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ORIGINAL ARTICLE

Monitoring of TNFR1, IL-2Rα, HGF, CCL8, IL-8 and IL-12p70 following HSCT and their role as GVHD biomarkers in paediatric patients

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No predictive factors are currently available to establish patient-specific GVHD risk. A panel of six serum cytokines (TNF receptor 1, IL-2 receptor alfa (IL-2Rα), hepatocyte growth factor (HGF), monocyte chemo-attractant protein-2, IL-8, IL-12p70) were monitored at established time points (days −1, +1, +7, +14, +21, +28 and +60) in 170 paediatric hematopoietic SCT (HSCT) recipients. We found that higher concentrations of IL-2Rα on days +14 and +21 together with HGF on days +14 and +21 were significantly associated at a higher probability of both grade II–IV GVHD (on day +14 it was: 60% vs 28%, $P=0.007$) and grade III–IV (on day +14 it was: 40% vs 15%, $P=0.001$). The higher IL-8 serum concentration on day +28 was associated with a lower probability of chronic GVHD being 4% vs 29% ($P=0.01$) for patients with higher vs lower IL-8 serum concentration. These findings were confirmed when the analysis was restricted to the the matched unrelated donor group. In conclusion, even if the serum cytokine levels were related to several variables associated with HSCT, we identified two cytokines as predictors of GVHD II–IV and III–IV, translating into a higher TRM risk (17% vs 3%, $P=0.004$).

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INTRODUCTION

Approximately 45 years have passed since Billingham[1] defined the essential elements of the GVHD reaction in the Harvey lecture. Following earlier studies showing the central role of T-lymphocytes contained in the graft promoting GVHD, T-cell immunosuppressive agents were added to reduce the incidence and severity of such complications: CYA ameliorated GVHD by inhibiting the increased expression of IL-2 and IL-2 receptor (IL-2R) by T-lymphocytes during activation, MTX kills proliferating T-lymphocytes in response to allo-Ag, while other agents selectively spare actively duplicating DNA cells, such as mofetil mycophenolate and others. Despite these achievements, both acute GVHD (aGVHD) and chronic GVHD (cGVHD) remain major complications following hematopoietic SCT (HSCT).[2]

Recently published papers have shown how the rise of TNFR1 levels correlates with the severity and incidence of GVHD, 1-year TRM and 1-year OS even within group stratifications for donor source.[3] Results on the monitoring of serum IL-2 receptor are less clear since it was initially proposed as a GVHD marker.[4,5] A murine model study showed how the early expression of monocyte chemo-attractant protein-2 (CCL8) in plasma predicts OS of GVHD in a pre-clinical model.[6] The IL-12 serial plasma concentration was studied in a series of 134 patients: an increased probability of relapse for patients having a low concentration was reported, but not increased aGVHD or cGVHD risk.[7] A panel of four cytokines was recently proposed as biomarkers for GVHD (IL-2 receptor alfa (IL-2Rα), TNF receptor-1 (TNFR-1), IL-8 and hepatocyte growth factor (HGF)), as they were able to discriminate between patients with and without GVHD and, more importantly, the same markers could predict GVHD response to therapy, TRM and OS.[8–10]

In this paper, we report our experience on 170 paediatric patients whose six cytokine serum concentrations were prospectively determined at seven established time-points. The primary end point of the study was to analyse the effect of selected cytokines on the cumulative incidence (CI) of aGVHD II–IV. The secondary end points were to analyse the effects of selected cytokines on CI of aGVHD III–IV, cGVHD, TRM, relapse incidence (RI), OS, and finally the cytokine serum concentrations according to the donor type, hematopoietic stem cell (HSC) source, preparative intensity conditioning, donor and patient age.

PATIENTS AND METHODS

Patients' characteristics

A total of 170 consecutive paediatric patients underwent T-cell-replete HSCT at our centre from March 1999 to October 2009. Details of the patient population are outlined in Table 1. In particular, we found that matched unrelated donor (MUD) recipients had significantly more AB0 incompatibility than matched family donor (MFD) recipients, while the probability of both donor and recipient being CMV negative was higher in the MFD group. These data are probably due to the paediatric age of both donor and recipient. This study was approved by the local Institute Review Board/ Ethic Committee; all patients or parents or legal guardians gave their consent to store biological materials before and after HSCT.

Sample collection

Serum samples from patients were prospectively collected at different time points: on days −1, +1, +7, +14, +21, +28 and +60. All aliquots

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Table 1. Patient and transplant-related characteristics

	MFD	*MUD*	P
Patients	63 (37%)	107 (63%)	
Age	9.2 (0.1–27.2)	8.6 (0.3–27.6)	NS
Gender (M/F)	30/33 (47%)	67/40 (63%)	NS
Diagnosis			
Acute leukaemia	27 (43%)	58 (54%)	NS
CML	3 (5%)	4 (4%)	NS
Lymphomas	6 (9%)	11 (10%)	NS
Myelodysplastic-Myeloproliferative Syndrome	3 (5%)	9 (8%)	NS
Solid tumour	12 (19%)	3 (3%)	<0.0001
Nonmalignant disease	12 (19%)	22 (20%)	NS
HSCT year			
1999–2002	18 (28%)	20 (19%)	0.069
2003–2006	29 (46%)	42 (39%)	
2007–2009	16 (25%)	45 (42%)	
Disease status			
Early disease	30 (48%)	58 (54%)	NS
Advanced disease	18 (28%)	25 (23%)	NS
Donor age	9 (0–42)	32 (0–54)	<0.0001
Donor gender F > M vs others	14/49 (22%)	20/87 (19%)	NS
Donor CMV serology neg > neg vs other	10/53 (16%)	6/101 (6%)	0.03
ABO major incompatibility	9/54 (14%)	41/66 (38%)	<0.0001
Myeloablative conditioning	45 (71%)	81 (76%)	NS
Reduced toxicity regimen	18 (28%)	26 (24%)	NS
GVHD prophylaxis			
CYA	36 (57%)	0	<0.0001
CYA + MTX	24 (38%)	0	
CYA + MTX + serotherapy	0	86 (80%)	
CsA + steroids	3 (5%)	0	
CYA + steroids + serotherapy	0	21 (19%)	
Stem cell source			
BM	57 (90%)	67 (62%)	0.008
PB	4 (6%)	19 (19%)	0.01
CB	2 (3%)	21 (20%)	0.002
Cell dose			
TNC BM	4.05×10^8/kg	5.2×10^8/kg	NS
Derived CD34+	6.9×10^6/kg	5.8×10^6/kg	NS
TNC PB	10.6×10^8/kg	13.8×10^8/kg	NS
Derived CD34+	9.5×10^6/kg	10.5×10^6/kg	NS
TNC CB	0.57×10^8/kg	0.53×10^8/kg	NS
Derived CD34+	0.19×10^6/kg	0.23×10^6/kg	NS

Abbreviations: CB = cord blood; MFD = matched family donor; MUD = matched unrelated donor; PB = peripheral blood; Serotherapy = ATG, ALG or Campath; TNC = total nucleated cells. Early disease was considered CR1 or CR2 for acute leukaemia or CP1 for CML patients, all the others were considered as advanced. Solid tumour patients were considered as early disease when they had HSCT in CR1, all the others were considered as advanced.

were immediately stored in pyrogen-free vials at $-80\,^{\circ}C$ and thawed on ice before use.

Measurement of cytokines

We used a human cytokine 12-Bio-Plex assay kit (Bio-Rad Laboratories, Milan, Italy), a bead-based multiplex immunoassay. This technology has the capacity to measure several cytokine/cytokine receptors simultaneously in a small volume of plasma or serum (12.5 μL) with a greater detection dynamic range (0.1–10 000 pg/mL) than single ELISAs with higher accuracy and sensitivity. Six cytokines were analysed: IL-2Rα, IL-8, CCL8, HGF, TNFR1 and IL-12p70. All the analyses were performed in duplicate to minimise any sample errors. A total of 6524 samples were analysed.

The samples were read on a Bio-Plex 200 instrument equipped with Bio-Plex Manager (version 6.0) software using five parameters in a nonlinear regression formula to compute sample concentrations from the standard curves.

Definitions and statistical analysis

The data were analysed as of 10 June 2012. Patient, donor and transplantation-related variables were expressed as medians and ranges, means, or percentages as appropriate.

In the first step of the study the univariate analysis of crude incidence of both aGVHD and cGVHD was performed as follows: each cytokine at each time-point (days +1, +7, +14, +21, +28 and +60) were first stratified at the 25th, 50th and 75th centiles, and then for each group the crude incidence of aGVHD[10,11] and cGVHD[12] by the χ^2 or Fischer test through SPSS software was analysed (www.spss.it). For cytokines that were significantly associated with aGVHD and cGVHD, the CI was calculated by NCSS software for Windows (www.ncss.com), while the *P* values were calculated by Gray's test.[13] The primary end point of the study was to analyse the effect of selected cytokines on the CI of aGVHD II–IV. The secondary end points were to analyse the effects of selected cytokines on CI of aGVHD III–IV, cGVHD, TRM, RI, OS, and finally the cytokine serum concentrations according to the donor type, HSC source, preparative intensity conditioning, donor and patient age. The CI of aGVHD was

calculated for patients surviving today +14, while the CI of cGVHD was calculated for patients surviving today +100. A separate analysis was conducted only for the MUD group to reduce the variability given by the use of serotherapy, GVHD prophylaxis, the BM as preferred stem cell source or any other variables that made these two groups different (see Table 1). TRM was defined as death unrelated to relapse or progression of underlying disease, while the relapse was diagnosed with the reappearance of neoplastic cells in BM, peripheral blood (PB) or cerebrospinal fluid (CSF) for haematological malignancy. For solid tumours computed tomography and magnetic resonance imaging were diagnostic tools for relapse or disease progression. OS was calculated as the interval from transplant to death or to the day of the last follow-up. RI and the TRM were considered the competitive events to calculate both the aGVHD and cGVHD incidence curves. The sensitivity and specificity of the selected cytokines to predict aGVHD II–IV and III–IV occurrence were calculated by receiver operating characteristic curves using NCSS software for Windows. Kaplan–Meier statistics[14] were used to calculate OS with SPSS software. The statistical differences between cytokine serum concentrations were determined according to the donor type (MFD vs MUD), the HSC source (BM vs PB and BM vs cord blood (CB), BM being the reference group), the preparative conditioning intensity (myeloablative conditioning regimen (MAC) vs reduced toxicity regimen), the patient's age ($\geqslant$9.7 vs $<$9.7 years) and donor's age ($\geqslant$21.9 vs $<$21.9 years). Stratification according to the serotherapy use was omitted because only patients of the MUD group received serotherapy. The differences between the groups were calculated by using the non-parametric Mann–Whitney test for $P<0.05$. The multivariate analysis was performed according to the multinomial logistic regression model and was calculated by SPSS software.

RESULTS

Transplant population outcome

The median follow-up for surviving patients was 6.7 years (4.8–15.6), while it was 0.64 years (25 days to 6.1 years) for deceased patients. The median interval between HSCT and aGVHD diagnosis was 21 days (14–80). The CI of aGVHD II–IV was 37% (95% CI, 31–45) and that of aGVHD 3–4 was 21% (95% CI, 15–28). The CI of GVHD II–IV and III–IV according to regimen intensity were 48% (95% CI, 36–54) and 21% (95% CI, 15–30), and 19% (95% CI, 9–35) and 18% (95% CI, 9–34) for MAC and reduced toxicity regimen recipients, respectively.

The median interval between HSCT and cGVHD diagnosis was 118 days (101–556). One-hundred and forty-one patients surviving and not relapsing by day +100 were evaluable for cGVHD incidence: the 10-year CI of cGVHD was 10% (95% CI, 6–17). Details of clinical outcome according to donor type are outlined in Table 2.

Table 2. HSCT outcome according to donor type for all patient population

	MFD N = *63*	*MUD* N = *107*
TRM-day 100	3% (95% CI, 0.8–12.4)	7% (95% CI, 3.8–14.5)
RI-day 100	13% (95% CI, 6.6–24.2)	13% (95% CI, 7.3–20.2)
Event-free Survival-day 100	84% (95% CI, 75–93)	80% (95% CI, 73–88)
OS-day 100	95% (95% CI, 90–100)	87% (95% CI, 80–93)
TRM-5 year	8% (95% CI, 3.4–18.4)	16% (95% CI, 10.2–24.5)
RI-5 year	30% (95% CI, 20.7–43.9)	30% (95% CI, 24–41.9)
Event-free Survival-5 year	62% (95% CI, 50–74)	52% (95% CI, 42–62)
OS-5 year	69% (95% CI, 58–81)	57% (95% CI, 48–67)
aGVHD II–IV	29% (95% CI, 19.6–42.4)	57% (95% CI, 46–65)
aGVHD III–IV	16% (95% CI, 9–28)	23% (95% CI,16.7–33.2)
cGVHD	13% (95% CI, 6.7–24.6)	9% (95% CI, 5.2–17.1)

Abbreviations: aGVHD = acute GVHD; cGVHD = chronic GVHD; HSCT = hematopoietic SCT; MFD = matched family donor; MUD = matched unrelated donor; RI = relapse incidence. TRM, RI, aGVHD and cGVHD are expressed as cumulative incidences.

Primary endpoint analysis

Crude incidence of aGVHD II–IV. The IL2-Rα higher serum concentrations on day +14 and day +21 ($\geqslant$568.9 and $\geqslant$579.7 pg/mL, respectively (50th centiles)) were significantly associated with a higher incidence of grade II–IV GVHD ($P=0.00$ and $P=0.04$). HGF showed a trend for a higher incidence of aGVHD on day +14 ($\geqslant$677.7 pg/mL (75th centiles) ($P=0.09$)) and it reached a statistically significant value on day +21 ($>$657 pg/mL (75th centiles, $P=0.03$)). The TNFR1, CCL8, IL-8 and IL-12p70 cytokines had no effect on aGVHD occurrence (Table 3). The receiver operating characteristic curve analysis for IL2-Rα and HGF combinations and grade II–IV GVHD as end point shows sensitivity as 87.4% and the specificity as 23% (data not shown).

Cumulative incidence of aGVHD. The grade II–IV and III–IV aGVHD CIs for patients with both IL-2Rα and HGF above the 75th centiles (day +14 $\geqslant$981.87 pg/mL and $\geqslant$673.6 pg/mL) were 60% (95% CI, 42–85) vs 28% (95% CI, 20–38, $P=0.007$) and 40% (95% CI, 24–68)

Table 3. Univariate analysis of aGVHD II-IV incidence according to cytokine serum concentrations

	P *values*					
	Day +1	*Day +7*	*Day +14*	*Day +21*	*Day +28*	*Day +60*
TNFR1						
$<$25th	0.16	0.61	0.23	0.52	0.76	0.06
25th–50th						
50th–75th						
$\geqslant$75th						
IL-2 Rα						
$<$25th	0.86	0.06	$<$0.000	0.04	0.16	0.11
25th–50th						
50th–75th						
$\geqslant$75th						
HGF						
$<$25th	0.689	0.551	0.098	0.038	0.19	0.08
25th–50th						
50th–75th						
$\geqslant$75th						
CCL8						
$<$25th	0.92	0.35	0.95	0.37	0.10	0.128
25th–50th						
50th–75th						
$\geqslant$75th						
IL-8						
$<$25th	0.86	0.14	0.87	0.67	0.91	0.11
25th–50th						
50th–75th						
$\geqslant$75th						
IL-12						
$<$25th	0.31	0.52	0.67	0.7	0.3	0.75
25th–50th						
50th–75th						
$\geqslant$75th						

Abbreviations: aGVHD = acute GVHD; CCL8 = monocyte chemo-attractant protein-2; HGF = hepatocyte growth factor; IL-2Rα = IL-2 receptor alfa; TNFR1 = TNF receptor 1. All patients were included. Each cytokine serum concentration was first divided at: $<$25th centiles, between 25th and 50th centiles, between 50th and 75th centiles and $\geqslant$75th centiles. Thereafter the crude incidence of GVHD II-IV was calculated between the groups through the X^2 test or the Fisher exact test, the *P* value is reported in each box.

vs 15% (95% CI, 9–24, $P = 0.007$, Figures 1 and 2), respectively. This observation was confirmed when we computed the grade II–IV and III–IV aGVHD CI according to relative cytokine increase.

We then analysed the grade II–IV and III–IV aGVHD CI according to IL-2Rα and HGF above the 75th centiles on day +21 (857.4 and 657 pg/mL, respectively). We found that the grade II–IV GVHD CI was 75% (95% CI, 56–99) compared to 35% (95% CI, 26–47, $P = 0.001$) and the grade III–IV GVHD CI was 38% (95 CI, 20–66) vs 18% (95% CI, 11–28, $P = 0.001$). The CIs of grade II–IV GVHD according to IL-2Rα and HGF above the 75th centiles over time (days +1, +7 and +14) are outlined in Supplementary Table 1.

We then focused our analysis on the MUD HSCT group confirming the role of day +14 IL-2Rα (⩾1161.91 pg/mL) and HGF (⩾687.83 pg/mL) serum concentration as a marker of aGVHD II–IV (64% (95% CI, 31–88) vs 32% (95% CI, 22–46), $P = 0.05$) and aGVHD III–IV (35.7% (95% CI, 17–72) vs 18% (95% CI, 10–30), $P = 0.13$ (see also Table 4 for aGVHD II-IV crude incidence)). The receiver operating characteristic curve analysis for IL-2Rα and HGF and GVHD III-IV as end point shows the sensitivity as 92% and the specificity as 66%. To exclude the HSCT-year and GVHD-prophylaxis effect, patients were then stratified according to HSCT-year (1999–2002, 2003–2006 and ⩾2007) and GVHD-prophylaxis (CYA + serotherapy + MTX or steroids vs CYA + MTX or steroids vs CYA alone): the higher serum concentration of IL-2Rα and HGF confirmed their role in discriminating aGVHD II-IV patients (data not shown). Finally, when we explore the liver and gut GVHD vs skin or mucosal involvement those cytokines were able to discriminate patients with higher incidence of visceral GVHD (40% (95% CI, 29–52) vs 17% (95% CI, 13–21), $P = 0.02$).

Secondary endpoint analysis

cGVHD incidence. We found that higher IL-8 serum concentrations on day +28 had a trend for a protective effect on cGVHD development (4% (95% CI, 0–22) vs 29% (95% CI, 18–36) $P = 0.01$), respectively (see also Table 5 for crude incidence). The IL-8 higher serum concentration maintained its protective role for cGVHD when we focused analysis on the MUD HSCT group.

The CI of cGVHD was 17% (95% CI, 6–47) and 11% (95% CI, 6–19, P = NS) for patients having IL-2Rα and HGF on day +14 above or below the 75th centiles (P = NS).

TRM. The day +100 TRM was 17% (95% CI, 5–46) and 3% (95% CI, 1–9, $P = 0.004$) for patients having IL-2Rα and HGF above or below the 75th centiles. The 5-year TRM was 22% (95% CI, 9–46) and 10% (95% CI, 6–18, P = NS) according to IL-2Rα and HGF serum concentrations.

RI. The 100-day RI was 5% (95% CI, 1–37) and 13% (95% CI, 8–21, P = NS) for patients having IL-2Rα and HGF above or below the 75th centiles. The 5-year RI was 16% (95% CI, 6–47) and 34% (95% CI, 26–45, P = NS) according to IL-2Rα and HGF serum concentrations.

OS. The 100-day OS was 83% (95% CI, 66–100) and 95% (95% CI, 91–99, P = NS) for patients having IL-2Rα and HGF above or below the 75th centiles. The 5-year OS was 61% (95% CI, 39–84) and 64% (95% CI, 55–74, P = NS) according to IL-2Rα and HGF serum concentrations.

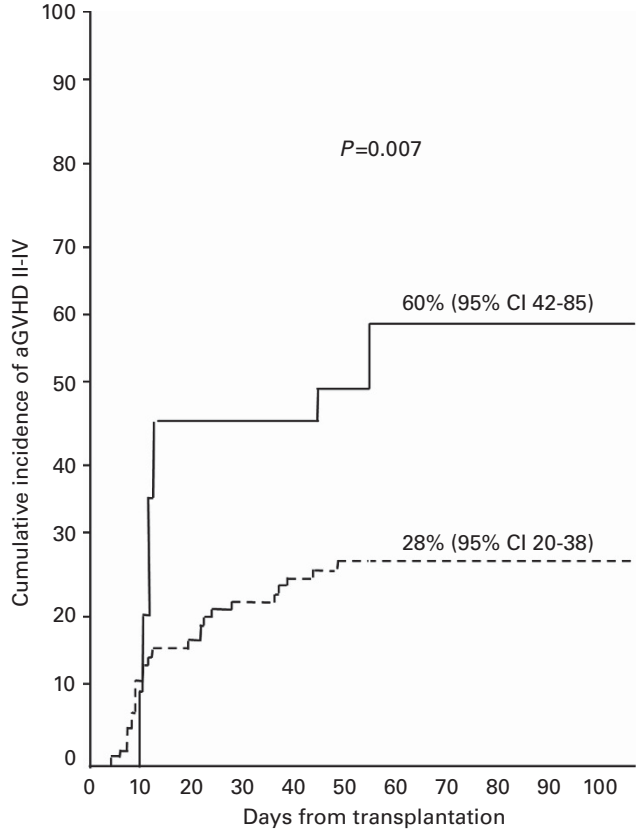

Figure 1. Cumulative incidence of grades II–IV acute GVHD according to day +14 IL-2Rα (⩾981.87 pg/mL, 75th centiles) and HGF (⩾673.66 pg/mL, 75th centiles) serum concentrations. Continuous line represents aGVHD II-IV cumulative incidence for patients having higher day −14 IL-2Rα and HGF values; dashed line represents aGVHD II-IV cumulative incidence for patients having lower day-14 IL-2Rα and HGF values.

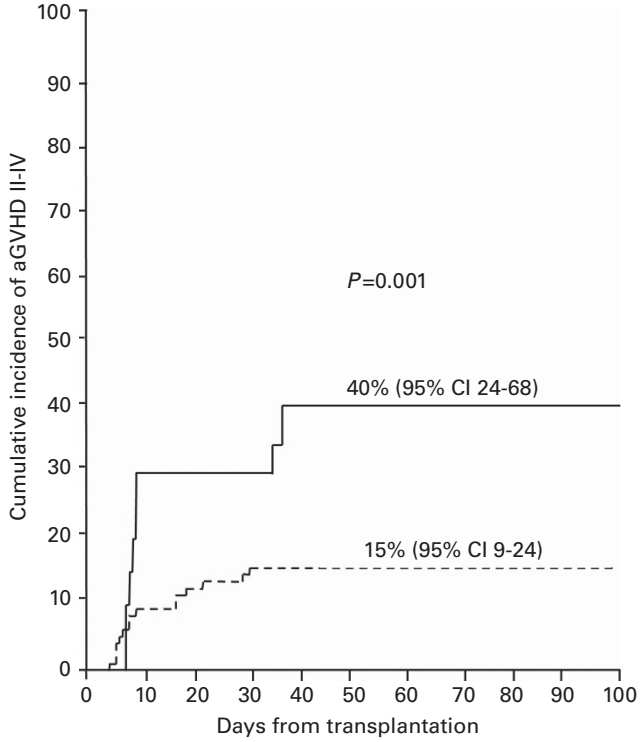

Figure 2. Cumulative incidence of grades III–IV acute GVHD according to day +14 IL-2Rα (⩾981.87 pg/mL, 75th centiles) and HGF (⩾673.66 pg/mL, 75th centiles) serum concentrations. Continuous line represents aGVHD II-IV cumulative incidence for patients having higher day −14 IL-2Rα and HGF values; dashed line represents aGVHD II-IV cumulative incidence for patients having lower day −14 IL-2Rα and HGF values.

Table 4. Univariate analysis of aGVHD II-IV incidence according to cytokine serum concentration for MUD HSCT only

	P *values*					
	Day +1	*Day +7*	*Day +14*	*Day +21*	*Day +28*	*Day +60*
TNFR1						
<25th	0.4	0.86	0.92	0.40	0.30	0.95
25th–50th						
50th–75th						
⩾75th						
IL-2 Rα						
<25th	0.24	0.83	0.03	0.67	0.40	0.64
25th–50th						
50th–75th						
⩾75th						
HGF						
<25th	0.83	0.60	0.80	0.79	0.32	0.38
25th–50th						
50th–75th						
⩾75th						
CCL8						
<25th	0.99	0.74	0.34	0.86	0.08	0.69
25th–50th						
50th–75th						
⩾75th						
IL-8						
<25th	0.67	0.89	0.28	0.07	0.93	0.08
25th–50th						
50th–75th						
⩾75th						
IL-12						
<25th	0.17	0.99	0.55	0.10	0.06	0.51
25th–50th						
50th–75th						
⩾75th						

Abbreviations: aGVHD = acute GVHD; CCL8 = monocyte chemo-attractant protein-2; HGF = hepatocyte growth factor; HSCT = hematopoietic SCT; IL-2Rα = IL-2 receptor alfa; MUD = matched unrelated donor; TNFR1 = TNF receptor 1. Each cytokine serum concentration was first divided at: <25th centiles, between 25th and 50th centiles, between 50th and 75th centiles and ⩾75th centiles. Thereafter the crude incidence of GVHD II-IV was calculated between the groups through the χ^2 test or the Fisher exact test, the *P* value is reported in each box.

Table 5. Univariate analysis of c GVHD according to cytokine serum concentrations

	P *values*					
	Day +1	*Day +7*	*Day +14*	*Day +21*	*Day +28*	*Day +60*
TNFR1						
<25th	0.07	0.98	0.88	0.21	0.99	0.15
25th–50th						
50th–75th						
⩾75th						
IL-2Rα						
<25th	0.18	0.7	0.96	0.13	0.82	0.49
25th–50th						
50th–75th						
⩾75th						
HGF						
<25th	0.79	0.09	0.78	0.10	0.43	0.45
25th–50th						
50th–75th						
⩾75th						
CCL8						
<25th	0.75	0.77	0.77	0.73	0.17	0.31
25th–50th						
50th–75th						
⩾75th						
IL-8						
<25th	0.77	0.40	0.21	0.38	0.005	0.95
25th–50th						
50th–75th						
⩾75th						
IL-12						
<25th	0.41	0.58	0.39	0.72	0.61	0.57
25th–50th						
50th–75th						
⩾75th						

Abbreviations: CCL8 = monocyte chemo-attractant protein-2; cGVHD = chronic GVHD; HGF = hepatocyte growth factor; IL-2Rα = IL-2 receptor alfa; TNFR1 = TNF receptor 1. All patients were included in this analysis. Each cytokine serum concentration was first divided at <25th centiles, between 25th and 50th centiles, between 50th and 75th centiles and ⩾75th centiles. Thereafter the crude incidence of cGVHD limited+extensive between groups was calculated through the χ^2 test or the Fisher exact test, the *P* value is reported in each box.

The donor type effect. When we compared the TNFR1 serum concentrations between MUDs and MFDs, we found a higher level in the former group early after transplantation and on day +21; thereafter we did not observe significant differences. The serum concentrations of IL-2Rα were significantly higher for MUDs on day +1 ($P<0.001$), +7 ($P=0.01$), +21 ($P=0.03$) and +28 ($P=0.01$). We only found higher HGF concentrations on day +1 for MFDs compared to MUD recipients ($P<0.001$).

The CCL8 concentration was significantly higher for MUD recipients on days +1 ($P=0.01$), +7 ($P=0.01$), +14 ($P<0.001$), +21 ($P<0.001$) and +28 ($P<0.001$). The MFD and MUD groups had significant differences of IL-8 concentration on day +1 ($P<0.001$), day +7 ($P<0.001$) and on day +21 ($P=0.03$, Supplementary Table 2).

The HSC source effect. The serum concentration of TNFR1 was significantly higher for patients receiving PB-derived HSCs compared to BM patients on days +1 ($P<0.001$), +7 ($P=0.05$) and +14 ($P<0.001$). The IL-2Rα serum concentration was significantly higher for PB-HSC patients compared to BM-derived HSCs on days +1 and +7 ($P<0.001$ and $P=0.01$), while no differences were observed at later time-points for each HSC source.

For the HGF serum concentration the only significant difference we observed was on day +14. It was higher for PB-HSC patients compared to both BM- or CB-HSCs ($P<0.001$, Supplementary Table 3).

The conditioning regimen effect. For TNFR1, we found a lower serum concentration of TNFR1 following MAC on day −1 ($P<0.001$) and on day +1 ($P<0.001$), while no later effects were observed. For IL-2Rα, we observed a statistically lower serum concentration for MAC on day −1 and on day +1 ($P<0.001$ and $P<0.001$), while no later effects were observed.

For HGF, we found lower serum concentration for MAC patients on days −1 and +1 ($P=0.01$ and $P=0.01$).

On day −1 IL-8 and IL-12p70 serum concentrations were lower following MAC regimens ($P<0.001$ and $P<0.001$). The IL-12p70 serum concentration following MAC regimen was also lower on day +1 ($P=0.02$) (Supplementary Table 3).

The patient and donor's age effect. For the TNFR1 analysis, we found a significant higher concentration for older patients on day −1 ($P<0.001$), +1 ($P=0.01$), +7 ($P=0.05$), +14 ($P=0.04$) and +21 ($P=0.04$), while no patient age effect was seen for IL-2Rα, IL-8 or CCL8. The HGF was higher for older patients on days −1 ($P=0.03$), +14 ($P=0.03$) and +21 ($P=0.04$).

The IL-12p70 serum concentration was higher for younger patients on day +21 ($P=0.01$).

When we stratified patients according to donor age, the TNFR1 was significantly higher for older donors on day +1 ($P=0.01$), +7 ($P=0.05$), +14 ($P=0.04$) and +21 ($P=0.04$), while IL-2Rα did not differ among these groups. The HGF concentration was significantly higher for older donors on day +14 ($P=0.02$). Finally, the IL-12p70 serum concentration was significantly higher for older donors on day +14 ($P=0.01$, Supplementary Table 4).

Multivariate analysis of factors affecting cytokine serum concentrations. We then performed a multinomial logistic regression (backward stepwise) analysis to evaluate the impact of factors predicting higher serum concentrations of IL-2Rα, HGF and IL-8. We were not able to find any independent factor for IL-2Rα either on day +14 or on day +21, while the HGF serum concentration on day +14 was significantly associated with the HSC source (PB vs others, RR 3.39 (95% CI, 1.18–10.2), $P=0.031$) and on day +21 it was significantly affected by the patient (RR 2.25 (95% CI, 1.09–4.65), $P=0.028$) and donor age (RR 3.52 (95% CI, 1.25–9.87), $P=0.017$). The IL-8 serum concentration was independently related to both donor type (RR 0.007 (95% CI, 0.002–0.02), $P<0.001$) and patient age (RR 0.29 (95% CI, 0.11–0.73), $P<0.001$, Supplementary Table 5).

DISCUSSION

A large amount of conflicting evidence in terms of cytokine and GVHD markers has been published. Mathias *et al*.[15] showed the IL-2Rα concentration was helpful in establishing the diagnosis of aGVHD, since they observed a marked increased value of IL-2Rα over baseline values in serial measurements. The Iranian group showed the effects of IL-18 and IL-2Rα in 39 patients. They observed that IL-18 concentration on day +10 correlated with the severity of GVHD, before clinical symptoms developed.[16] More recently other published papers have reported different results on IL-12p70. In a series of reduced intensity conditioning, Mohty's study reported that, among a panel of 10 cytokines, only the IL-12p70 level was significantly associated with grade II–IV aGVHD.[17] In the same year, the paper by Reddy *et al*. reported no differences of aGVHD according to IL-12 levels.[7] Regarding HGF, the Japanese group showed, in 38 patients, that HGF correlated significantly with the grade of GVHD.[18] Moreover, the HGF serum concentration was found to be correlated to gastrointestinal GVHD together with regenerating islet-derived 3-alpha (REG3a[19]) and cytokeratin-18.[20] The IL-7 serum concentration was also monitored in a study of 31 patients who underwent reduced intensity MFD-HSCT. Acute GVHD was significantly associated with a higher IL-7 concentration on days +7 and +14 together with the CD34+ cell dose, with high sensitivity and specificity.[21] Finally, proteonomic studies showed that elafin was significantly associated to skin GVHD.[9]

Some considerations may be extrapolated from our study.

The first step of our study was to analyse the role of six cytokines in GVHD occurrence. We found that two cytokines were related to grade II–IV and III-IV GVHD, the IL-2Rα and HGF. The IL-2R is a heterotrimeric protein expressed on the surface of certain immune cells that binds and responds to IL-2. The IL-2R is made up of three non-covalently associating subunits—α (CD25), β (CD122) and γ (CD132). The α and β chains are involved in binding IL-2, while signal transduction following cytokine interaction is carried out by the γ-chain, along with the β subunit. The β and γ chains of the IL-2R are members of the type I cytokine receptor family. HGF is a paracrine cellular growth, motility and morphogenic factor. It is secreted by mesenchymal cells and targets and acts primarily upon epithelial cells and endothelial cells, but also acts on hemopoietic progenitor cells. It has been shown to have a major role in embryonic organ development, in adult organ regeneration and in wound healing.[22–24]

As reported above, IL-2Rα might be considered as a marker of skin GVHD, while, interestingly, HGF was found to be significantly higher in patients with visceral-only and skin-visceral GVHD compared to skin-only GVHD.[19] When we later combined these cytokines, the CI of grades II–IV and III–IV GVHD was significantly higher. Furthermore, when we compared the visceral GVHD we found that day +14 IL-2Rα and HGF were higher in patients with visceral GVHD ($P=0.02$). The TRM was significantly associated with higher serum concentrations of IL-2Rα and HGF, and despite a lower RI, OS was similar for patients having lower serum concentrations of IL-2α and HGF. Despite the low specificity of the IL-2Rα-HGF combination calculated by the receiver operating characteristic curve analysis, the sensitivity was 87%, indicating that a very high proportion of patients developing GVHD could be diagnosed. All these data were confirmed when the analysis was restricted to the MUD group. However, it has to be underlined that, over the years, the use of more intensive conditioning regimens such as TBI has changed altogether the *in vivo* GVHD prophylaxis (Supplementary Table 6), and potentially this could have an impact on GVHD occurrence. However, we were able to confirm the independent value of IL-2α and HGF irrespective of the HSCT-year and GVHD prophylaxis effects. Interestingly, we found that the day +28 lower IL-8 serum concentration was significantly associated with cGVHD even when the analysis was restricted to MUD recipients. When we conducted the multivariate analysis we found that both the donor type (MUD) and patient's age (<9.7 years) were independently associated with a higher IL-8 serum concentration and finally with lower cGVHD CI.

Secondly, we were able to demonstrate the effect of several variables on paediatric-HSCT serum cytokine concentrations such as the donor type, the HSC source, the conditioning regimen intensity, and the patient- and donor-age effect. These factors had different effects on cytokines and on the timing of their effects. In particular we observed: (a) the CCL8 serum concentration was significantly higher following MUD HSCT; (b) the PB-derived HSCT group had a high concentration of TNFR1 and IL-2Rα early after transplantation; (c) the serum concentrations of TNFR1 and IL-2Rα were significantly lower for MAC on days −1 and +1 following HSCT (This might be due to a combination of factors: patient and donor's age and probably also due to a higher cell number given to a reduced toxicity regimen patient group.); (d) the older patient and donor groups had high TNFR1 serum concentrations until day +21. The multinomial logistic regression analysis showed that the HGF day +14 and +21 serum concentrations were related to patient and donor age, while the unrelated donor cytokine serum concentrations were significantly associated with a lower IL-8 serum concentration. Unfortunately, while we cannot extrapolate any independent factor affecting IL-2Rα serum concentrations, it has to be stated that the role of PB-derived HSCs was mainly evaluated in the MUD group because, in Italy, the G-CSF administration to donors who are minors is prohibited.

In conclusion, our study confirms the role of HGF as a gastrointestinal aGVHD marker as reported by the Japanese group[18] and the combination of cytokines as factors related to GVHD, as reported by the University of Michigan group.[8–10]

In other words, although advanced GVHD remains an unresolved issue, we now have the possibility to study the biological mechanisms before it is of clinical relevance.

These data have to be confirmed in a larger prospective study and, if confirmed, a two-biomarker analysis might limit the invasive procedure for GVHD diagnosis, and also discriminate patients with high-risk features and, consequently, provide an individualized therapy for each patient that would improve the prognosis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Author contributions: MB wrote the paper and analysed the data, ES and MM carried out research, PQ, EB, FN, and EV analysed the data, FF designed research.

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