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The Netherlands

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## ARTICLE



# Pre-transplant inflammation and its associations with acute GvHD and mortality in pediatric allogeneic hematopoietic stem cell transplantation patients

Anne B. Verbeek<sup>1</sup>✉, Erik G. J. von Asmuth<sup>1</sup>, Erik B. van den Akker<sup>2</sup>, Anja M. Jansen-Hoogendijk<sup>1</sup>, Marco W. Schilham<sup>1</sup>, Arjan C. Lankester<sup>1</sup>, Gertjan Lugthart<sup>1</sup>, Alexander B. Mohseny<sup>1</sup> and Emilie P. Buddingh<sup>1</sup>✉

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In this explorative study we aimed to identify inflammatory serum proteins measured before allogeneic hematopoietic stem cell transplantation (HSCT) that are associated with acute Graft-versus-Host Disease (aGvHD) and mortality in pediatric HSCT recipients. We measured 28 inflammatory serum proteins in 384 pediatric patients (2010–2022) with malignant (30%) and non-malignant (70%) indications for allogeneic HSCT. A sample before the start of the conditioning (T1) was included, as well as a sample on the day of HSCT (T2). For patients who developed aGvHD we also included a sample at the time of diagnosis, before initiation of systemic treatment (TP-GvHD). Associations with aGvHD, steroid-refractory aGvHD, non-relapse mortality (NRM) and overall survival (OS) were analyzed using robust (cause-specific) Cox models. At T1, TNFR1, sIL-2R $\alpha$  and TNFR2 were among the most strongly associated proteins for the development of aGvHD. Multiple inflammatory proteins, such as CXCL16, TNFR1 and SCF at T1 were highly associated with NRM and OS. At TP-GvHD, ST2 levels were significantly associated with the development of steroid-refractory aGvHD and NRM. This study shows that inflammatory serum protein levels before HSCT are associated with aGvHD and mortality and lays the groundwork for further validation and use in risk-adapted therapy for pediatric HSCT recipients.

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## INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) is a curative treatment for severe hematological and immunological diseases, such as hematological malignancies, hemoglobinopathies and inborn errors of immunity (IEI) [1]. Its success, however, is hampered by the occurrence of potentially lethal complications, the most common being acute Graft-versus-Host Disease (aGvHD) [2, 3].

The identification of patients at greater risk of HSCT-related complications has been the focus of many biomarker studies since this could allow for preemptive interventions to minimize tissue damage and inflammation and improve outcomes (Supplementary Table 1). However, these biomarkers have mostly been studied in adult populations. Reports from pediatric cohorts show inconsistent results, mainly due to small sample size [4]. Recently, Qayed et al. reported on a large pediatric biomarker cohort with over 300 GvHD cases, however, only samples after GvHD onset were included [5]. Evaluation of prognostic biomarkers in pediatric HSCT populations is important because underlying conditions, transplantation strategies and complications such as aGvHD and mortality rates differ significantly from those in adults [6].

Few biomarkers have been studied in a pre-transplantation setting [7–15]. As tissue damage caused by the underlying disease and/or by the conditioning regimen is associated with the

development of aGvHD and mortality [16–18], the evaluation of pro-inflammatory cytokines and other serum proteins released before HSCT may identify children at risk and allow for timely therapeutic interventions to improve outcomes.

In the current explorative study, we evaluated the pre-HSCT levels of serum proteins associated with inflammation and endothelial activation in children scheduled for HSCT. We studied whether levels of these serum proteins were associated with adverse outcomes and whether they were affected by the conditioning regimen. Our aim was to discover serum proteins that could be used as prognostic biomarkers in pediatric HSCT patients.

## METHODS

Pediatric patients who received their first allogeneic HSCTs at the Willem Alexander Children's Hospital in Leiden between 01-01-2010 and 31-01-2022 were eligible for inclusion in this study. Informed consent for the biobanking of blood samples was obtained for all patients included. All patient data were pseudonymized before use. The study protocol was in line with the principles of the Declaration of Helsinki. The institutional research committee and the Medical Research Ethics Committee Leiden the Hague Delft reviewed and approved of the study protocol (B21.067). All methods were performed in accordance with the relevant guidelines and regulations.

<sup>1</sup>Willem-Alexander Children's Hospital, Department of Pediatrics, Pediatric Stem Cell Transplantation unit and Laboratory of Pediatric Immunology, Leiden University Medical Center, Leiden, The Netherlands. <sup>2</sup>Molecular Epidemiology, Department of Biomedical Data Sciences, Leiden University Medical Center, Leiden, The Netherlands.

✉email: a.b.verbeek@lumc.nl; E.P.Buddingh@lumc.nl

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The primary outcome for the study was the association of serum protein levels before HSCT with aGvHD (Grade II-IV), overall survival (OS) and non-relapse mortality (NRM). Secondary outcomes for this study were the change in serum protein levels in response to the conditioning regimen and, for patients who developed aGvHD, the association of serum protein levels before initiation of aGvHD treatment with the development of steroid-refractory (SR) aGvHD and NRM. AGvHD was graded according to the modified Glucksberg criteria [19]. NRM was defined as death without preceding relapse. SR-aGvHD was defined as progression of aGvHD within 3–5 days after start of first-line treatment with  $\geq 2$  mg/kg/d of prednisone or no improvement within 5–7 days or incomplete response after >28 days of immunosuppressive treatment including steroids [20].

For each patient, biobanked serum samples were retrospectively selected at two timepoints: before conditioning (T1) and after conditioning (within 2 days before HSCT up until the day of HSCT, T2). For patients who developed aGvHD, we also included a sample at time of diagnosis of aGvHD, before the initiation of first-line treatment with systemic steroids (TP-GvHD). Samples were stored in  $-80^{\circ}\text{C}$  until use. A customized multiplex fluorescent microbead immunoassay (Luminex Discovery Assay, R&D System, Minneapolis, MN, USA) was used according to the manufacturer's instructions [21]. We selected 28 serum proteins, previously associated with aGvHD, other inflammatory complications or mortality in HSCT (Supplementary Table 1): Angiopoietin 2 (ANG2), C-C motif ligand (CCL) 2, CCL20, CCL8, C-X-C motif ligand (CXCL) 9, CXCL13, CXCL16, E-Selectin, Intercellular adhesion molecule 1 (ICAM-1), Interleukin (IL) 5, IL-6, IL-6R $\alpha$ , IL-10, IL-17, Macrophage migration inhibitory factor (MIF), Pentraxin-related protein (PTX3), P-Selectin, Receptor for advanced glycosylation end products (RAGE), S100 calcium-binding protein A9 (S100A9), Soluble IL-2 receptor subunit alpha (sIL-2R $\alpha$ ), Stem cell factor (SCF), Suppression of tumorigenicity 2 (ST2), Tissue Factor (TF), Tumor necrosis factor receptor (TNFR) 1, TNFR2, Tyrosine kinase with Ig and EGF homology domains-2 (TIE-2), Vascular cell adhesion molecule 1 (VCAM-1) and Vascular endothelial growth factor C (VEGF-C). Out of range values were imputed by adding 10% to the highest value of that specific serum protein (for values above the upper detection level) or by subtracting 10% from the lowest value (for values below the lower detection level). IL-5 was excluded from all analyses because more than 85% of measurements were below the lower limit of detection. Percentages of imputed data are displayed in Supplementary Table 2. Clinical data were collected retrospectively from electronic health records.

Statistical analysis was performed using R software (version 4.0.3 and 4.4.3) [22]. All serum protein data were log-transformed prior to analyses. Serum protein data were graphically analyzed using heatmaps with unsupervised k-means clustering to cluster patients and Spearman's rank correlation coefficients to cluster serum proteins. Univariate associations of serum protein levels with NRM, aGvHD and SR aGvHD were calculated using robust Cause-Specific-Cox (CSC) models, in order to deal with competing events and outliers in our data [23]. Univariate robust Cox-models were used for OS. These data were graphically analyzed in volcano plots and forest plots using ggplot2 [24].

We performed a sensitivity analysis to investigate whether the associations between serum protein levels and outcomes were influenced by associations between cytokines and underlying disease and whether these associations were consistent among different underlying disease subgroups, specifically: bone marrow failure (BMF, both congenital and acquired), hemoglobinopathies and other non-malignant hematological disease, hematologic malignancies and IEI. For this analysis auto-immune disease and metabolic disorders were excluded ( $n = 3$ ). Furthermore, we calculated the log2 fold changes (FC) of the serum protein levels in response to the conditioning treatment and compared levels before and after the conditioning regimen using the Wilcoxon signed-rank test. We evaluated whether log2 fold changes of serum protein levels differed between Busulfan and Treosulfan based conditioning regimens, which were the two most common regimens. False discovery rate (FDR) correction was performed on all  $p$ -values using the Benjamini-Hochberg procedure.

## RESULTS

### Patient characteristics

384 pediatric patients who received consecutive HSCTs were eligible for inclusion in this study (Supplementary Fig. 1). Stored serum samples were available for 98% of patients at T1 and for 95% of patients at T2. For 66 out of 68 patients who developed

**Table 1.** Patient and transplant characteristics.

| Characteristic                                                   | N = 384 <sup>a</sup> |
|------------------------------------------------------------------|----------------------|
| <b>Age</b>                                                       | 8.2 (0.2–18.6)       |
| <b>Patient sex</b>                                               |                      |
| Male                                                             | 235 (61%)            |
| Female                                                           | 149 (39%)            |
| <b>Diagnosis</b>                                                 |                      |
| Bone marrow failure                                              | 81 (21%)             |
| Immunodeficiency                                                 | 59 (15%)             |
| Thalassemia                                                      | 53 (14%)             |
| Sickle cell disease                                              | 48 (13%)             |
| Acute Myeloid Leukemia                                           | 39 (10%)             |
| Acute Lymphoblastic Leukemia                                     | 38 (9.9%)            |
| Hemophagocytic Lymphohistiocytosis                               | 22 (5.7%)            |
| Myelodysplastic syndrome                                         | 21 (5.5%)            |
| Other hematologic malignancy                                     | 17 (4.4%)            |
| Other non-malignant hematological disease                        | 3 (0.8%)             |
| Inborn error of metabolism                                       | 2 (0.5%)             |
| Auto-immune/auto-inflammatory conditions                         | 1 (0.3%)             |
| <b>Donor type</b>                                                |                      |
| Matched unrelated donor (10/10)                                  | 129 (34%)            |
| Mismatched unrelated donor                                       | 111 (29%)            |
| Matched related donor (10/10)                                    | 105 (27%)            |
| Mismatched related donor                                         | 38 (9.9%)            |
| Unrelated donor (matching unknown)                               | 1 (0.3%)             |
| <b>Graft type</b>                                                |                      |
| Bone marrow                                                      | 295 (77%)            |
| Umbilical cord blood                                             | 50 (13%)             |
| Peripheral blood stem cells                                      | 39 (10%)             |
| <b>Conditioning</b>                                              |                      |
| Treosulfan based                                                 | 179 (47%)            |
| Busulfan based                                                   | 120 (31%)            |
| Fludarabine, Cyclofosfamide                                      | 54 (14%)             |
| Etoposide + TBI                                                  | 17 (4.4%)            |
| Other                                                            | 14 (3.6%)            |
| <b>Serotherapy</b>                                               |                      |
| ATG                                                              | 276 (72%)            |
| Alemtuzumab                                                      | 66 (17%)             |
| No serotherapy                                                   | 42 (11%)             |
| <b>GvHD prophylaxis</b>                                          |                      |
| Calcineurin inhibitor only                                       | 25 (6.5%)            |
| Calcineurin inhibitor with MTX and/or MMF and/or steroids        | 276 (72%)            |
| PT-CY with calcineurin inhibitor and MMF                         | 32 (8.3%)            |
| T-cell depletion (post-transplant, in the bag or CD34 selection) | 15 (3.9%)            |
| Other                                                            | 36 (9.4%)            |

<sup>a</sup>Median (Minimum-Maximum);  $n$  (%).

aGvHD grade II-IV, a sample was available at TP-GvHD (before initiation of systemic treatment).

Patient and transplant characteristics are summarized in Table 1. The majority of patients received bone marrow grafts (71%). Most patients received transplants from an unrelated donor (63%), of whom 54% were matched (10/10) and 46% were mismatched ( $\leq 9/$



**Fig. 1** Explorative analysis of inflammatory proteins at T1. **a** Heatmap of pre-HSCT (T1) inflammatory serum protein levels in all patients with k-means clustering. **b–d** Histograms of aGvHD prevalence, diagnosis and clinical outcomes per cluster.

10). Almost all patients (89%) were treated with serotherapy as part of the conditioning regimen, with 72% receiving anti-thymocyte globulin (ATG/Thymoglobulin) and 17% receiving alemtuzumab. The 1-year incidence of grade II–IV aGvHD was 18%. OS at 1-year post-HSCT was 86% and NRM was 10% (Supplementary Table 3). Cumulative incidence plots for aGvHD and NRM, and overall survival curves for the four main disease subgroups are displayed in Supplementary Fig. 2.

### Explorative analysis

We performed an explorative analysis by creating a heatmap and performing unsupervised k-means clustering [25]. This showed distinct inflammatory serum protein profiles at T1 that could be divided into 5 clusters, associated with different outcomes and underlying diseases (Fig. 1a–d). Cluster 1 ( $n = 46$ ) had the lowest expression of inflammatory serum proteins overall and consisted mainly of BMF patients with a low incidence of aGvHD and low NRM. Cluster 2 ( $n = 49$ ) mainly consisted of patients with hematologic malignancies and had increased levels of serum proteins including sIL-2R $\alpha$ , TNFR1, TNFR2, ST2, ANG2, CXCL16 and CCL2 and had the highest incidence of aGvHD and high NRM. Patients in cluster 3 ( $n = 143$ ) and 4 ( $n = 110$ ) were enriched for patients with hemoglobinopathies and had intermediate levels of inflammatory serum proteins and low incidence of aGvHD and NRM. Cluster 5 ( $n = 29$ ) had the highest expression of inflammatory serum proteins overall and consisted mainly of patients with IELs and had a higher incidence of aGvHD and highest NRM.

### Associations with clinical outcomes

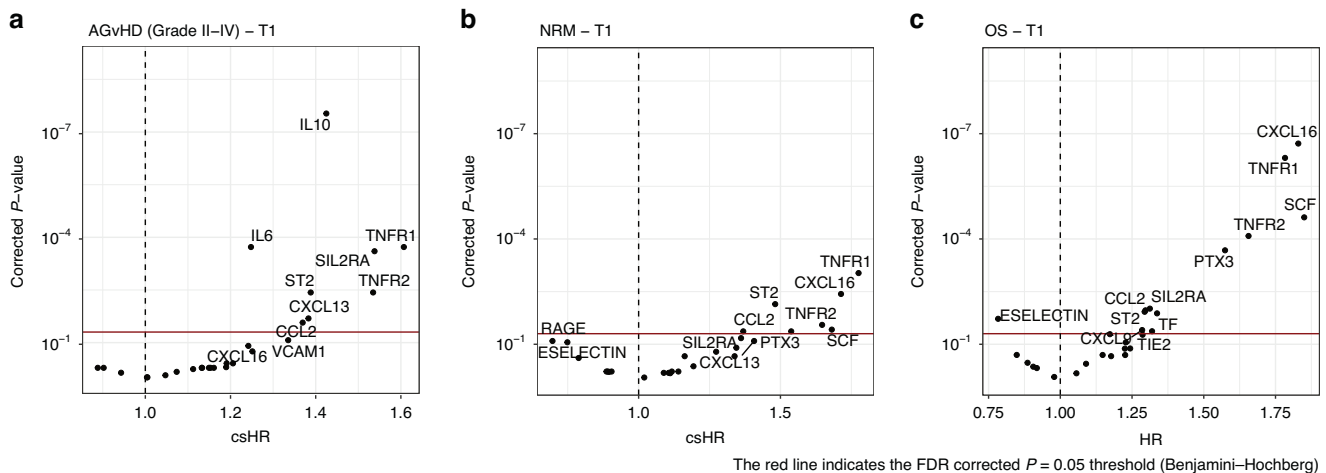
To further study the association between pre-transplant serum protein levels and the development of aGvHD, NRM and OS, we calculated cause-specific hazard ratios (csHR) (Fig. 2). This showed

that elevated levels of several inflammation-associated serum proteins prior to start of the conditioning regimen (T1) were associated with poor outcome. In particular, TNFR1, sIL-2R $\alpha$ , TNFR2, IL-10, ST2, CXCL13, CCL2 and IL-6 levels prior to start of conditioning were significantly associated with the occurrence of aGvHD (Fig. 2a), whereas high levels of TNFR1, CXCL16, SCF, TNFR2, PTX3, ST2 and CCL2 were associated with worse NRM (Fig. 2b) and OS (Fig. 2c). In addition, sIL-2R $\alpha$  and TF were associated with OS, but not with NRM. Although many of the elevated serum proteins were interrelated, the strongest correlations with outcome were observed for TNFR1. Figure 3 shows the cumulative incidence and survival plots for aGvHD, NRM and OS stratified by quartiles of TNFR1 at T1. Similar graphs are displayed in Supplementary Fig. 3 with a cut-off value of 1279 pg/ml for TNFR1, which is based on the optimal combination of sensitivity and specificity for aGvHD using the Youden Index.

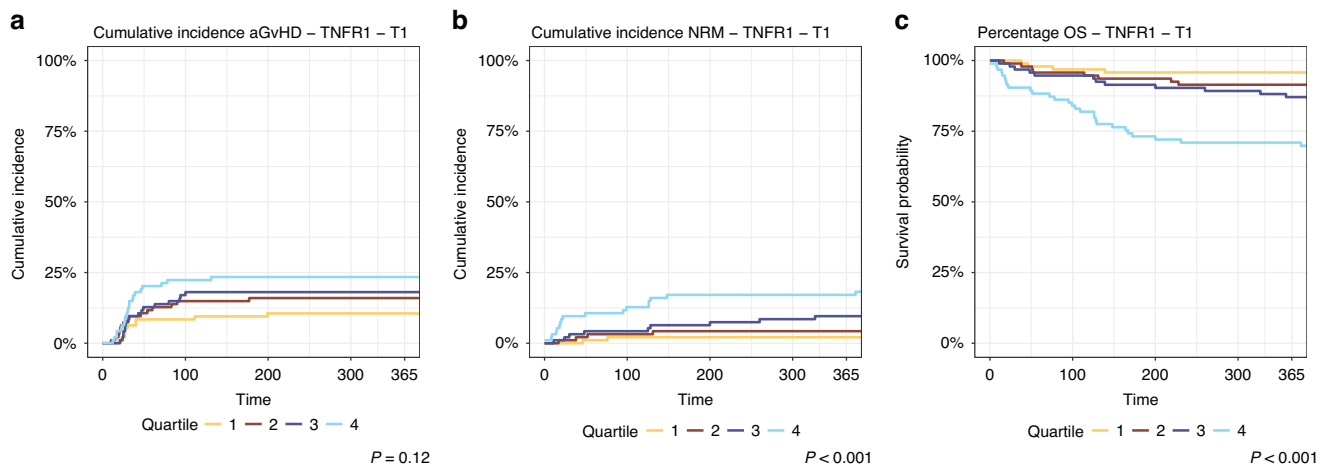
Next, we evaluated whether the conditioning therapy affected the correlation of inflammatory serum proteins with outcome. The associations between serum protein levels and aGvHD were reduced after conditioning (T2) compared to before conditioning (T1) (Supplementary Figs. 4 and 5). However, higher inflammatory profiles were generally more strongly associated with survival at T2 as compared to T1 (Supplementary Figs. 4 and 5).

### Sensitivity analysis

We performed a sensitivity analysis for underlying disease subgroups. For all inflammatory serum proteins and all outcomes, the confidence intervals (CI) of the BMF, hemoglobinopathy, hematologic malignancy and IEL subgroups overlapped with the CIs of the overall group, suggesting that the overall effect was not caused by a specific subgroup (Supplementary Figs. 6 and 7). However, the associations of serum protein levels at T1 with



**Fig. 2 Associations of inflammatory serum proteins with clinical outcomes.** Volcano plots of cause-specific hazard ratios (csHR) for aGvHD (a), NRM (b), and OS (c) at T1.



**Fig. 3 Clinical outcomes stratified by TNFR1 levels.** Cumulative incidence plots for aGvHD (a) and NRM (b), and survival plot for OS (c) with patients divided in quartiles based on serum TNFR1 level at T1.

aGvHD occurrence were generally the strongest in IEI, and the weakest in BMF. For NRM and OS no such trends were observed.

### Conditioning

Next, we evaluated the dynamics in serum protein levels before and after conditioning (Fig. 4). CXCL16, CCL2 and PTX3 showed a more than twofold increase after conditioning ( $p < 0.001$ ) and S100A9 showed a more than twofold reduction after conditioning ( $p < 0.001$ ). The change in serum protein levels during the conditioning was significantly different between Treosulfan and Busulfan based regimens for multiple serum proteins. For example, the increase in ST2 was significantly larger in patients treated with busulfan-based as compared to Treosulfan-based conditioning (Supplementary Figs. 8 and 9). Supplementary Fig. 10 displays the use of different conditioning regimens stratified by disease subgroup.

### Steroid refractory acute graft-versus-host disease

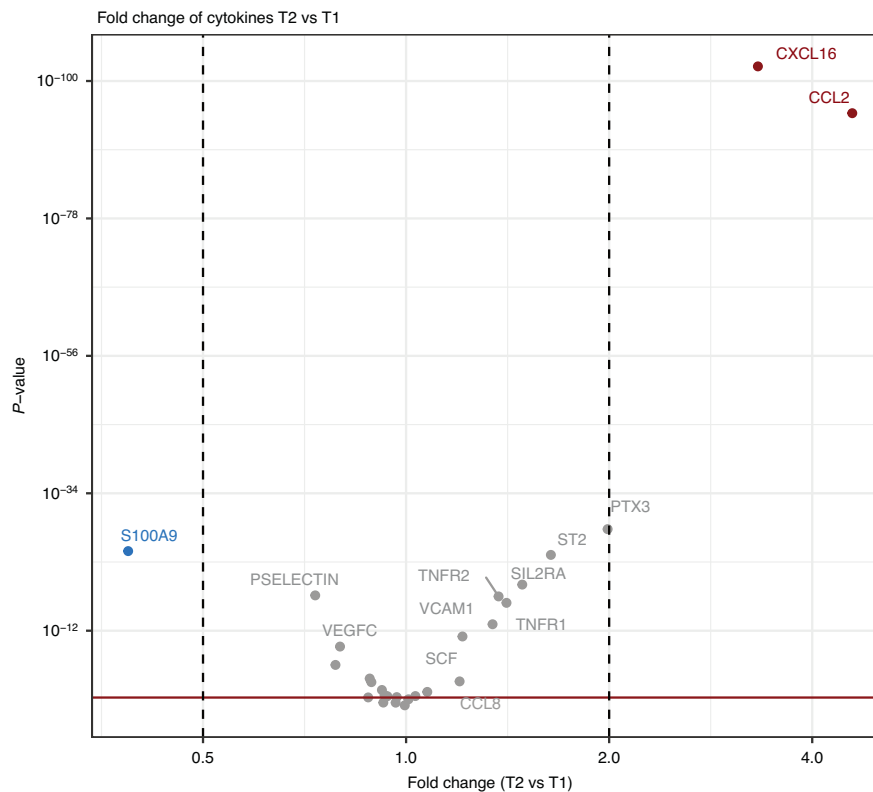
We also investigated whether serum protein levels at the time of diagnosis of aGvHD were associated with the development of SR-aGvHD. ST2 at TP-GvHD was significantly associated with the occurrence of SR-aGvHD (csHR 2.21,  $p < 0.001$ ) (Fig. 5a). In Fig. 5b, an overview of ST2 dynamics over time is shown for patients without aGvHD, patients with steroid responsive aGvHD (non-SR-aGvHD) and patients with SR-aGvHD. Additionally, ST2 was strongly correlated with NRM after GvHD onset (csHR 4.34,  $p < 0.001$ ) (Fig. 5c). Since ST2

is a validated predictive biomarker for SR-aGvHD and NRM after aGvHD onset [26–28], we calculated the area under the receiver operating curve (AUROC), which was 0.84 for SR-aGvHD development (95% CI 0.74–0.93) and 0.85 for NRM (95% CI 0.73–0.97) at 100 days after TP-GvHD. Using the Youden index we determined the optimal cut-off values for ST2 which resulted in a cut-off of 85,000 pg/ml for SR-aGvHD (sensitivity 0.89, specificity 0.67), and a cut-off of 44,000 pg/ml for NRM (sensitivity 0.63, specificity 0.95). Supplementary Table 4 lists the affected organs for all aGvHD patients.

### DISCUSSION

In this explorative study, we investigated inflammatory serum protein levels before HSCT and their associations with clinical outcomes. We discovered multiple potential biomarkers measured before HSCT that were associated with (SR) aGvHD, NRM and OS. To our knowledge, this is the first study to find significant associations between these serum proteins before the start of the conditioning regimen, and the occurrence of aGvHD weeks later.

Our main findings are that higher levels of inflammatory serum proteins pre-HSCT, prior to start of the preparative chemotherapy regimen, are associated with the later occurrence of aGvHD, NRM and OS. TNFR1 and TNFR2 were among the most significantly associated serum proteins for all outcomes. This highlights the central role of TNF-alpha signaling in HSCT-related complications,



**Fig. 4 Inflammatory serum protein levels in response to the conditioning regimen.** Median fold change of inflammatory serum proteins after the conditioning regimen as compared to before the start of the conditioning regimen (T2 vs. T1).

as has been described previously [9, 10, 12, 16, 29–31]. Additionally, sIL-2R $\alpha$ , IL-10, ST2, CXCL13, CCL2 and IL-6 at T1 were significantly associated with aGvHD. Most of these markers have been described before in association with the onset of aGvHD in adults but have rarely been studied in children.

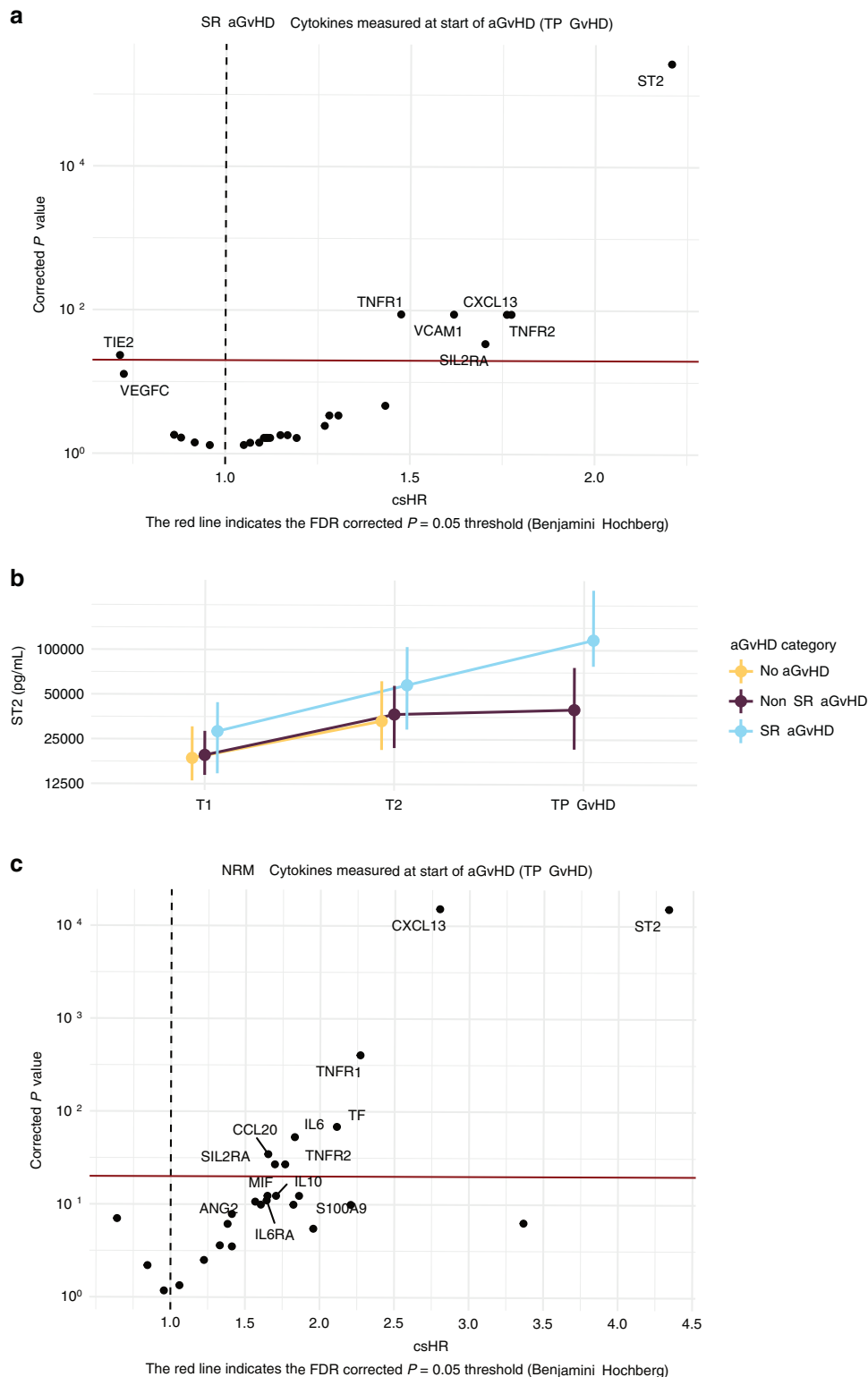
We found that the inflammatory profile before start of conditioning (T1) was more strongly associated with aGvHD, than the inflammatory profile after conditioning. This was surprising, since the inflammation caused by the tissue damage from the conditioning regimen is known to be an important etiological factor in the development of aGvHD [16, 29, 30]. Apparently, pre-existing high levels of inflammatory proteins are an even more important factor in determining the individual risk of developing aGvHD. For OS and NRM, the impact of the conditioning regimen was not as clear; multiple serum proteins were associated with these outcomes both before and after conditioning.

We report on a large cohort of 384 pediatric patients with a variety of underlying diseases and transplantation characteristics. Although our study was not powered to find significant associations within underlying disease subgroups, the effects were mostly consistent between subgroups and did not rely on an association between diagnosis and clinical outcome. We observed a trend in the associations with aGvHD, i.e., stronger associations in IEI and malignancies and weaker associations in BMF. This is likely due to the underlying pathophysiology and the intensity of treatment prior to HSCT for these diseases.

We also showed the impact of the conditioning regimen on inflammatory serum proteins, which differed between different regimens. In particular, the increase of ST2, a well-known biomarker for aGvHD, was greater after Busulfan-based conditioning regimens compared to Treosulfan-based regimens. However, since this is a non-interventional retrospective study, there may be other factors that differ between these subgroups (e.g., year of HSCT and ATG timing) that are associated with inflammatory complications and survival.

Finally, we were able to confirm ST2 as a predictive biomarker for SR-aGvHD and NRM in children, with an AUROC of 0.84 and 0.85 respectively. Since SR-aGvHD is a major cause of morbidity and mortality, studies to investigate whether outcomes will improve by treating children with high levels of ST2 at diagnosis of aGvHD with additional first-line treatment with non-steroidal agents, such as infliximab or ruxolitinib, are urgently needed.

Strengths of this study are the relatively high number of children included, high level of data completeness and inclusion of a wide variety of patients. A limitation of the study is its retrospective, observational nature, resulting in different transplantation approaches for patients over time, such as serotherapy timing and post-transplantation cyclophosphamide use. In this study we did not include a multivariate analysis, since this meant performing and correcting for many more tests, limiting our discovery power. Furthermore, patients with specific diseases may have had pre-treatments of chemotherapy or immunosuppressants, or had an infection before the conditioning regimen which may have impacted the cytokine levels and the outcomes after HSCT, although our sensitivity analysis showed consistent results between different disease subgroups. Another limitation is the absence of some validated post-HSCT biomarkers in our biomarker panel, such as amphiregulin and REG3a [5, 32]. These biomarkers were not compatible with our immunoassay and could therefore not be included. Additionally, we describe a relatively low incidence of aGvHD (18% 1-year post-HSCT) and NRM (10% 1-year post-HSCT), and a high OS (83%) as compared to other studies, which limits the power of our study [33–35]. This is why our findings should be validated in a separate patient cohort to confirm the potential clinical use of these biomarkers. This step is crucial before implementing biomarkers for the identification of high-risk patients in practice and eventually adjusting the clinical management of these patients. Ultimately, clinical trials will need



**Fig. 5 Associations of inflammatory serum proteins with SR-aGvHD. a** Volcano plot of cause-specific hazard ratios for SR-aGvHD at the time of aGvHD diagnosis (TP-GvHD). **b** ST2 levels over time stratified by controls (no aGvHD), steroid responsive aGvHD (non-SR aGvHD) and SR aGvHD patients. Error bars display the 25th to 75th percentile range at each timepoint. **c** Volcano plot of cause-specific hazard ratios for NRM at the time of aGvHD diagnosis (TP-GvHD).

to show if biomarker-guided pre-transplant control of immune dysregulation will improve post-transplant outcomes.

In conclusion, this explorative biomarker study in children shows that inflammatory serum protein levels before HSCT are

associated with aGvHD and mortality and lays the groundwork for further validation and use in risk-adapted therapy to improve the care for children undergoing this complex and high-risk procedure.

## DATA AVAILABILITY

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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## AUTHOR CONTRIBUTIONS

ABV was responsible for study design, literature review, clinical data collection, data cleaning and statistical analysis, interpretation of results and drafting of the manuscript. EGJA assisted with study design, statistical analysis, and interpretation. EBA provided expert advice on data cleaning and statistical analysis, and interpretation of results. AMJH performed all laboratory tests and collected and cleaned the laboratory data. MWS provided expert advice on study design and laboratory data collection. ACL, GL and ABM were involved in study design and interpretation of results. EPB was responsible for study design, interpretation of results and supervised the research. All authors contributed to writing the manuscript.

## COMPETING INTERESTS

The authors declare no competing interests.

## ADDITIONAL INFORMATION

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**Correspondence** and requests for materials should be addressed to Anne B. Verbeek or Emilie P. Buddingh.

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