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ORIGINAL ARTICLE

Profile of Inflammation-Associated Proteins in Early Post-Transplant Samples of Patients After Allogeneic Hematopoietic Stem Cell Transplantation: a Preliminary Study

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Abstract Allogeneic hematopoietic stem cell transplantation (aHSCT) is used as a curative treatment in severe hematological and immunological disorders. Despite clear improvement of the aHSCT outcome, substantial proportion of patients still suffers from severe complications, including graft-versus-host disease (GvHD). The aim of this study was, therefore, to identify inflammation-associated molecules deregulated in the early serum samples of the patients after aHSCT and nominate markers associated with particular aHSCT parameters/complications. Serum concentrations of 92 inflammation-associated proteins were measured in samples obtained from 80 aHSCT patients 14 days after transplantation and from 23 healthy control subjects by a novel sensitive proximity extension assay technology using Proseek Multiplex Inflammation I kit. Serum profiles of inflammatory proteins in patients after aHSCT were substantially different from those observed in control subjects and related to underlying disease status before transplantation. Particularly, the difference between aHSCT patients and controls reached significance level for 57 analytes (40 upregulated, 17 downregulated in aHSCT patients). The concentration of several markers was associated with the level of donor/recipient HLA match (TGF-α: $p_{corr} = 0.025$, HGF: $p_{corr} = 0.036$) and with complete donor chimerism at day +30 after allografting (DNER: $p_{corr} = 0.042$). None of the markers was significantly associated with acute and chronic GvHD after correction. More than half of investigated proteins significantly differed between the samples from aHSCT patients and healthy control subjects as a consequence of the "cytokine storm" after aHSCT. Comparisons of patient's subgroups based on specific biological/clinical parameters revealed much less evident differences; nevertheless, we nominated several markers associated with the level of donor/recipient HLA match and post-transplant chimerism.

Keywords Hematopoetic stem cell transplantation · Graft-versus-host disease · Protein profiles · Biomarkers · Inflammation

Introduction

Allogeneic hematopoietic stem cell transplantation (aHSCT) is used as curative treatment in severe hematological malignancies and primary immunodeficiencies (Gratwohl et al. 2013). Despite substantial developments of approaches in aHSCT (from donor selection and "tailored" conditioning regimens up to complex supportive care), the morbidity and mortality of aHSCT are still unsatisfactorily high (MacMillan et al. 2008).

Numerous biological, clinical, and demographic factors were previously associated with the overall outcome of aHCST. It is widely accepted that "high-risk" diseases,

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long period from diagnosis to transplantation, higher age of both the patient and stem cell donor, and severe co-morbidities are associated with worse prognosis of aHSCT patients. Apart from the relapse of underlying disease, graft-versus-host disease (GvHD), infections, and regimen-related toxicity (RRT) are the most frequent causes of aHSCT failure. It is apparent that all these major aHSCT complications are tightly associated with the functions and mechanisms of the immune system and inflammation, particularly: (1) beneficial role of immune alloreactivity in control of relapses of hematological malignancies (graft versus leukemia effect), (2) the early reconstitution of the immune system as a prevention of life-threatening infections, (3) immune alloreactivity manifested in a destructive manner—GvHD (Choi and Reddy 2014), and (4) pathways of inflammation and DNA damage response in RRT complications. Accordingly, balancing the immune response after the transplantation seems to be critical for the control of severe aHSCT complications.

The beneficial effect of HLA match between donor and recipient in aHSCT was repeatedly confirmed (Petersdorf 2008). Enormous interest in further gene variants associated with aHSCT outcome resulted in the development of "non-HLA" genetics of aHSCT in the last decade (Chien et al. 2012). The group of candidate genes important for particular processes of immune alloreactivity comprises minor histocompatibility antigens, cytokines, adhesion molecules (Ambruzova et al. 2009), integrins, receptors of NK cells (KIRs), and others. Recent reports enabled to apply genotyping of KIR genes for the selection of optimal donor for aHSCT in acute myeloblastic leukemia (Cooley et al. 2010).

At present, our real possibilities to identify the recipients in high risk of severe aHSCT complications and for the pre-emptive diagnostics of them are very limited. In this respect, great effort has been focused in the past two decades to the search for biomarkers of aHSCT complications using classical approaches and complex proteomic analysis (Levine et al. 2015; Paczesny et al. 2013) that lead to the identification of several promising molecular targets. It is widely accepted that application of various methods based on different principles may significantly enlarge the chance to identify relevant markers of aHSCT complications (Fiema et al. 2012; Frampton et al. 2014). Using the novel sensitive multiplex technique, proximity extension assay (PEA) (Assarsson et al. 2014; Lundberg et al. 2011), our preliminary study aims, therefore, to reveal inflammation-associated proteins differentially expressed in the early samples of patients after aHSCT and markers associated with particular aHSCT parameters and complications (e.g., GvHD).

Materials and Methods

Study Population

Eighty adult patients who underwent indicated aHSCT in a single tertiary hemato-oncological centre (University Hospital Olomouc) and 23 healthy control subjects were involved into this study. Clinical and demographic characteristics of aHSCT patients are listed in Table 1. Involvement of the patient to the study was not limited by the main diagnosis, type of donor (related/unrelated), type of conditioning regimen, nor level of HLA match. The diagnosis of acute GVHD (aGVHD) and chronic GVHD (cGVHD) was made in regard to clinical manifestations and histological findings of biopsied tissue. It was graded and staged according to established criteria (Cahn et al. 2005; Przepiorka et al. 1995; Sullivan et al. 1991). Informed consent for the blood sampling as well as protein expression testing was obtained from each investigated subject. The study was approved by the Ethics Committee of the Medical Faculty of Palacky University and the Faculty Hospital in Olomouc.

Analysis of Serum Protein Profiles

Serum profiles of 92 inflammation related proteins were determined in the samples obtained from the patients

Table 1 Characteristics of the patients after aHSCT involved in this study ($N = 80$)

Parameters	Recipients	Donors	Parameters	*N*
Gender (M/F)	48/32	57/23	*Stem cells source*	
Age (median, range)	49 (20–64)	35.5 (18–63)	Bone marrow	6
Diagnosis[a]			Peripheral blood	74
AML	30	NA	*Acute GvHD (grade)*	
ALL	16	NA	0–I	55
MDS/MPD	13	NA	II	13
CLL	9	NA	III	11
CML	4	NA	IV	1
Other	8	NA	*Chronic GVHD*	
Donor type			No	43
Related	24 (30%)		Limited	6
Unrelated	56		Extensive	15
HLA match (A, B, C, DRB1, DQB1)			Not evaluated	16
10/10	57 (71%)		Living (last follow-up)	57
9/10	23		Departeds (last follow-up)	23

NA not applicable

[a] Patients were allografted for acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), myelodysplastic/myeloproliferative syndrome (MDS/MPD), chronic lymphocytic leukemia (CLL), chronic myeloid leukemia (CML) and other hematological disorders

14 days after application of hematopoietic stem cell allograft and from the healthy control subjects. PEA (Assarsson et al. 2014; Lundberg et al. 2011) using the Proseek Multiplex Inflammation I kit (Olink Bioscience, Uppsala, Sweden) was performed according to the manufacturer's instructions (for investigated markers, please refer to the Supplementary Table 1). Briefly, serum samples (1 µl) were incubated with 92 oligonucleotide labelled antibody probe pairs that bind to their respective target present in the sample. A PCR reporter sequence was formed by a proximity dependent DNA polymerization event and was subsequently detected and quantified using high-throughput real-time PCR (BioMark^{TM} HD System, Fluidigm Corporation). Data analysis was performed by employing a pre-processing normalization procedure. For each data point, delta Cq (dCq) values were obtained by subtracting the value for the extension control, thus normalizing each sample for technical variation within one run. Normalization between runs was then performed by subtraction of the interplate control for each assay. In the final step of the pre-processing procedure, the values were set relative to a correction factor determined by Olink. The generated Normalized Protein eXpression (NPX) unit is on a log2 scale where a larger number represents a higher protein level in the sample. Linearization of the protein expression data (linear ddCq) was performed by the mathematical operation 2^{NPX}.

Statistical Analysis

For each analysed group (subgroup) of aHSCT patients and healthy control subjects, mean values with 95% group confidence interval of linearized protein expression of 92 investigated inflammation-associated markers were determined. The comparisons between groups (aHSCT patients versus control subjects and subgroups of patients according to GvHD, chimerism and HLA match) were performed by the Mann–Whitney–Wilcoxon test. Nominal p values were corrected for the multiple comparisons using the Benjamini–Hochberg procedure (Benjamini and Hochberg 1995). Corrected p values less than 0.05 were considered as statistically significant.

Results and Discussion

To compare concentrations of 92 inflammation-associated molecules between the patients after aHSCT and healthy control subjects, we investigated samples obtained from 80 patients 14 days after allografting and 23 controls by PEA technology using Proseek Multiplex Inflammation I kit. As expected, serum profiles of inflammatory proteins in patients after aHSCT were substantially different from those observed in control subjects. Out of 92 compared markers, the differences were statistically significant (with corrected p values <0.05) for 57 of them; 40 molecules were upregulated (e.g., CSF-1, IL-6, CX3CL1, and CCL23) and 17 downregulated (e.g., SCF, CXCL5, and TRANCE) in aHSCT patients by comparison with healthy control subjects (Table 2). Importantly, for 20 markers, the difference between aHSCT patients and controls reached significance level at $p_{corr} < 10^{-7}$. Such deeply different protein profiles in the early samples of aHSCT patients reflect "cytokine storm" protein signature, but may also involve signals from already developing alloimmune reaction originating from the graft/recipient interaction.

The "cytokine storm" after aHSCT is a well-known phenomena originating primarily from tissue damage and inflammation due to the underlying disease, conditioning regimen and donor T cells activation after interaction with host antigen-presenting cells (Ferrara et al. 2009). Both in animal models of aHSCT and clinical transplantation in humans, conditioning regimen usually leads to the potent induction and release of pro-inflammatory and several anti-inflammatory (as a reflection of severe systemic inflammation) cytokines. Consistent with the previous observations in aHSCT (Hill et al. 1997; Toubai et al. 2012; Xun et al. 1994), we found significant upregulation of numerous pro-inflammatory and regulatory (anti-inflammatory) cytokines, chemokines, and growth factors (IL-6, IFN-γ, IL-18, IL-18R1, CCL23, CX3CL1, CCL3, MCP-1, IL-10, IL-10RB, IL-17A, etc.). Not surprising, we observed strong upregulation of macrophage colony-stimulating factor 1 (CSF-1) which reflects induction of haematopoiesis from the graft cells, and of Caspase 8, a cysteine protease which is involved in apoptosis, activation of T, B, and NK cells, and macrophage differentiation. In this respect, promoter polymorphism of the gene encoding for Caspase 8 was recently associated with the survival following unrelated aHSCT, particularly in the context of T-cell depletion with Alemtuzumab (Shaw et al. 2015).

Inconsistent with expectations, no significant increase of the "classical cytokine storm" associated pro-inflammatory cytokines TNF and IL-1 (IL-1α) was observed in this study. Nevertheless, there are the reports from animal models showing that conditioning regimen causes rapid but temporary increase of tumor necrosis factor (TNF) and IL-1α with subsequent normalization within 3–5 days after conditioning regimen (Xun et al. 1994). Despite the release of these pleiotropic cytokines may later be augmented in relation with ingoing aHSCT complications (aGVHD, infections, and veno-occlusive disease), in samples obtained 2 weeks after aHSCT, the levels of TNF and IL-1 may appear unchanged in the majority of patients. Furthermore, cytokine storm depends on the type (total body irradiation, chemotherapy) and intensity (myeloablative

Table 2 Fifty-seven inflammation-associated molecules with significantly different concentration between the serum samples obtained from aHSCT patients 14 days after allografing and those obtained from healthy control subjects

Analyte	Change[a]	Nominal *p*	Corrected *p*	Analyte	Change[a]	Nominal *p*	Corrected *p*
CSF-1	U	4.91e−13	3.6e−11	CCL3	U	6.36e−6	1.93e−5
IL-6	U	7.91e−13	3.6e−11	FGF-23	U	8.08e−6	2.37e−5
CX3CL1	U	1.76e−12	5.34e−11	FGF-21	U	2.35e−5	6.59e−5
SCF	D	3.47e−12	7.9e−11	CXCL9	U	2.39e−5	6.59e−5
CCL23	U	1.82e−11	3.32e−10	OPG	U	4.61e−5	1.23e−4
CXCL5	D	2.52e−11	3.83e−10	ADA	U	7.63e−5	1.98e−4
IL-10RB	U	5.62e−11	7.3e−10	VEGF-A	U	1.56e−4	3.94e−4
CASP-8	U	1.21e−10	1.32e−9	STAMPB	U	1.86e−4	4.56e−4
CD40	U	1.45e−10	1.32e−9	LAPTGF-β1	D	1.97e−4	4.73e−4
IFN-γ	U	1.57e−10	1.32e−9	CCL28	D	2.61e−4	6.09e−4
CDCP1	U	1.65e−10	1.32e−9	MCP-4	U	4.06e−4	9.25e−4
TRANCE	D	1.74e−10	1.32e−9	MCP-3	U	4.65e−4	1.03e−3
IL-10	U	3.94e−10	2.76e−9	TNFRSF9	U	5.8e−4	1.26e−3
TWEAK	D	7.97e−10	5.18e−9	PD-L1	U	9.67e−4	2.05e−3
IL-15RA	U	1.44e−9	8.71e−9	OSM	D	2.52e−3	5.21e−3
DNER	D	1.63e−9	9.26e−9	MCP-1	U	3.52e−3	7.12e−3
TNFB	D	1.8e−9	9.64e−9	IL-17A	U	3.64e−3	7.2e−3
IL-18	U	6.92e−9	3.5e−8	IL-18R1	U	3.99e−3	7.73e−3
TRAIL	D	8.35e−9	4e−8	IL-24	U	4.09e−3	7.75e−3
CD5	U	1.46e−8	6.64e−8	AXIN1	U	4.29e−3	7.96e−3
Flt3L	U	2.36e−8	1.02e−7	SLAMF1	D	5.69e−3	1.04e−2
CCL19	U	6.49e−8	2.68e−7	EN-RAGE	D	6.52e−3	1.16e−2
CD244	D	1.12e−7	4.43e−7	ST1A1	U	1.03e−2	1.79e−2
NT-3	D	3.79e−7	1.44e−6	TGF-α	D	1.04e−2	1.79e−2
CD6	D	1.16e−6	4.22e−6	4e−BP1	U	2.08e−2	3.5e−2
CST5	U	1.59e−6	5.58e−6	CXCL11	U	2.45e−2	4.06e−2
SIRT2	U	1.66e−6	5.59e−6	CCL11	D	2.85e−2	4.64e−2
CXCL10	U	2.65e−6	8.62e−6	IL-7	U	3.12e−2	4.99e−2
β-NGF	U	3.96e−6	1.24e−5				

[a] U—"upregulated": concentration of the analyte is significantly higher in aHSCT patients than in control subjects. D—"downregulated": concentration of the analyte is significantly lower in aHSCT patients than in control subjects. A *p* value "4.91e−13" means 4.91×10^{-13}

and reduced intensity) of the conditioning regimen. We are aware that also other pre-transplant biological parameters, namely diagnosis and status of underlying disease, may significantly affect the profile of inflammatory molecules in heterogeneous population of aHSCT patients. Accordingly, it has been shown that the pre-transplantation serum cytokine profile in recipients differs from healthy individuals and shows considerable variation even between the patients with complete hematological remission (Reikvam et al. 2012). Although we could not compare the protein profiles before and after transplantation (pre-transplant samples were not available for comparison), we revealed significant differences in post-transplant (day +14) samples between the patients allografted at complete remission ($N = 44$) and those transplanted in active disease ($N = 33$, Supplementary Table 2), which further emphasizes the importance of underlying disease for early post-transplantation protein profiles.

Apart from 40 molecules significantly upregulated in the samples of aHSCT patients, other 17 analytes showed lower levels by comparison with healthy control subjects (Table 2). These molecules are involved in several different pathways of apoptosis, haematopoiesis, chemotaxis, and inflammation. Stem cell factor (SCF, c-Kit-ligand) is a cytokine that plays an important role in haematopoiesis and has recently been shown to inversely correlate with T-cell reconstitution and clinical outcomes after cord blood transplantation in adults (Politikos et al. 2015). The most numerous group of downregulated markers belongs to the TNF superfamily, such as TNF-related activation-induced cytokine (TRANCE, RANKL, TNFSF11), lymphotoxin α (TNFB, TNFSF1), TNF (ligand) superfamily, member 12

(TWEAK, TNFSF12), and TNF-related apoptosis-inducing ligand (TRAIL, TNFSF10). Whether the lower concentration of these markers in serum samples two weeks after aHSTC may reflect regulation of response to tissue damage, environment of developing haematopoiesis together with GvHD prophylaxis (immunosuppression) or another unknown cause, remains unclear.

We were also interested whether any differences in protein profiles could be identified in our relatively small aHSCT cohort between subgroups of patients based on particular clinical/biological parameters. In general, comparisons of patient's subgroups based on these parameters revealed much less evident differences in profile of inflammation markers. First, when we compared aHSCT patients later developing acute GvHD ($N = 27$; grade I: 2, grade II: 13, grade III: 11, and grade IV: 1) with those without this complication ($N = 53$), the subgroups differed nominally only in three proteins (IL2-RB, CCL19, and CXCL6), but none was significant after correction (Table 3). Similarly, in comparison of patients with chronic GvHD ($N = 21$; extensive: 15, limited: 6) with those without cGvHD ($N = 43$; 16 patients not classified), a trend to deregulation of six proteins (CX3CL1, SLAMF1, MCP-4, DNER, IL-10RA, IL-17A) was observed, but with insignificant corrected p value.

Interestingly, comparison between patients with completely HLA matched donor and those with mismatched donor revealed two markers (TGF-α: $p_{corr} = 0.025$, HGF: $p_{corr} = 0.036$) significantly different between subgroups after correction. For example, transforming growth factor (TGF)-α which was found in lower concentrations in patients after aHSCT than in control subjects (see Table 2) seems to be even more downregulated in patients with HLA-mismatched donor (Table 3). Both TGF-α and hepatocyte growth factor (HGF) are growth factors that play important roles, e.g., in angiogenesis and tissue regeneration. Circulating TGF-α acts via its receptor epidermal growth factor receptor (EGFR). It has been shown that another EGFR ligand, namely EGF, was decreased in aHSCT patients at the onset of aGvHD (Holtan et al. 2015). With regard to this finding, one can speculate that downregulation of TGF-α in patients with HLA-mismatched donor may by analogy reflect incipient alloreaction based on HLA incompatibility.

Furthermore, we also identified one marker, namely Delta and Notch-like epidermal growth factor-related receptor (DNER), which was more abundant in the early samples of aHSCT patients who reached complete donor chimerism at day +30 after allografting ($N = 61$) by comparison to those with mixed chimerism ($N = 18$) (Fig. 1). DNER is a transmembrane protein primarily found in the central nervous system and previously described as an activator of the NOTCH1 pathway which is a well-conserved cell-fate determining factor in embryo development (Gao et al. 2016). Nevertheless, Greene et al. (2016) has very recently shown that DNER is not a Notch ligand and its true function remains to be elucidated. Accordingly, we are not able to provide plausible explanation for our original observation which deserves further clarification.

Table 3 Subanalysis of the serum concentration of inflammation-associated molecules 14 days after allografting between the subgroups of aHSCT patients based on acute and chronic GvHD, donor chimerism at day +30 (mixed versus complete), and donor/recipient HLA match. All nominally significant differences are reported for aGVHD and cGVHD (none significant after correction)

Subgroup 1 (*N*)	Subgroup 2 (*N*)	Analyte	Change[a]	Nominal *p*	Corrected *p*
aGvHD (27)	No aGvHD (53)	IL-2RB	D	8.16e−3	0.74
		CCL19	U	2.12e−2	0.79
		CXCL6	U	4.72e−2	0.79
cGVHD (21)	No cGVHD (43)	CX3CL1	D	4.23e−3	0.39
		SLAMF1	U	1.11e−2	0.39
		MCP-4	U	1.28e−2	0.39
		DNER	D	2.21e−2	0.50
		IL-10RA	U	3.01e−2	0.55
		IL-17A	U	4.77e−2	0.71
Mix. chimer. (18)	Compl. chimer. (61)	DNER	D	4.63e−4	**0.042**
HLA match (57)	HLA mismatch (23)	TGFA	U	2.72e−4	**0.025**
		HGF	U	7.96e−4	**0.036**

Differences significant after correction are presented with corrected p values in bold

N the number of patients analysed in particular subgroup

[a] U—"upregulated": concentration of the analyte is higher in the subgroup 1 than in subgroup 2. D—"downregulated": concentration of the analyte is higher in the subgroup 2 than in subgroup 1. A p value "4.63e−4" means 4.63×10^{-4}

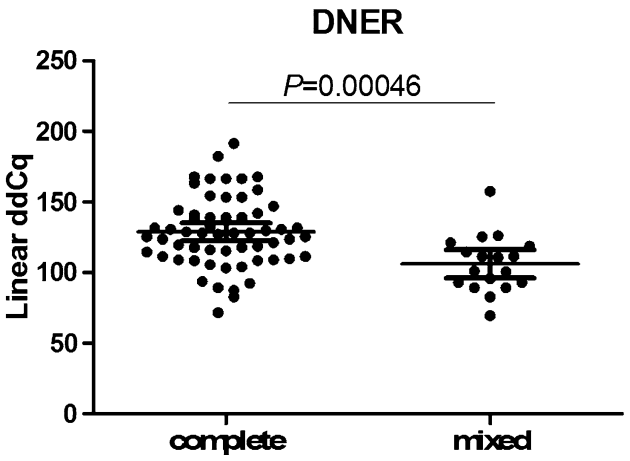

Fig. 1 Linearized protein expression (linear ddCq, see "Materials and methods" section) of the Delta and Notch-like epidermal growth factor-related receptor (DNER) in the patients after aHSCT with complete donor chimerism ("complete", $N = 61$) and mixed chimerism ("mixed", $N = 18$) at day +30 after transplantation (chimerism was not evaluated in one patient). *Data points* represent the amount of the protein in each individual sample; the *lines* represent mean value (longer horizontal line) with 95% confidence interval for subgroup mean value. For comparison of "complete" versus "mixed" subgroups: $p_{nom} = 0.00046$, $p_{corr} = 0.042$

Based on our current literary knowledge, the important immune/inflammation mediators and cells are for long term investigated in the studies focused on the identification of systemic and local biomarkers of aHSCT outcome. The previous reports revealed, e.g., predictive value of the panel of molecules IL-2Rα, TNFR1, HGF, and IL-8 for acute GvHD (Paczesny et al. 2009). Chemokine IL-8 (CXCL8) was identified as a potential diagnostic marker for GvHD also in the Japanese proteomic study (Hori et al. 2008). Another paper revealed tight association of decreased number of the regulatory T lymphocytes ($CD4^+CD25^{hi}FOXP3^+$) with the higher occurrence and severity of GvHD (Magenau et al. 2010). Furthermore, novel biomarkers for particular organ manifestations of GvHD, e.g., elafin for skin GvHD have been reported (Paczesny et al. 2010). Recently, a prognostic score for acute GvHD prediction based on biomarkers TNFR1, ST2, and REG3α was proposed and evaluated in a multicenter study (Levine et al. 2015). Out of above-mentioned aGVHD markers, we were not able to concordantly replicate association with aGVHD for two markers involved in our protein panel (IL-8 and HGF). The reason for that may be that our preliminary study was based on lower number of aHSCT patients (with only 27 those with aGvHD) than in the previous proteomic studies (Paczesny et al. 2009). Another explanation of this inconsistency may lean on single fixed timepoint of sample collection (+14 days after allografting) in this study by comparison to sequential series of samples, including those obtained within 24 h after diagnosis of aGvHD in the study of Paczesny et al. (2009).

In conclusion, here, we present our preliminary data obtained by measurement of large panel of inflammatory-associated proteins in the early post-transplant serum samples from patients after aHSCT using a novel sensitive PEA. Based on our findings, we can sum up that PEA may be considered as reliable technique for monitoring of inflammatory protein profiles after aHSCT. We revealed that concentration of more than half of investigated proteins significantly differed between samples from aHSCT patients and healthy control subjects, the majority of deregulated markers were increased in the patients. Comparisons of patient's subgroups based on specific biological/clinical parameters revealed much less evident differences in particular markers of inflammation; however, we nominated several markers associated with the level of donor/recipient HLA match and chimerism at day +30 post-transplant. It is important to note that when aiming at one endpoint (e.g., aGvHD), one has to consider further variables that may significantly affect protein profiles (e.g., underlying disease status). Our planned follow-up study will concentrate in the enlargement of the patient's group, assessment of profiles in serial samples after aHSCT, and predictive models based on combined clinical and systemic markers.

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