses of transplanted CD34<sup>+</sup> and CD3<sup>+</sup> cells lead to improved survival without an increased risk of severe GVHD, provided that in vivo T cell depletion is used.

## MATERIALS AND METHODS

### Patients

Between the years 2000 and 2008, 187 children and adolescents (112 males and 75 females) with malignant (n = 155) and nonmalignant disorders (n = 32)

underwent unmanipulated allogeneic UD HSCT at our center (Table 1). Their median age at transplant was 9.6 years (range: 0.6-20.2 years). All patients with malignant disorders were given disease-specific myeloablative (MA) conditioning regimens according to European protocols, except for those receiving a second transplant, who were grafted with disease-adapted reduced-toxicity regimens including treosulfan (instead of busulfan). Total body irradiation (TBI) was used in 51 patients. Patients with nonmalignant disorders were conditioned according to the guidelines of the European Blood and Marrow Transplant (EBMT) Inborn Errors Working Party except for patients with severe aplastic anemia (SAA) conditioned mostly with treosulfan and cyclophosphamide. For GVHD prophylaxis, rabbit ATG (Fresenius, median dose 30 mg/kg) was used in 160 patients, whereas 22 children received Thymoglobuline (Genzyme, median dose 10 mg/kg) and 5 other patients Campath-1H (median dose 1 mg/kg). Standard Cyclosporine A (CsA) and short methotrexate (MTX) were added to GVHD prophylaxis. The risk status of the patients at transplant was assessed by our own modification of the classification proposed by Meisel et al. [12]: patients with acute lymphoblastic leukemia (ALL)/acute myelogenous leukemia (AML) in first and second complete remission (CR), chronic myelogenous leukemia (CML) in the first chronic phase, myelodysplastic syndrome (MDS)-refractory cytopenia, non-Hodgkin lymphoma (NHL) in CR, and any nonmalignant disease were considered standard risk (n = 118), whereas children with ALL/AML in equal to or greater than the third CR or nonremission, CML equal to or greater than the accelerated phase, MDS-refractory anemia with excess of blasts, juvenile myelomonocytic leukemia (JMML), NHL in nonremission, and patients undergoing any second transplant were classified as high risk (n = 69).

The grafted cells were obtained from HLA-allele matched or mismatched UDs (age 19-56 years, median

**Table 1. Primary Diagnosis (n = 187 Patients)**

Malignant	N	Nonmalignant	N
ALL	67	SAA	13
AML	33	Fanconi Anemia	5
CML	25	X-ALD	4
MDS	18	SCID	3
NHL	5	WAS	2
JMML	4	Omenn Syndrome	2
HD	2	X-CGD	2
LCAL	1	Griselli Syndrome	1
Total	155	Total	32

ALL indicates acute lymphoblastic leukemia; AML, acute myelogenous leukemia; CML, chronic myelogenous leukemia; MDS, myelodysplastic syndrome; NHL, non-Hodgkin lymphoma; JMML, juvenile myelomonocytic leukemia; HD, Hodgkin disease; LCAL, large cell anaplastic lymphoma; SAA, severe aplastic anemia; X-ALD, X-linked adrenoleukodystrophy; SCID, severe combined immunodeficiency disease; WAS, Wiskott-Aldrich syndrome; X-CGD, X-linked chronic granulomatous disease.

34 years). HLA typing was performed at the high-resolution level (4 digits) in A\*, B\*, Cw\*, DRB1\*, and DQB1\* alleles. There were 159 matched- and 28 mismatched donor-recipient pairs, respectively (according to ALL international BFM SCT 2008 criteria, all 10 alleles were high-resolution typed). Table 2 presents the detailed distribution of different combinations of HLA-mismatched donor-recipient pairs. Table 3 provides details on the patient sex, age, diagnosis, risk status at transplant, donor age, donor/recipient CMV-status and blood group compatibility, year of transplant (2000-2004 versus 2005-2008), TBI use, degree of HLA-match and data on neutrophil and platelet recovery within the 3 groups divided according to stem cell source (peripheral blood [PB] versus BM), and number of infused CD34<sup>+</sup> and CD3<sup>+</sup> cells in the graft. Median number of transplanted CD34<sup>+</sup> cells was  $11.3 \times 10^6/\text{kg}$  (range: 0.8-57.8) in the PBSC group and  $3.4 \times 10^6/\text{kg}$  (range: 0.8-11.0) in the BM group ( $P < .001$ ). Median number of transplanted CD3<sup>+</sup> cells was  $5.2 \times 10^8/\text{kg}$  (range: 0.2-20.0) in the PBSC group and  $0.4 \times 10^8/\text{kg}$  (range: 0.04-10.1) in the BM group ( $P < .001$ ).

The study was approved by the local Ethics Committee, and patients over 18 years old or parents of patients under 18 years signed informed consents.

### Aims of the Study

As the endpoints of this prospective study, hematologic recovery, OS, DFS, the incidence of severe acute

GVHD (aGVHD grade III-IV) and extensive chronic GVHD (cGVHD) were compared within 3 study cohorts of patients:

1. Recipients of PBSC versus BM;
2. Patients grafted with higher CD34<sup>+</sup> cell dose ( $\geq 10 \times 10^6/\text{kg}$ ) versus those grafted with lower dose ( $< 10 \times 10^6/\text{kg}$ ).
3. Patients grafted with higher CD3<sup>+</sup> cell dose ( $\geq 4 \times 10^8/\text{kg}$ ) versus those grafted with lower dose ( $< 4 \times 10^8/\text{kg}$ ).

The cell dose cutoffs were chosen by taking 2 approximately similar (in numbers) cohorts of children. The cell dose of  $10 \times 10^6$  CD34/kg was slightly higher than median ( $9.72 \times 10^6$  CD34/kg), so we rounded the division value up to  $10 \times 10^6$ . Similarly, the cell dose of  $4 \times 10^8$  CD3/kg was slightly lower than the median ( $4.26 \times 10^8/\text{kg}$ ), so we rounded the division value down to  $4 \times 10^8/\text{kg}$ . We concluded that values close to medians were good cutoff points for cell doses (as in NMDP study) [17].

To avoid a possible bias in favor of higher CD34<sup>+</sup> cell doses in younger patients, we analyzed separately the age groups 0-10 years and 10-20 years and then 0-5 years, 5-10 years, 10-15 years, and older. We looked at the influence of CD34<sup>+</sup> or CD3<sup>+</sup> cell dose (above and below age-adjusted medians) on OS, DFS, and both severe aGVHD or extensive cGVHD in relatively homogenous smaller age groups. Ten years was chosen to be the age cutoff because it was close to the median (9.6 years), so we rounded up the cutoff value to 10.

In addition, a parallel subanalysis of patients grafted with different CD34<sup>+</sup> and/or CD3<sup>+</sup> cell doses was performed using a method (analysis of quartiles) similar to the one described in a recently published NMDP study [16].

Furthermore, the secondary aim of our study was to analyze the impact of HLA mismatch on the outcomes.

Hematologic recovery was defined as the achievement of an absolute neutrophil count (ANC)  $> 0.5 \times 10^9/\text{L}$  and/or platelet count  $> 50 \times 10^9/\text{L}$  for 3 consecutive days, respectively. For the analysis of cGVHD, only patients surviving beyond day +100 were taken into consideration. GVHD was assessed according to well-known clinical symptoms, laboratory tests, and histopathology, as applicable. aGVHD and cGVHD were graded according to the criteria described previously and adapted for children [18,19]. aGVHD was treated mostly with methylprednisolone and CsA. In case of steroid resistance nonstandardized fashion including ATG, etanercept, or mycophenolate mofetil (MMF) or a combination of 3-4 immunosuppressive drugs was used. Extensive cGVHD was treated with methylprednisolone, CsA, etanercept, and/or MMF.

**Table 2. Types of HLA-Mismatched Donor-Recipient Pairs**

Full allele match (10/10) – A*B*Cw*DRB1*DQB1*	94/187 (50.26%)
One antigen or allele mismatch (9/10)	65 (47+18) / 187 (34.76%)
A*-mismatch	14 (9+5)
B*-mismatch	8 (2+6)
Cw*-mismatch	33 (30+3)
DRB1*-mismatch	2 (0+2)
DQB1*-mismatch	8 (6+2)
Two allele/antigen mismatch (8/10)	23/187 (12.29%)
Two antigen mismatch (8 ant)	
B*+Cw*	3
A*+Cw*	3
Cw*+Cw*	1
Two allele or two antigen + allele mismatch	
B*+Cw*	6
C*+DQB1*	3
B*+DQB1*	2 (1+1)
A*+B*	2 (1+1)
Cw*+Cw*	1
A*+Cw*	1
A*+A*	1
Three allele/antigen mismatch (7/10)	5/187 (2.67%)
B*+Cw*+Cw*	1
B*+Cw*+Cw*	1
A*+B*+Cw*	1
B*+B*+DQB1*	1
B*+B*+Cw*	1

8 ant indicates abbreviation for the group of two HLA antigen-mismatched recipient pairs; antigen mismatches are depicted as first number in brackets.

**Table 3. Characteristics of the Study Cohorts: Univariate Analysis of Multiple Variables**

Variables	Stem Cell Source		P*	CD34 <sup>+</sup> Cell Dose		P†	CD3 <sup>+</sup> Cell Dose		P‡
	PB	BM		N	Median (SE)		N	Median (SE)	
Recipient sex			.680			.487			.535
male	88 (60.7)	24 (57.1)		75	9.42 (0.86)		73	4.33 (0.35)	
female	57 (39.3)	18 (42.9)		112	9.78 (1.27)		110	4.11 (0.48)	
Recipient age			.256			.000			.000
≥10 years	65 (44.8)	23 (54.8)		89	7.20 (0.56)		86	3.07 (0.27)	
<10 years	80 (55.2)	19 (45.2)		98	12.13 (1.17)		97	5.64 (0.44)	
Donor age			.883			.870			.741
≥35 years	64 (44.1)	18 (42.9)		82	9.90 (1.09)		81	3.70 (0.45)	
<35 years	81 (55.9)	24 (57.1)		105	9.37 (0.98)		102	4.36 (0.37)	
Sex match			.399			.013			.813
female to male	23 (15.9)	9 (21.4)		32	6.77 (1.26)		31	4.97 (0.73)	
other	122 (84.1)	33 (78.6)		155	9.97 (0.83)		152	4.13 (0.31)	
Recipient CMV-status			.579			.883			.859
negative	57 (43.5)	18 (48.7)		75	9.81 (1.11)		72	4.49 (0.43)	
positive	74 (56.5)	19 (51.4)		93	9.47 (1.00)		92	4.27 (0.39)	
Donor CMV-status			.178			.912			.389
negative	76 (58.0)	26 (70.3)		102	9.55 (0.96)		99	3.55 (0.41)	
positive	55 (42.0)	11 (29.7)		66	9.64 (1.17)		65	4.94 (0.36)	
ABO match			.174			.915			.966
matched	43 (29.7)	8 (19.1)		51	9.37 (1.28)		49	3.70 (0.51)	
mismatched	102 (70.3)	34 (81.0)		136	9.76 (0.88)		134	4.53 (0.34)	
Diagnosis			.309			.001			.014
nonmalignant	27 (18.6)	5 (11.9)		32	15.64 (1.88)		31	5.24 (0.79)	
malignant	118 (81.4)	37 (88.1)		155	8.98 (0.77)		152	3.80 (0.29)	
Risk status‡			.855			.480			.614
standard	92 (63.5)	26 (61.9)		118	9.84 (0.83)		115	3.94 (0.37)	
high risk	53 (36.6)	16 (38.1)		69	9.17 (1.37)		68	4.81 (0.45)	
Degree of HLA match			.701			.465			.678
10\10	71 (49.0)	23 (54.8)		94	9.18 (0.89)		93	3.75 (0.40)	
9\10	50 (34.5)	15 (35.7)		65	9.72 (1.21)		63	4.46 (0.52)	
8\10	20 (13.8)	3 (7.1)		23	9.11 (2.80)		23	4.97 (0.67)	
7\10	4 (2.8)	1 (2.4)		5	11.27 (6.82)		4	5.71 (1.03)	
BFM match status			.261			.525			.346
matched (≥9/10)	121 (83.5)	38 (90.5)		159	9.47 (0.72)		156	4.02 (0.32)	
mismatched (≤8/10)	24 (16.6)	4 (9.5)		28	10.19 (2.58)		27	4.97 (0.59)	
Stem cell source						.000			.000
peripheral blood				145	11.27 (0.84)		143	5.15 (0.30)	
bone marrow				42	3.43 (0.39)		40	0.40 (0.29)	
Year of transplant			.000			.038			.002
2000-2004	43 (62.3)	26 (37.7)		69	7.00 (1.58)		65	2.45 (0.45)	
2005-2008	102 (86.4)	16 (13.6)		118	10.06 (0.69)		118	4.94 (0.35)	
TBI			.317			.014			.103
TBI	37 (72.5)	14 (27.4)		51	8.63 (0.73)		51	3.55 (0.28)	
non-TBI	108 (79.4)	28 (20.6)		136	10.57 (0.93)		132	4.72 (0.36)	

PB indicates peripheral blood; BM, bone marrow; CMV, cytomegalovirus; ABO, blood group; HLA, human leukocyte antigen; BFM, stem cell transplant protocol from Berlin-Frankfurt-Münster group; TBI, total body irradiation; ANC, day of absolute neutrophil count recovery  $>0.5 \times 10^9/L$ ; PLT, day of platelet count recovery  $>50 \times 10^9/L$ .

Univariate analysis was performed by  $\chi^2$ -test\* and Mann-Whitney U/Kruskal-Wallis test† used for nonparametric variables.

‡Standard risk: ALL/AML in first and second complete remission (CR), CML in first chronic phase, MDS-refractory cytopenia, NHL in CR, any nonmalignant disease; high risk: ALL/AML in equal to or greater than third CR or nonremission, CML equal to or greater than accelerated phase, MDS-refractory anemia with excess of blasts, JMML, NHL in nonremission, any second transplant.

## Statistical Analysis

For statistical comparisons between the 3 studied cohorts of patients, a chi-square test was used for categorical parameters, whereas Mann-Whitney *U* or Kruskal-Wallis tests were performed for nonparametric continuous variables. Kaplan-Meier/cumulative incidence methods and log-rank/Gray tests were used for comparison of time-dependent endpoint outcome parameters within the studied patient groups described earlier [20,21]. Variables were analyzed for prognostic

impact on OS, DFS, and the incidence of severe GVHD (aGVHD grade III-IV and extensive cGVHD) and included: recipient sex, sex match, donor and recipient age, donor and recipient CMV status, ABO blood group compatibility, patients' diagnosis and risk group, stem cell source (PB versus BM), infused CD34<sup>+</sup> and CD3<sup>+</sup> cell dose per kg, year of transplant (2000-2004 versus 2005-2008), conditioning regimen (TBI versus non-TBI) and degree of donor-recipient HLA match. Only variables with

a  $P$ -value  $<.2$  in univariate analysis were included into the final multivariate analysis-proportional hazard (Cox) regression with a backward stepwise approach [22]. For aGVHD and cGVHD, death without an event, as well as graft rejection were the competing risks. Because of a strong correlation between  $CD34^+$  and  $CD3^+$  cell doses, two possible models of multivariate analysis for OS and DFS are presented. Furthermore, two possible models of multivariate analysis for aGVHD grade III-IV are shown, one including BFM match status (matched versus mismatched) and the other including HLA match variant (graft mismatched at 2 HLA antigens or 3 antigen/alleles versus other). For the cell dose variables, the optimal cut point was determined by taking age-adjusted medians. Because of a well-known correlation of  $CD34^+$  cell doses with age, probabilities of survival were calculated within groups of patients 0-5 years, 5-10 years, 10-15 years, and 15-20 years old, and 0-10 years and 10-20 years old. The  $P$ -values are 2-sided and a significance level of .05 was used as relevant. Statistical analyses were performed using Statistica for Windows 8.0 and R-2.10.1 statistical software package [23].

## RESULTS

### Study Cohorts

One hundred eighty-seven patients were prospectively included in the study: 145 in the PB, and 42 in the BM groups. The decision about the type of transplant material was made by the donor together with the local harvest center, and was only in part influenced by the transplant center's preference. Patient, donor, and transplant characteristics were fully comparable between the 2 cohorts except for the expected higher infused  $CD34^+$  and  $CD3^+$  cell dose in the PB group and the year of transplant (Table 3). Patients in the PB group were given statistically significant 3.3 times more  $CD34^+$  cells/kg and 12.9 times more ( $>1$  log)  $CD3^+$  cells/kg when compared with the BM group ( $P < .001$ ). The cohorts of patients analyzed according to infused  $CD34^+$  cell dose/kg were comparable in terms of patient, donor, and transplant characteristics except for sex match, diagnosis, year of transplant, the use of TBI, and, as expected, recipient age and stem cell source (Table 3). The cohorts of patients analyzed according to infused  $CD3^+$  cell dose were comparable except for diagnosis, year of transplant and, as expected, recipient age and stem cell source (Table 3). The cohorts of patients analyzed according to diagnosis (malignant versus nonmalignant) were comparable except for, as expected, the age ( $P < .05$ ),  $CD34^+$  and  $CD3^+$  cell doses ( $P < .05$ ), risk group ( $P < .01$ ), and the use of TBI ( $P < .001$ ) (data not shown).

### Hematologic Recovery

The median day of ANC recovery ( $>0.5 \times 10^9/L$ ) was 14 for PBSC and 18 for BM recipients ( $P < .001$ ). Five patients died without achieving neutrophil reconstitution on days +6, +10, +11, +15, and +26 post-transplant, respectively, because of sepsis in 3 cases and heart failure or renal insufficiency in the remaining 2 patients. The median day of platelet recovery was 18 for PBSC and 30 for BM recipients ( $P < .001$ ). Time to neutrophil engraftment was significantly shorter in patients receiving higher doses of both  $CD34^+$  ( $\geq 10 \times 10^6$ ) and  $CD3^+$  ( $\geq 4 \times 10^8$ ) cells/kg (median day 14 and 16;  $P < .001$  and  $P = .003$ , respectively). Similarly, platelet recovery in patients who received higher doses of both  $CD34^+$  and  $CD3^+$  cells/kg was achieved 9 and 6 days earlier, respectively (median day 16 and 18 versus 25 and 24;  $P < .001$  and  $P = .002$ , respectively).

### OS and DFS (Tables 4 and 5)

With a median follow-up of 3.2 years (range: 0.8-7.6 years, PB group) and 5.4 years (range: 2.3-8.8 years, BM group) there was a statistically significant advantage in DFS of patients given PBSC versus BM ( $0.55 \pm 0.04$  versus  $0.4 \pm 0.08$ ,  $P < .05$ , log-rank, Figure 1). In terms of OS, there was a trend toward better OS in the PB group ( $0.56 \pm 0.04$  versus  $0.43 \pm 0.08$ ,  $P = 0.065$ , log-rank, Figure 1). As expected, patients at high risk had a poorer DFS in comparison with standard risk patients ( $0.41 \pm 0.06$  versus  $0.58 \pm 0.05$ ,  $P < .05$ ). There was no difference in OS and DFS between patients transplanted from MUDs or mismatched UD (including separately 10/10, 9/10, 8/10, allele matched transplants, Table 4) despite the fact that patients transplanted from mismatched donors were mostly (57.1%) high risk patients according to BFM criteria, whereas the group of children transplanted from matched donors included significantly fewer high risk individuals (33.3%,  $P = .016$ ,  $\chi^2$ -test).

The group of 7/10-allele matched transplanted children was inconclusive in terms of statistics because of the low number of patients. The most striking statistically significant differences in OS and DFS were observed when comparing groups receiving higher and lower doses of both  $CD34^+$  and  $CD3^+$  cells in the graft. Patients receiving  $\geq 10 \times 10^6$  and  $\geq 15.4 \times 10^6$  ( $>75$ th percentile)  $CD34^+$  cells/kg had OS (and DFS) probability of 0.64 and 0.69 (0.60 and 0.67), respectively versus 0.44 and 0.47 (0.44 and 0.46) when given less than the previously mentioned amounts. Similarly patients given  $\geq 4 \times 10^8$  and  $\geq 6.9 \times 10^8$  ( $>75$ th percentile)  $CD3^+$  cells/kg had OS (and DFS) probability of 0.61 and 0.71 (0.60 and 0.69), respectively, versus 0.45 and 0.48 (0.43 and 0.46) when infused less than the earlier-mentioned amounts.

**Table 4. Influence of Multiple Variables on Clinical Outcome by Univariate Analysis (Log-Rank\* or Gray Test†)**

Variables	Overall Survival*				Disease-Free Survival*				aGVHD Grades III-IV†				Extensive cGVHD†			
	N	%	SE	P	N	%	SE	P	N	%	SE	P	N	%	SE	P
Recipient sex				.658				.552				.297				.504
male	112	0.51	0.05		112	0.49	0.05		112	0.25	0.04		93	0.16	0.04	
female	75	0.56	0.06		75	0.55	0.06		75	0.18	0.05		63	0.20	0.05	
Recipient age				.028				.050				.085				.755
≥10 years	89	0.44	0.06		89	0.43	0.05		89	0.16	0.04		70	0.16	0.05	
<10 years	98	0.62	0.05		98	0.59	0.05		98	0.27	0.05		86	0.14	0.04	
Donor age				.142				.202				.903				.578
≥35 years	82	0.49	0.06		82	0.48	0.06		82	0.22	0.05		62	0.13	0.05	
<35 years	105	0.56	0.05		105	0.54	0.05		105	0.21	0.04		94	0.16	0.04	
Sex match				.775				.994				.267				.542
female to male	32	0.45	0.12		32	0.48	0.11		32	0.28	0.08		28	0.11	0.07	
other	155	0.54	0.04		155	0.52	0.04		155	0.20	0.03		128	0.16	0.04	
Recipient CMV status				.796				.742				.896				.391
negative	75	0.55	0.06		75	0.53	0.06		75	0.21	0.05		64	0.18	0.05	
positive	93	0.55	0.05		93	0.52	0.05		93	0.22	0.04		80	0.13	0.04	
Donor CMV status				.541				.486				.107				.780
negative	102	0.54	0.05		102	0.51	0.05		102	0.25	0.04		86	0.15	0.04	
positive	66	0.56	0.07		66	0.55	0.07		66	0.15	0.05		58	0.14	0.05	
ABO match				.774				.965				.260				.201
matched	51	0.50	0.07		51	0.50	0.07		51	0.27	0.06		44	0.21	0.07	
mismatched	136	0.54	0.05		136	0.52	0.04		136	0.19	0.04		112	0.13	0.04	
Diagnosis				.273				.165				.172				.240
nonmalignant	32	0.66	0.08		32	0.66	0.08		32	0.12	0.06		27	0.08	0.06	
malignant	155	0.50	0.04		155	0.48	0.04		155	0.23	0.03		129	0.16	0.04	
Risk group‡				.082				.046				.575				.820
standard	118	0.59	0.05		118	0.58	0.05		118	0.20	0.04		99	0.15	0.04	
high risk	69	0.43	0.06		69	0.41	0.06		69	0.23	0.05		57	0.14	0.05	
Year of transplant				.184				.214				.425				.372
2000-2004	69	0.50	0.06		69	0.46	0.06		69	0.25	0.05		55	0.18	0.06	
2005-2008	118	0.60	0.05		118	0.54	0.05		118	0.19	0.04		101	0.13	0.04	
TBI				.858				.850				.330				.461
TBI	51	0.50	0.04		51	0.51	0.04		51	0.25	0.06		44	0.11	0.03	
non-TBI	136	0.50	0.04		136	0.52	0.04		136	0.20	0.04		112	0.16	0.04	
Degree of HLA-match				.628				.228				.055				.720
10\10	94	0.54	0.06		94	0.55	0.05		94	0.16	0.04		78	0.22	0.04	
9\10	65	0.51	0.06		65	0.48	0.06		65	0.23	0.05		56	0.16	0.05	
8\10	23	0.56	0.10		23	0.52	0.10		23	0.30	0.10		19	0.22	0.11	
7\10	5	0.40	0.22		5	0.20	0.18		5	0.60	0.22		3	no ext cGVHD		
BFM HLA-match status				.692				.334				.039				.645
matched (≥9/10)	159	0.53	0.04		159	0.52	0.04		159	0.19	0.03		134	0.14	0.03	
mismatched (≤8/10)	28	0.53	0.10		28	0.46	0.09		28	0.36	0.09		22	0.19	0.10	
HLA-match variants				.189				.024				.008				.439
8/10ant + 7/10	12	0.42	0.14		12	0.25	0.13		12	0.50	0.15		8	0.25	0.20	
other	175	0.54	0.04		175	0.53	0.04		175	0.19	0.03		148	0.14	0.03	

(Continued)

Table 4. (Continued)

Variables	Overall Survival*				Disease-Free Survival*				aGVHD Grades III-IV†				Extensive cGVHD†			
	N	%	SE	P	N	%	SE	P	N	%	SE	P	N	%	SE	P
Stem cell source				.065				.047				.922				.275
peripheral blood	145	0.56	0.04		145	0.55	0.04		145	0.21	0.04		127	0.14	0.04	
bone marrow	42	0.43	0.08		42	0.40	0.08		42	0.21	0.07		29	0.21	0.08	
Infused CD34 <sup>+</sup> cell dose				.009				.018				.889				.780
≥10 × 10 <sup>6</sup> /kg	86	0.64	0.05		86	0.60	0.05		86	0.22	0.05		77	0.14	0.05	
<10 × 10 <sup>6</sup> /kg	101	0.44	0.05		101	0.44	0.05		101	0.21	0.04		79	0.15	0.05	
CD34 <sup>+</sup> dose per kg				.030				.038				.093				.842
Q1	47	0.41	0.07		47	0.41	0.07		47	0.19	0.06		34	0.18	0.07	
Q2	47	0.43	0.08		47	0.43	0.08		47	0.23	0.06		39	0.13	0.06	
Q3	47	0.59	0.08		47	0.55	0.08		47	0.23	0.06		41	0.18	0.07	
Q4	46	0.69	0.07		46	0.67	0.07		47	0.20	0.06		42	0.12	0.05	
Infused CD3 <sup>+</sup> cell dose				.016				.012				.609				.981
≥4 × 10 <sup>8</sup> /kg	96	0.61	0.06		96	0.60	0.05		96	0.20	0.04		83	0.15	0.04	
<4 × 10 <sup>8</sup> /kg	87	0.45	0.05		87	0.43	0.05		87	0.23	0.05		71	0.14	0.05	
CD3 <sup>+</sup> dose per kg				.062				.035				.888				.632
Q1	46	0.41	0.07		46	0.39	0.07		46	0.22	0.06		34	0.12	0.07	
Q2	46	0.52	0.08		46	0.49	0.08		46	0.22	0.06		41	0.15	0.06	
Q3	46	0.50	0.09		46	0.49	0.09		46	0.17	0.06		40	0.20	0.07	
Q4	45	0.71	0.07		45	0.68	0.07		45	0.24	0.06		39	0.10	0.05	

aGVHD indicates acute graft-versus-host disease; cGVHD, chronic graft-versus-host-disease; HLA, human leukocyte antigen; TBI, total body irradiation; BFM, stem cell transplant protocol from Berlin-Frankfurt-Münster group; 8/10 ant, two HLA-antigen mismatch; ext, extensive; Q, quartiles of CD34<sup>+</sup> dose per kg: Q1 <5.33, Q2 5.33-9.72, Q3 9.73-15.42, Q4 >15.42; quartiles of CD3<sup>+</sup> dose per kg: Q1 <1.71, Q2 1.71-4.26, Q3 4.27-6.85, Q4 >6.85.

‡Standard risk: ALL/AML in first and second complete remission, CML in first chronic phase, MDS-refractory cytopenia, NHL in complete remission, any nonmalignant disease; high risk: ALL/AML in equal to or greater than third complete remission or nonremission, CML equal to or greater than accelerated phase, MDS-refractory anemia with excess of blasts, JMML, NHL in nonremission, any second transplant.

\*Log-rank test.

†Gray test.

**Table 5. Survival Analyses in Age Groups According to Age-Adjusted Medians of Infused CD34<sup>+</sup> and CD3<sup>+</sup> Cell Doses per kg (Log-Rank)**

		Infused CD34 <sup>+</sup> Cell Dose/kg							Infused CD3 <sup>+</sup> Cell Dose/kg						
		<Me for Age Group			≥Me for Age Group				< Me for Age Group			≥Me for Age Group			
Survival	Age Group	N	%	SE	N	%	SE	P	N	%	SE	N	%	SE	P
OS	0-5	23	0.47	0.11	24	0.83	0.08	<u>.006</u>	23	0.61	0.10	23	0.74	0.09	<u>.364</u>
	5-10	26	0.50	0.11	25	0.62	0.10	<u>.560</u>	26	0.55	0.10	25	0.56	0.11	<u>.965</u>
	10-15	25	0.46	0.10	25	0.59	0.10	<u>.421</u>	25	0.43	0.10	24	0.59	0.11	<u>.290</u>
	15-20	20	0.15	0.08	19	0.58	0.13	<u>.001</u>	19	0.31	0.11	18	0.44	0.14	<u>.225</u>
DFS	0-5	23	0.38	0.10	24	0.83	0.08	<u>.001</u>	23	0.52	0.10	23	0.74	0.09	<u>.150</u>
	5-10	26	0.52	0.10	25	0.58	0.10	<u>.703</u>	26	0.56	0.10	25	0.54	0.10	<u>.774</u>
	10-15	25	0.46	0.10	25	0.59	0.10	<u>.435</u>	25	0.43	0.10	24	0.60	0.11	<u>.276</u>
	15-20	20	0.15	0.08	19	0.54	0.13	<u>.002</u>	19	0.31	0.11	18	0.40	0.13	<u>.344</u>
aGvHD grade III-IV	0-5	23	0.31	0.10	24	0.21	0.08	<u>.359</u>	19	0.06	0.05	21	0.10	0.07	<u>.898</u>
	5-10	25	0.36	0.10	24	0.22	0.09	<u>.218</u>	25	0.33	0.10	20	0.11	0.07	<u>.303</u>
	10-15	24	0.13	0.07	25	0.12	0.06	<u>.944</u>	22	0.14	0.07	21	0.16	0.09	<u>.391</u>
	15-20	19	0.32	0.11	18	0.12	0.08	<u>.093</u>	12	0.36	0.15	14	0.16	0.10	<u>.669</u>
ext cGvHD	0-5	17	0.07	0.06	24	0.13	0.07	<u>.538</u>	19	0.06	0.05	21	0.10	0.07	<u>.618</u>
	5-10	24	0.29	0.09	21	0.15	0.08	<u>.236</u>	25	0.33	0.10	20	0.11	0.07	<u>.076</u>
	10-15	21	0.20	0.09	23	0.11	0.08	<u>.297</u>	22	0.14	0.07	21	0.16	0.09	<u>.964</u>
	15-20	10	0.33	0.16	16	0.20	0.10	<u>.308</u>	12	0.36	0.15	14	0.16	0.10	<u>.258</u>

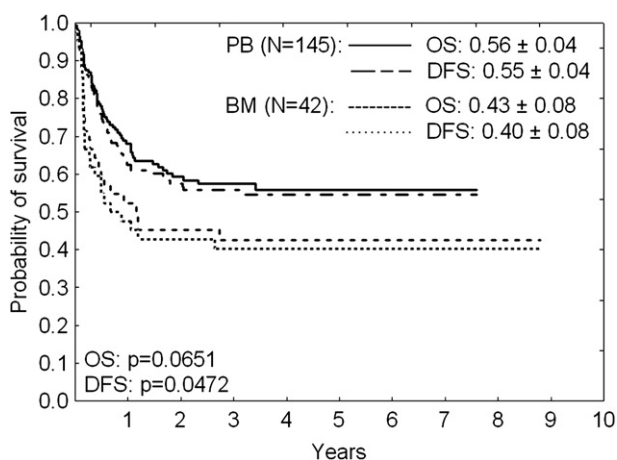
Me indicates median for age groups (CD34<sup>+</sup> cell dose: 0-5 years,  $16.02 \times 10^6$ ; 5-10 years,  $11.68 \times 10^6$ ; 10-15 years,  $8.77 \times 10^6$ ; 15-20 years,  $6.41 \times 10^6$ ; CD3<sup>+</sup> cell dose: 0-5 years,  $6.96 \times 10^8$ ; 5-10 years,  $5.15 \times 10^8$ ; 10-15 years,  $3.60 \times 10^8$ ; 15-20 years,  $2.14 \times 10^8$ ); OS, overall survival; DFS, disease-free survival; aGVHD, acute graft-versus-host disease; ext cGVHD, extensive chronic graft-versus-host disease.

In addition, a subanalysis of different CD34<sup>+</sup> and CD3<sup>+</sup> doses using quartiles revealed the best OS and DFS rates in the fourth quartile and the worst in the first one. Furthermore, we analyzed the impact of CD34<sup>+</sup> and CD3<sup>+</sup> cell doses (higher and lower than age-adjusted medians) on OS and DFS in smaller age cohorts, showing the survival advantage when using higher doses of CD34<sup>+</sup> cells/kg (Table 5 and Figure 2). In age groups 0-10 years and 10-20 years old, the survival advantage of patients who received higher doses of CD34<sup>+</sup> (Figure 2) and CD3<sup>+</sup> cells (data not shown) was clearly statistically significant.

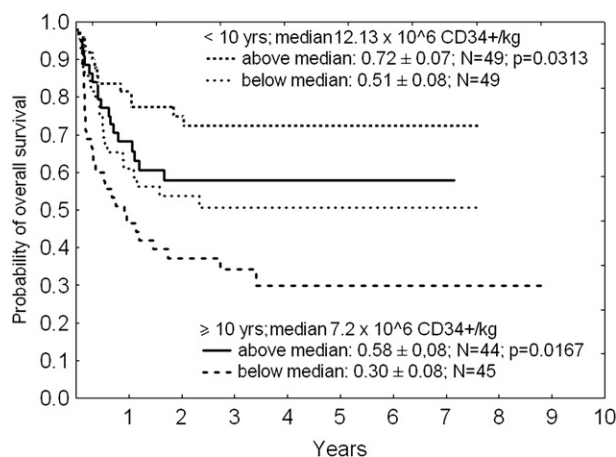
These clinically important results were confirmed in a multivariate analysis showing that both infused CD34<sup>+</sup> and CD3<sup>+</sup> cell doses lower than  $10 \times 10^6$ /kg

and  $4 \times 10^8$ /kg, respectively, were significant, independent risk factors for mortality (relative risk, RR 1.8, 95% confidence interval [CI] 1.2-2.8,  $P = .009$ , and RR 1.7, CI 1.1-2.7, respectively; Table 6 and Figure 3A). Regarding DFS, the multivariate analysis has revealed another independent risk factor for poor outcome apart from the 2 just-mentioned grafted cell doses, which was the use of donors mismatched at 2 HLA antigens or 3 HLA allele/antigens (Table 6 and Figure 3B).

With a median follow-up of 3.5 years (range: 0.8-8.8 years), 103 patients (55.1%) remain alive. Eighty-four patients died because of relapse or early/



**Figure 1.** Probability of OS and DFS according to stem cell source. DFS and OS are depicted for the PB group (upper lines) and BM group (lower lines). N, number of patients in each arm of the study cohort, P (log-rank).



**Figure 2.** Probability of OS according to infused age-adjusted median dose of CD34<sup>+</sup> cells/kg. Dotted and short-dashed lines depict patients younger than 10 years given CD34<sup>+</sup> cell dose below and above age-adjusted median ( $12.13 \times 10^6$  CD34<sup>+</sup> cells/kg), whereas dashed and solid lines depict patients older than 10 years given CD34<sup>+</sup> cell dose above and below age-adjusted median ( $7.2 \times 10^6$  cells/kg). N, number of patients in each arm of the study cohort, P (log-rank).

**Table 6. Multivariate Analysis of Risk Factors for Overall-, Disease-Free Survival, and the Incidence of aGVHD Grades III-IV**

Clinical Outcome		Variable*	Relative Risk	95% CI		P (Partial)	P (Model)
Overall survival	I	CD34 <sup>+</sup> cell dose (<10 × 10 <sup>6</sup> /kg)	1.80	1.15	2.81	.010	.009
	or						
	II	CD3 <sup>+</sup> cell dose (<4 × 10 <sup>8</sup> /kg)	1.71	1.10	2.67	.017	.016
Disease-free survival	I	8/10ant + 7/10	2.54	1.27	5.08	.008	.004
		CD34 <sup>+</sup> cell dose (<10 × 10 <sup>6</sup> /kg)	1.68	1.09	2.59	.019	
	or						
	II	8/10ant + 7/10	2.44	1.18	5.07	.017	.005
		CD3 <sup>+</sup> cell dose (<4 × 10 <sup>8</sup> /kg)	1.76	1.14	2.71	.010	
Incidence of acute GVHD grades III-IV	I	BFM match status (8/10 + 7/10)	2.19	1.07	4.48	.032	.046
	or						
	II	8/10ant + 7/10	3.72	1.56	8.90	.003	.011

CI indicates confidence interval; GVHD, graft-versus-host disease

\*Variables analyzed for prognostic impact on survival and incidence of aGVHD grade III-IV: recipient sex, sex match, donor and recipient age, donor and recipient cytomegalovirus status, ABO blood group compatibility match, patients' diagnosis and risk group, stem cell source (peripheral blood versus bone marrow), infused CD34<sup>+</sup> and CD3<sup>+</sup> cell dose per kg, donor-recipient HLA matching: degree of HLA-match or BFM match status (matched versus mismatched) or 8/10 ant (2 antigen mismatch) + 7/10 versus other. Only variables with a *P*-value < .2 in univariate analysis (log-rank test) were included in the final multivariate analysis: proportional hazard (Cox) regression with a backward stepwise approach.

late treatment-related mortality (TRM). TRM until day +100 was 16.0% and overall TRM reached 31.0%. The reasons for death were: relapse *n* = 21 (11.2%), secondary malignancies *n* = 2 (1.1%), sepsis *n* = 14 (7.5%), other infection (viral) *n* = 7 (3.7%), aGVHD *n* = 12 (6.4%), cGVHD *n* = 9 (4.8%), thrombotic thrombocytopenic purpura (TTP) *n* = 6 (3.2%), invasive fungal infection *n* = 3 (1.6%), pulmonary embolism *n* = 3 (1.6%), veno-occlusive liver disease *n* = 2 (1.1%), brain infarct *n* = 2 (1.1%), aorta thrombosis *n* = 1 (0.5%), heart failure *n* = 1 (0.5%) and renal insufficiency *n* = 1 (0.5%). There were no statistically significant differences in the TRM causes between the studied patients' cohorts. Year of transplant (2000-2004 versus 2005-2008) influenced neither OS, DFS, or the incidence of severe GVHD.

#### Severe aGVHD Grade III-IV (Table 4)

An analysis of the incidence of moderate-to-severe aGVHD grade II-IV (59.9%) was not the aim of this study, because moderate aGVHD grade II no longer seems to be a problem in treatment when compared with severe aGVHD grade III-IV. In a recently published large pediatric study, there was absolutely no difference in TRM between children developing aGVHD grade II and those with none or grade I aGVHD [24].

The cumulative incidence of clinically important severe aGVHD grade III-IV was comparable between the recipients of PB versus BM (Figure 4A). As shown in Tables 4 and 5, infused CD34<sup>+</sup> or CD3<sup>+</sup> cell dose had absolutely no impact on the occurrence of severe aGVHD (Figure 4B and C). As expected, the more HLA mismatches were in the donor-recipient pairs, the more frequent the cumulative incidence of severe aGVHD was (Figure 5A). There was a significantly higher incidence of severe aGVHD in the mismatched group (0.36 ± 0.09) versus matched group (0.19 ± 0.03, *P* = .039, log-rank) when using ALL international

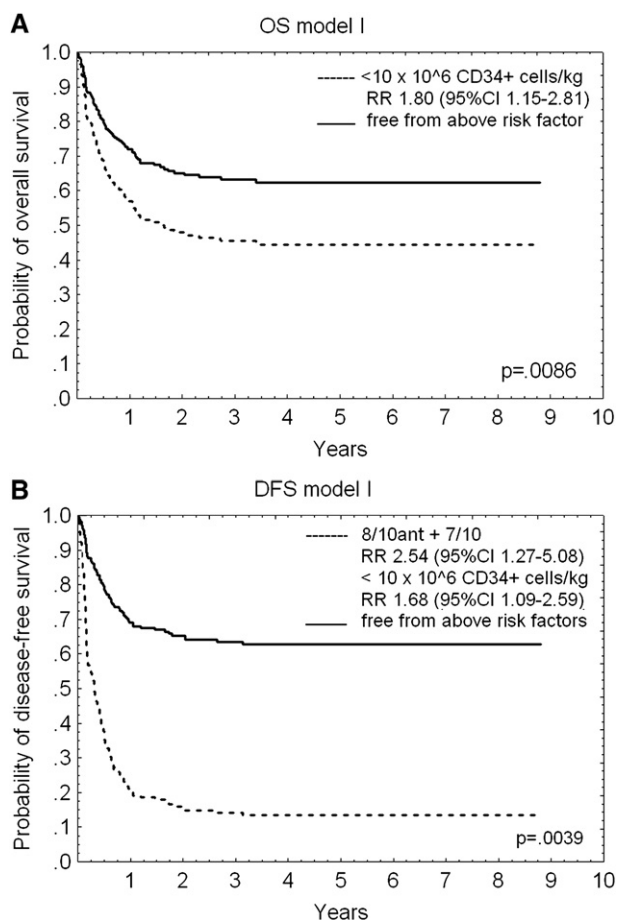
BFM SCT 2008 study criteria. However, this did not adversely affect OS or DFS, as shown previously (Table 4). Within the mismatched group the highest risk of severe aGVHD was associated with the use of donor-recipient pairs mismatched at 2 HLA antigens (not 2 alleles or 1 antigen + 1 allele) and 3 HLA allele/antigens (Figure 5B). These clinically relevant results were confirmed in a multivariate analysis showing that either the use of mismatched donors according to BFM criteria (RR 2.2, 95% CI 1.1-4.5, *P* = .046, Table 6) or, in another model, the use of donors mismatched at 2 HLA antigens or 3 HLA allele/antigens (RR 3.7, 95% CI 1.6-8.9, *P* = .011) were significant, independent risk factors for incidence of severe aGVHD.

#### cGVHD (Table 4)

The cumulative incidence of clinically relevant extensive cGVHD was comparable between the recipients of PB and BM (0.14 ± 0.04 for PB versus 0.21 ± 0.08 for BM groups, Figure 6A). As shown in Tables 4 and 5, infused CD34<sup>+</sup> or CD3<sup>+</sup> cell dose had no impact on the occurrence of extensive cGVHD (Figure 6B and C). There was a visible trend toward a higher cumulative incidence of extensive cGVHD in patients whose donors were mismatched at 2 HLA antigens (not 2 alleles or 1 antigen + 1 allele) and 3 HLA allele/antigens (*P* = .074). However, the multivariate analysis disclosed no significant risk factors for the development of extensive cGVHD.

## DISCUSSION

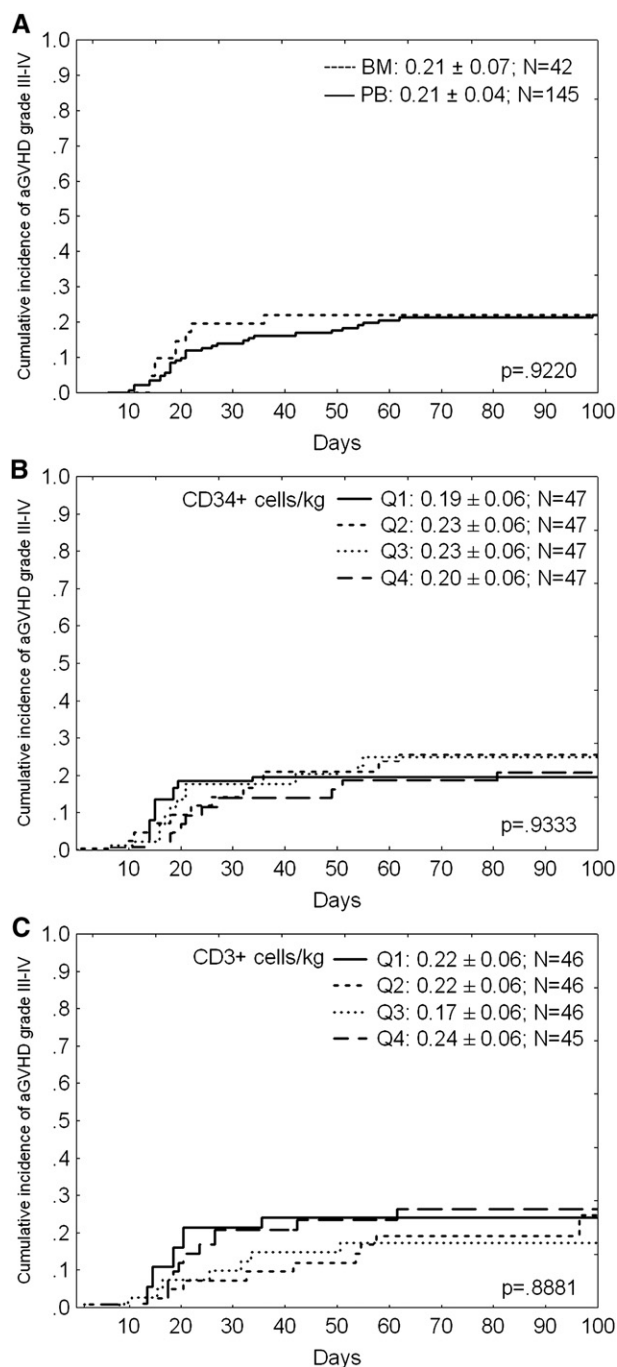
There is a worldwide concern whether to limit the dose of infused CD34<sup>+</sup> and CD3<sup>+</sup> cells/kg in recipients of both related and unrelated donor grafts. It is well recognized that doses of different cellular subsets within grafts can affect the clinical outcome following



**Figure 3.** Independent risk factors associated with probability of OS and DFS. (A) Dashed line depicts lower OS in patients grafted with  $<10 \times 10^6$  CD34<sup>+</sup> cells/kg, whereas solid line shows better survival in children who are free from the above-mentioned independent risk factor. (B) Dashed line depicts lower probability of DFS in patients with both independent risk factors: CD34<sup>+</sup> cell dose per kg  $<10 \times 10^6$  and the graft mismatched at 2 HLA antigens or 3 antigen/alleles, whereas solid line presents patients without the above-mentioned risk factors. RR, relative risk, CI, confidence interval, *P* (hazard Cox regression model).

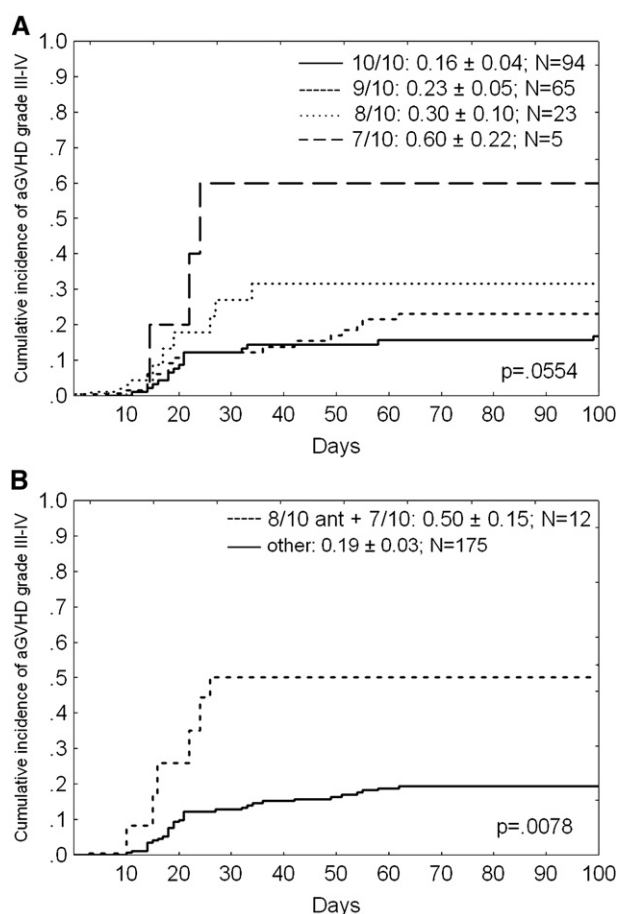
allogeneic HSCT. In MA setting in adults, a clear association between higher CD34<sup>+</sup> cell dose and improved survival after HLA-identical BMT has been reported [25]. The impact of higher CD34<sup>+</sup> (and presumably CD3<sup>+</sup>) cell dose in the graft on the risk of cGVHD is controversial [26], as some studies have reported only distinct correlations [27] or none at all [16,28].

There are only a few studies analyzing the impact of cell dose in the graft on the outcome of UD PBSCT. In a large single-center study Nakamura et al. [13] showed a correlation between higher CD34<sup>+</sup> cell doses and faster lymphocyte recovery leading to a decrease in relapse rate. A recently published large multicenter prospective study clearly showed that NMDP-facilitated UD PBSCTs [16] result in rapid engraftment and survival comparable to previous studies on UD BMT [29]. The key finding of that study was



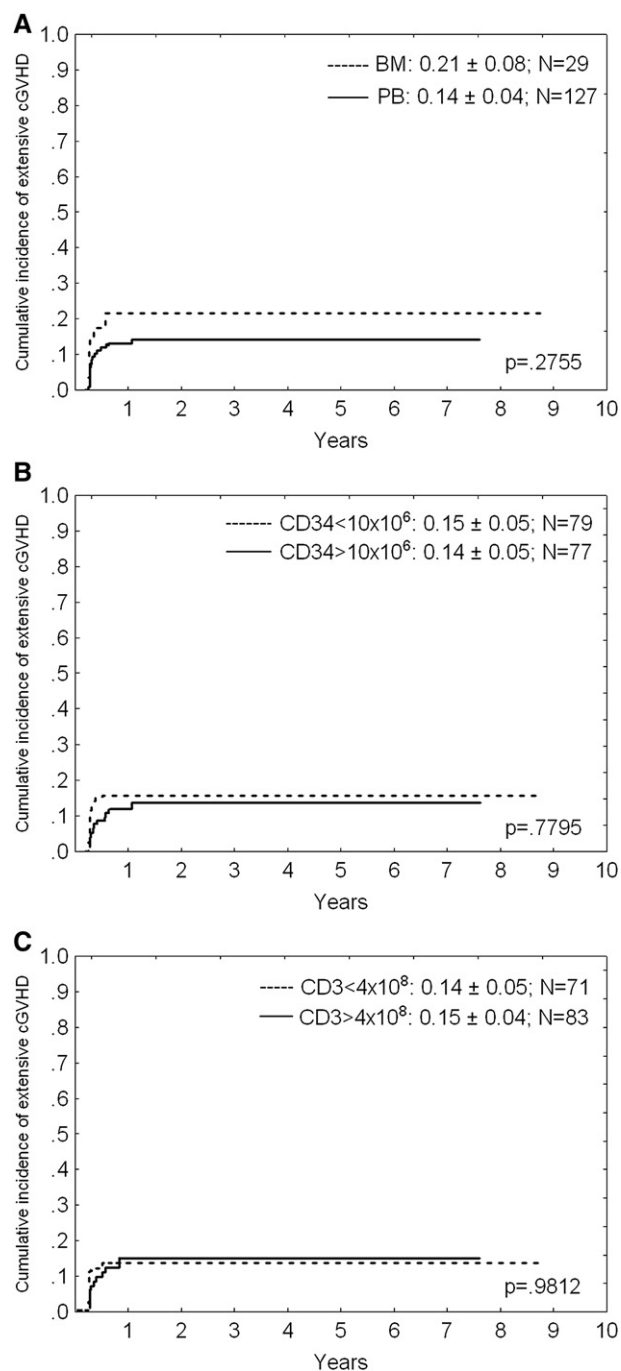
**Figure 4.** Incidence of severe aGVHD grade III-IV. (A) Comparison between PB (solid line) and BM recipients (dotted line). (B) Comparison between patients grafted with Q1 (solid line), Q2 (short-dashed line), Q3 (dotted line), and Q4 (long-dashed line); Q, quartile dose of CD34<sup>+</sup> cells/kg. (C) Comparison between patients grafted with Q1 (solid line), Q2 (short-dashed line), Q3 (dotted line), and Q4 (long-dashed line); Q, quartile dose of CD3<sup>+</sup> cells/kg. N, number of patients in each group of patients; quartiles of CD34<sup>+</sup> dose per kg  $\times 10^6$ : Q1  $<5.33$ , Q2 5.33-9.72, Q3 9.73-15.42, Q4  $>15.42$ ; quartiles of CD3<sup>+</sup> dose per kg  $\times 10^8$ : Q1  $<1.71$ , Q2 1.71-4.26, Q3 4.27-6.85, Q4  $>6.85$ , *P* (log-rank, cumulative incidence).

the independent predictive value of the higher CD34<sup>+</sup> cell dose for significant improvement in patients' outcome including faster hematologic recovery and better OS [16]. CD34<sup>+</sup> cell doses between 4.5 and  $9.5 \times 10^6$ /kg



**Figure 5.** Incidence of severe aGVHD grade III-IV, N, number of patients in each arm of the study cohort. (A) Incidence of severe aGVHD according to degree of HLA match, 10/10, full allele match; 9/10, single HLA allele/antigen mismatch; 8/10, 2 HLA allele/antigen mismatches; 7/10, three HLA allele/antigen mismatches. (B) Incidence of severe aGVHD in patients transplanted from donors mismatched (dotted line) at 2 HLA antigens (8/10 ant) or 3 HLA allele/antigens (7/10) versus others, *P* (log-rank).

recipient weight resulted in a significant 12% improvement in 3-year OS in recipients of MA conditioning [16]. Doses greater than  $9.5 \times 10^6$ /kg did not further improve OS in the earlier mentioned study [16], which stands in contrast to our data, showing best OS and DFS in patients receiving CD34<sup>+</sup> cell doses above the 75th percentile ( $\geq 15.4 \times 10^6$ /kg), but it should be noted that the highest CD34<sup>+</sup> cell doses in our study were given mostly to younger patients. These patients generally have better prognosis, but there were still a few high-risk patients in the youngest cohort including children after previous unsuccessful haploidentical transplants. Another confirmation of our results may be found in the most recently published study by Collins et al. [17], showing higher survival rates in patients grafted with PBSCs from UD containing  $>5 \times 10^6$  CD34<sup>+</sup> cells/kg. There is, however, one important point that cannot be missed: all previously cited studies included patients who were not given in vivo T cell depletion as



**Figure 6.** Incidence of extensive cGVHD, N, number of patients in each arm of the study cohort. (A) Comparison between PB (solid line) and BM recipients (dotted line). (B) Comparison between patients grafted with  $\geq 10 \times 10^6$  CD34<sup>+</sup> cells/kg (solid line) and  $<10 \times 10^6$  CD34<sup>+</sup> cells/kg (dotted line). (C) Comparison between patients grafted with  $\geq 4 \times 10^8$  CD3<sup>+</sup> cells/kg (solid line) and  $<4 \times 10^8$  CD3<sup>+</sup> cells/kg (dotted line), *P* (log-rank).

GVHD prophylaxis. On the contrary, our study is based on patients who received rabbit ATG or CAMPATH. The dose of rabbit ATG we used was not high: the majority of our patients were given ATG-Fresenius at a dose of 30 mg/kg, which is lower than the dose recommended by BFM group (60 mg/kg) or Sedlacek et al. [2,3] (40 mg/kg). Another point is

the use of rabbit ATG (Thymoglobuline). Here, the recommended dose is around 7.5 mg/kg, and our dosage of Thymoglobuline in those few patients was similar or slightly higher (10 mg/kg). The majority of children, however, were given the standard (rather low) dose of ATG-Fresenius (30 mg/kg).

In our study, we demonstrated a higher survival rate among the patients who had received higher doses: the more CD34<sup>+</sup> cells were given, the better survival rates were achieved. Of course, the just-mentioned result is slightly biased by the fact that younger children with more chances to receive higher dose of CD34<sup>+</sup> cells/kg usually had a better outcome. To avoid this possible bias, we analyzed the age groups 0-10 years and 10-20 years, and 0-5 years, 5-10 years, 10-15 years, and older separately, looking at the impact of high and low doses in different age cohorts. Our age-matched analysis (Table 5) proved that the megadose concept was working within almost all age cohorts in terms of survival. Because of a lower number of cases, in age groups 5-10 years and 10-15 years, the survival advantage was not statistically significant, yet visible. When looking at the larger age groups of 0-10 years and 10-20 years old, the survival advantage of patients who received higher doses was clearly statistically significant (Figure 2). We analyzed lower dose cutoffs to find a cell amount that is more achievable for adolescents and adults by looking at the median values for different age groups (Table 5 and Figure 2). For the age group of 0-10 years, the dose over  $12 \times 10^6$  CD34<sup>+</sup> cells/kg favored outcome, whereas in older children >10 years old the dose  $>7 \times 10^6$  CD34/kg promoted survival. Those cell doses are achievable in pediatric patients when PBSC is used. The dose of  $5 \times 10^6$  CD34<sup>+</sup>/kg obtainable in adult studies [16,17] might be equivocal to the dose of  $10 \times 10^6$  CD34/kg in children. In conclusion, we think that the easily achievable dose of  $10\text{--}12 \times 10^6$  CD34/kg for children <10 years or  $7 \times 10^6$  for older children may be desired in pediatric patients undergoing unrelated donor PB SCT (provided that in vivo T cell depletion is used). Multicenter randomized studies are required, however, to further prove this concept.

One may ask whether the year of transplant or diagnosis influenced our results. Indeed, more BM than PBSC transplants were performed in the years 2000-2004 (Table 3); however, there was absolutely no difference in outcomes between the studied periods (Table 4). The major reason for this might be a well-standardized "high-resolution" HLA typing used in our center since the beginning and a uniform use of in vivo T cell depletion.

Despite some differences between patients with nonmalignant and malignant disorders, the majority of variables in both groups were comparable and those 2 groups can be included together. In the nonmalignant

group, we had both older patients at risk of transplant-related complications (children with SAA, Fanconi anemia, or metabolic disorders) and some very young patients (severe combined immunodeficiency [SCID]), who had their second transplants after previous early haploidentical graft rejection or late graft failure. Patients with nonmalignant diseases tended to be younger, but had the same HLA matching levels accepted, the same GVHD prophylaxis and the same use of BM versus PBSC.

The major concern when using higher doses of CD34<sup>+</sup> and CD3<sup>+</sup> cells is the increased risk of GVHD. We have found absolutely no correlation between the CD34<sup>+</sup> and CD3<sup>+</sup> cell dose and the incidence of severe aGVHD and cGVHD, which remains in line with the NMDP studies [16,17]. In an adult study on UD HSCT using reduced-intensity conditioning (RIC) patients receiving a CD34<sup>+</sup> cell dose  $\geq 17 \times 10^6$  per kg had a higher incidence of GVHD [14]. In our study, such a high dose of grafted CD34<sup>+</sup> cells ( $\geq 15.4 \times 10^6$ /kg, ie, >75th percentile) promoted a significantly better OS and DFS, and did not influence GVHD. The majority of our patients (except for patients with SAA or Fanconi anemia) were conditioned, however, with an MA regimen, which may make an important difference and they were children, not adults (median age in Remberger et al. [14] study was 50 years). It was not the first time that PBSC appeared to have similar cGVHD outcomes to BM in children: Meisel et al. [12] showed comparable risk of both aGVHD and cGVHD in a small pediatric cohort of PBSC and BM recipients. Most patients in that study received ATG/ALG serotherapy as GVHD prophylaxis. All children in our study received ATG or CAMPATH as GVHD prophylaxis, and beside the well-known lower risk for GVHD in children it appeared to be the major factor for comparable rates of cGVHD among PBSC and BM recipients.

To the best of our knowledge, there are virtually no studies on the effect of cell dose on the outcome in pediatric patients undergoing UD HSCT. Furthermore, little is known about the impact of CD3<sup>+</sup> cell dose on OS, DFS, and the incidence of GVHD even in adult UD transplant patients.

In our opinion, the use of ATG is the most important factor enabling successful use of UD PB SCT in children. The results of our study, showing improved OS and DFS and no difference in severe GVHD in patients receiving higher CD34<sup>+</sup> and CD3<sup>+</sup> cell doses, make PBSC transplants with high CD34<sup>+</sup> and CD3<sup>+</sup> cell doses a feasible and perhaps desirable option. The dose above our 25th percentile, that is,  $5 \times 10^6$  CD34/kg may be easily achieved for any children when using PBSC. Even in an adult study of Remberger et al. [14] median CD34<sup>+</sup> cell dose in UD PBSC was  $7.5 \times 10^6$ /kg. Our study results remain in

line with 2 very recent NMDP papers, confirming the beneficial effects of higher CD34<sup>+</sup> cell dose in unrelated donor transplants in prevalent adult populations [16,17]. Our data comes from a pediatric cohort of patients and cannot be easily compared with adult data, but the message remains the same. It is important to note that PBSCs containing 1 log more T cells and higher amounts of CD34<sup>+</sup> cells are not worse (if not better) for the outcome. Furthermore, there seems to be no need to drastically limit the infused dose of CD34<sup>+</sup> or CD3<sup>+</sup> cells/kg in case a transplant center is given high amounts of CD34<sup>+</sup> cells from an unrelated good mobilizer.

HLA disparity is one of the most important factors governing the severity of both aGVHD and cGVHD [29,30]. An important finding of our study is the similar outcome of transplants using HLA allele mismatched (7-8/10) and matched (9-10/10) UD's (according to BFM criteria). Our concluding remarks reflect the results of the Sedlacek study [2,3]. The use of in vivo T cell depletion is certainly a prerequisite in GVHD prophylaxis, as shown by Sedlacek et al. [2].

In summary, we have demonstrated for the first time in a large pediatric cohort that higher doses of transplanted CD34<sup>+</sup> and CD3<sup>+</sup> cells lead to improved survival without an increased risk of severe aGVHD or cGVHD. The study findings may be limited to the population of patients receiving in vivo T cell depletion, which is now broadly used in unrelated donor settings in Europe. With regard to the degree of HLA match, it seems that transplants from 8 of 10 allele matched donors (with maximum 1 mismatched antigen) do not influence OS, whereas one should avoid performing transplants from 2 HLA-antigens or 3 allele/antigen mismatched donors.

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## AUTHORSHIP STATEMENT

Contribution: K.K. had primary responsibility for study design, data analysis, data interpretation, and manuscript writing, and had primary responsibility for the entire article as an accurate and verifiable report.

J.P. had primary responsibility for data collection, data file preparation, data analysis, and manuscript writing; M.M. and D.T. had primary responsibility for data analysis and interpretation, including statistical analysis and for manuscript writing; for example, had primary responsibility for provision of study patients, data collection, data analysis, and manuscript writing; J.O-L participated in extensive data collection, data file preparation, and data analysis; M.U., A.D., J.M., and D.P. participated in data collection, data analysis, and interpretation and manuscript writing, A.C. had responsibility for study design and for the entire article as an accurate and verifiable report.

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