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ORIGINAL ARTICLE

Elevated plasma CXCL9 and CHI3L1 prior to HCT predict post-HCT lung complications in children

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Abstract

Objectives. Lung complications occur commonly post-haematopoietic stem cell transplant (HCT) in children and contribute significantly to mortality. The development of biomarkers predictive of post-HCT lung complications may improve outcomes for these children. Therefore, the primary objective of this study was to identify pre-transplant plasma biomarkers predictive of lung complications in children undergoing HCT. **Methods.** Plasma samples from 117 pre-HCT patients were used to measure 78 soluble immune analytes, and extensive clinical metadata were retrospectively collected from the electronic medical record, including clinical characteristics of the post-HCT lung complications. **Results.** Firstly, we showed that the plasma cytokine signature of children undergoing HCT differed significantly between children depending on the indication for HCT (malignant vs non-malignant). Secondly, we found that the plasma cytokine signature changed with the age of the patient. Thirdly, we showed that elevated pre-HCT plasma levels of CXCL9 and Chitinase 3-like 1, both induced by IFN-γ, were predictive of post-HCT lung complications in patients with a malignant indication for HCT [area under the curve (AUC): 0.70, $P = 0.0007$ and AUC: 0.68, $P = 0.0016$, respectively]. **Conclusion.** In conclusion, we have identified two potential biomarkers of post-HCT lung disease in paediatric patients and these require validation in future cohorts.

Keywords: biomarker, CHI3L1, CXCL9, haematopoietic stem cell transplant, paediatric, pulmonary complications

INTRODUCTION

Haematopoietic stem cell transplant (HCT) is a cellular therapy used in children with a range of haematological malignancies, inborn errors of immunity and metabolic disorders. Whilst HCT aims to cure the child's underlying condition, it has a range of serious complications that can occur.[1] Lung complications occur in approximately 25–50% of children following HCT and contribute a high proportion of non-relapse mortality.[2–4]

Risk factors for the development of lung complications post-HCT include human leukocyte antigen (HLA) donor-recipient mismatch, total body irradiation (TBI), pre-existing lung disease and specific chemotherapy agents, for example busulfan and cyclophosphamide.[5–11] Strategies pre-transplant to determine patients at increased risk of post-HCT lung complications include lung imaging and lung function tests (e.g. spirometry). There are several limitations of these strategies, including interpretation of non-specific imaging findings that may be related to previous therapy and limitations of lung function in children < 7 years of age. The same approach is used post-HCT with serial lung function testing to identify post-HCT lung complications. This standardised approach relies on significant lung function impairment to develop before identifying lung complications, which in turn relates to the poor outcomes associated with lung complications. A recent multicentre study showed that children who develop a severe post-HCT lung complication that requires intensive care unit admission have an 11-fold increased risk of death.[4] This highlights the urgent need for the development of predictive biomarkers that can identify children at risk of these lung complications before HCT.

Several studies have identified pre-HCT signatures predictive of post-HCT lung disease, including specific ACE genotype variants, pulmonary microbiome patterns and BAL cellular profiles.[5,12–13] Several cytokines have also been identified as potential candidate biomarkers for the prediction of post-HCT lung disease,[14] including bronchoalveolar lavage (BAL) levels of TNF, GM-CSF, IL-1β, IL-8 and G-CSF.[5,15] The following plasma cytokines have also been associated with post-HCT lung disease: TNF family, MCP-1, IL-6, sCD14, IL-8, Ang-2 and ST2.[14,16–19] Limitations of some of these studies include a lack of pre-HCT plasma profiling to predict lung disease, combining paediatric and adult cohorts, investigation of only some types of complications, for example influenza, and relatively small panels of ELISA-based cytokine assays. Multiplex bead-based immunoassays enable the quantification of larger numbers of cytokines which can assist both in identifying predictive biomarkers and in understanding the pathophysiology underlying lung complications post-HCT.

The primary aim of this study was to identify plasma cytokine signatures measured in paediatric patients prior to HCT that predict the development and characteristics of post-HCT lung complications. The hypothesis is that systemic immunological profiling would characterise signatures of high-risk patients prior to HCT.

RESULTS

Patient demographics and lung complication outcomes

Overall, 117 (56%, 117/203) of children who underwent HCT during the study period had plasma samples analysed for 78 different immune analytes. This included 53% (62/117) who developed post-HCT lung complications (Figure 1a). Of the 78 immune analytes measured, 50 were reliably detected and included herein.

Table 1 shows the pre-HCT characteristics of patients who developed post-HCT lung complications compared to those who did not. Transplant characteristics of the group with lung complications showed a lower proportion of patients receiving bone marrow as the stem cell source (24% vs 45%) and matched sibling donor (27% vs 54%) in keeping with known risk factors for the development of lung complications.[4] The types of lung complications that occurred are shown in Figure 1b, showing higher proportions of early (n = 40, 65%) and infectious lung complications (n = 37, 60%).

Figure 1c and Supplementary table 1 show the aetiology of the 53 episodes (n = 49 patients) of lung infection in patients who developed an infective lung complication. There were 20 episodes of bacterial pneumonia which were confirmed microbiologically in 40% (8/20) and clinically defined in 60% (12/20) of episodes. The most common causes of viral pneumonitis were

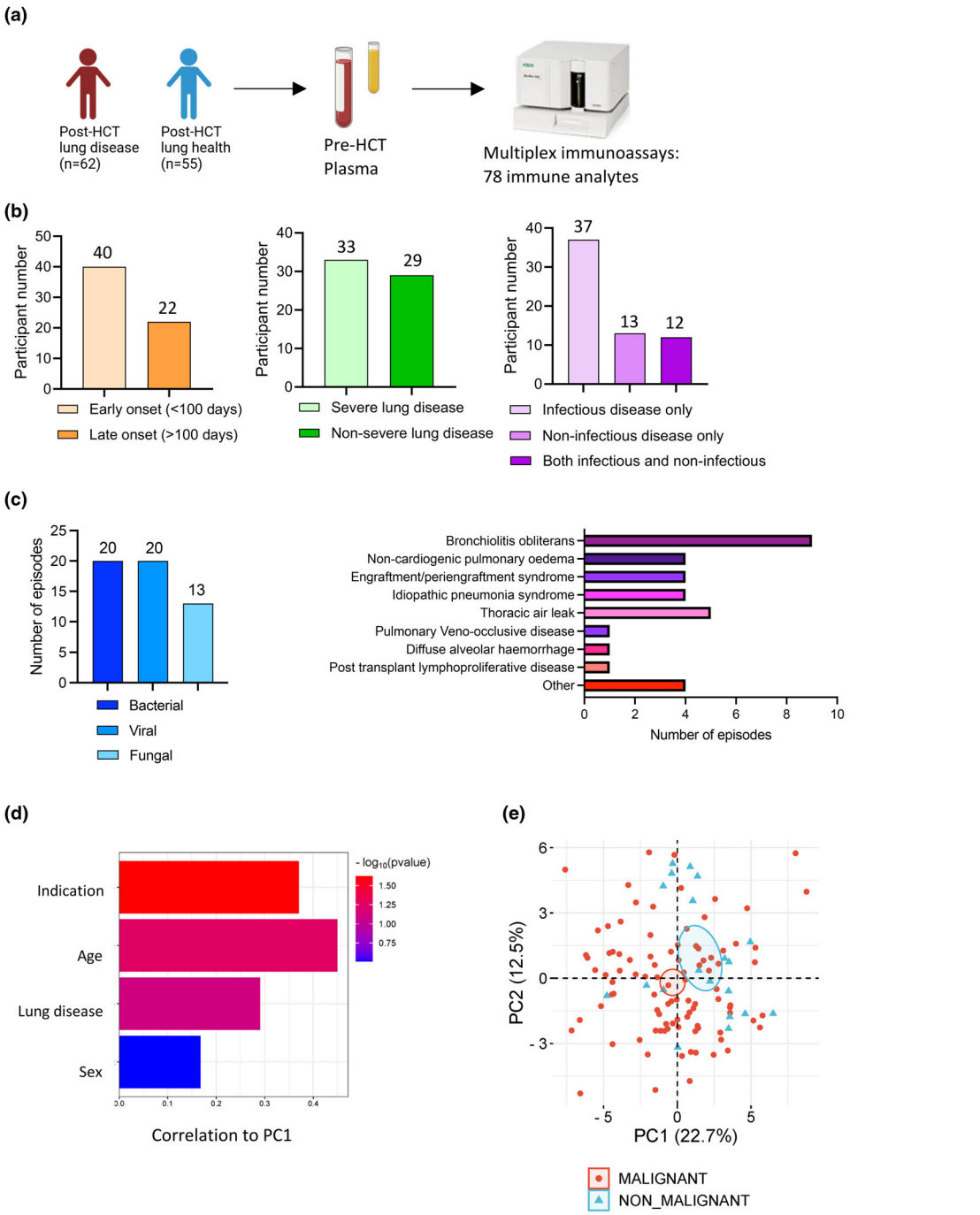

Figure 1. Patient cohort and lung complication characteristics. **(a)** Overview of cohort and study design utilising multiplex immunoassays to measure cytokines in pre-HCT plasma samples from 117 children. **(b)** Bar graphs showing the characteristics of pulmonary complications; onset of pulmonary complication (early; defined as occurring within the first 100 days of HCT), severity of lung complication (severe defined as requiring intensive care unit admission, developing chronic lung disease, requiring oxygen therapy or dying because of pulmonary disease) and type of lung complication (infectious, non-infectious or both). **(c)** Bar graphs showing the types of infective and non-infective lung disease by episode frequency. **(d)** Clinical variables associated with PC1 of the analyte data and **(e)** graphical representation of the principal components analysis coloured by underlying indication (malignant and non-malignant indications for HCT). HCT, haematopoietic stem cell transplant.

Table 1. Patient characteristics undergoing HCT

Characteristic	Pulmonary complication *N* = 62 (%)	No pulmonary complication *N* = 55 (%)
Age ([a])		
< 2.99 years (*n*, %)	10 (16)	9 (16)
3–0.5.9 years	11 (18)	14 (25)
6–12 years	16 (26)	14 (25)
> 12.1 years	25 (40)	18 (33)
Male, *n* (%)	38 (61)	26 (47)
Diagnosis, *n* (%)		
Malignant	53 (85)	44 (80)
Non-malignant	9 (15)	11 (20)
Specific diagnoses		
B-Acute lymphoblastic leukaemia	16 (26)	16 (29)
T-Aacute lymphoblastic leukaemia	5 (8)	5 (9)
Acute myeloid leukaemia	16 (26)	14 (25)
Juvenile myelomoncytic leukaemia	5 (8)	0 (0)
Mixed phenotype acute leukaemia	4 (6)	0 (0)
Other malignant	7 (11)	9 (16)
Primary immunodeficiency (any)	3 (5)	1 (2)
Aplastic anaemia	1 (2)	6 (11)
Other non-malignant	5 (8)	4 (7)
Stem cell source, *n* (%)		
Bone marrow	15 (24)	25 (45)
Peripheral blood	38 (61)	29 (53)
Cord blood	9 (15)	1 (2)
Donor type		
Haploidentical	13 (21)	12 (22)
Matched unrelated	32 (52)	18 (33)
Matched sibling	17 (27)	25 (45)
Conditioning regimen, *n* (%)		
Myeloablative	56 (90)	47 (85)
Conditioning details		
Busulfan-based regimen	30 (48)	24 (44)
Treosulfan-based regimen	9 (15)	6 (11)
Total body irradiation based, *n* (%)	21 (34)	19 (35)
Serotherapy, *n* (%)		
Alemtuzumab	3 (5)	3 (5)
Antithymoglobulin	32 (52)	25 (45)
GVHD prophylaxis, *n* (%)		
Methotreaxte + calcineurin inhibitor	19 (31)	15 (27)
Mycophenylate + calcineurin inhibitor	14 (22)	15 (27)
Methylprednisolone + calcineurin inhibitor	3 (5)	0 (0)
Calcineurin inhibitor only	18 (29)	18 (33)
T cell receptor α/β depletion	6 (10)	6 (11)
Other combination	2 (3)	1 (2)
Other, *n* (%)		
Chemotherapy pre-HCT	51 (82)	44 (80)

[a]At time of blood sample within 3 months of Day 0.

CMV 30% (6/20), parainfluenza 25% (5/20) and adenovirus 15% (3/20). Fungal infections were classified as possible, probable and proven according to EORTC criteria and identified in 46% (6/13), 23% (3/13) and 38% (5/13), respectively. Figure 1c also shows the 34 episodes of non-infectious lung disease that were identified in 25 patients. The most common types of non-infectious diagnoses included bronchiolitis obliterans 26% (9/34) and idiopathic pneumonia syndrome 12% (4/34).

PCA analyses identified the following clinical variables associated with PC1: underlying indication (malignant vs non-malignant, $r = 37$, $P = 0.02$), age (infancy through adolescence, $r = 44$, $P = 0.05$) and lung complication outcome ($r = 29$, $P = 0.07$) (Figure 1d and e). Given the significant contribution of disease indication to the pre-HCT cytokine levels (Figure 1d and e), the malignant and non-malignant cohorts were analysed separately moving forward.

Indication for HCT and age influence pre-HCT plasma cytokine levels

We first compared pre-HCT plasma cytokine levels between children with a malignant ($n = 97$) and non-malignant ($n = 20$) indication (Figure 2a). This showed that patients with a malignant indication for HCT had reduced levels of TNFSF12 (TWEAK) and elevated levels of TNFSF13β (BAFF), M-CSF, sTNFR1, Chitinase 3-like 1 (CHI3L1), IL-18, IL-1β, sTNFR2, CXCL10 and SCF, compared to patients with a non-malignant indication for HCT (all $P < 0.05$). Figure 2b shows the distribution of the most significantly elevated and reduced cytokines between the two groups: TNFSF13β (BAFF) ($P < 0.000001$) and TNFSF12 (TWEAK) ($P = 0.003$), respectively.

Because of age significantly contributing to PC1, the plasma cytokine data for each underlying indication were also analysed by age group (infant; 0–2.9 years, preschool; 3–5.9 years, childhood; 6–10.9 years and adolescent; 11–16 years). Supplementary table 2 (malignant indication) and Supplementary table 3 (non-malignant indication) show the median concentrations (pg/mL) of all analytes across each age group, as well as statistical comparisons between preschool vs infant, childhood vs infant, and adolescent vs infant (*P*-values determined by Mann–Whitney *U*-test). This is also represented visually in the two heatmaps in Figure 2c, which

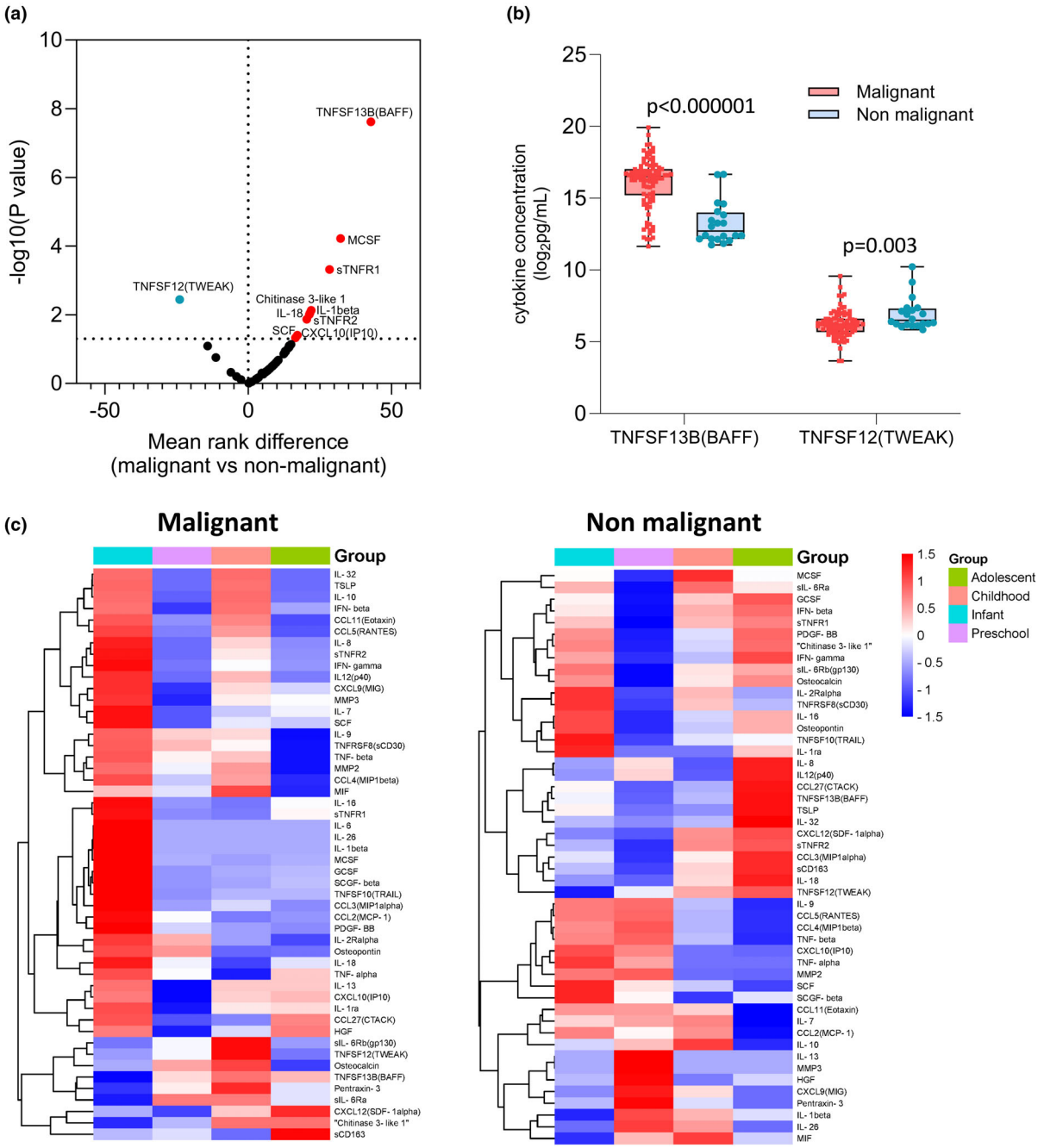

Figure 2. Indication and age influence pre-HCT plasma cytokine levels. **(a)** Volcano plot depicting statistical analysis of pre-HCT plasma analytes in children with a malignant indication (n = 97) versus non-malignant indication (n = 20) for HCT. **(b)** Box plot the top showing the two most significant analytes contributing variation between malignant and non-malignant cohorts: TNFSF13B (BAFF) and TNFSF12 (TWEAK). **(c)** Heatmaps depicting median plasma cytokine levels for each age group (infant, preschool, childhood and adolescent) for children with a malignant indication and a non-malignant indication. Unadjusted *P*-values are shown; light red/light blue dots represent analytes that passed an unadjusted *P*. HCT, haematopoietic stem cell transplant.

show the median cytokine concentration in each age category and highlight age-related changes in the malignant and non-malignant cohorts. In the malignant cohort ($n = 97$ total), we found, infants had significantly elevated levels of 24 cytokines compared to at least one other older age group (Figure 2c, Supplementary table 2). Eight of these cytokines [G-CSF, IFN-γ, IL-7, IL-16, CCL3, S-CGF-β, TNF-α and TNFSF10 (TRAIL)] were elevated in infants relative to all three other age groups (Figure 2c, Supplementary table 2). Conversely, CXCL12 (SDF-1α) and TNFSF13β (BAFF) were significantly reduced in infants relative to older children (Figure 2c, Supplementary table 2).

Age-related trends were also identified in the non-malignant cohort; however, this analysis is limited because of the smaller numbers in this group ($n = 20$ total) (Figure 2c, Supplementary table 3). We found that IL-9, CCL4 (MIP1β) and TNF-β were significantly elevated in infants compared to adolescents with a non-malignant indication (Figure 2c, Supplementary table 3). Like their malignant counterparts, infants with a non-malignant indication also tended to show elevated levels of IL-1rα ($P = 0.057$), IL-2Rα ($P = 0.057$) and TNF-α ($P = 0.057$), as well as reduced levels of CXCL12 (SDF-1α) ($P = 0.071$), compared to older children; however, these findings did not reach statistical significance.

Pre-HCT levels of CXCL9 and chitinase 3-like 1 (CHI3L1) are associated with post-HCT lung disease in children with a malignant indication

Given the above contributions of disease indication (malignant vs non-malignant) to analyte variability, the primary aim of determining whether pre-HCT cytokines could predict post-HCT lung disease was tested in both groups. In patients who underwent HCT for a malignant indication ($n = 97$), 54% ($n = 53$) developed a lung complication post-HCT. The key findings in this group are shown in Figure 3a and b, showing that elevated pre-HCT plasma CXCL9 (MIG) and Chitinase 3-like 1 (CHI3L1) were strongly associated with post-HCT lung complication outcomes ($P = 0.0005$ and $P = 0.0014$, respectively, and FDR-$P = 0.02$, and FDR-$p = 0.03$, respectively). The following additional three pre-HCT plasma cytokines were elevated in the lung complication group when analysed using an uncorrected *P*-value threshold of $P < 0.05$: sCD163, IL-2Rα and sTNFR2. Given that CXCL9 and CHI3L1 were most strongly associated with post-HCT lung disease, we assessed the sensitivity and specificity of these markers using area under the curve analyses (Figure 3c). This showed that pre-HCT plasma levels of CXCL9 had significant predictive value (AUC = 0.7, $P = 0.0007$) and a threshold > 7.075 $\log_2$ pg/mL (134.83 pg/mL) has a sensitivity of 69.81% and specificity of 61.36% ($P = 0.0007$) to predict post-HCT lung disease. Similarly, pre-HCT levels of CHI3L1 had significant predictive value (AUC = 0.68, $P = 0.0016$) and a threshold > 11.15 $\log_2$ pg/mL (2272.39 pg/mL) has a sensitivity of 71.7% and specificity of 56.82% ($P = 0.013$).

We also examined whether biomarkers were associated with specific characteristics of lung complications (i.e. severity, infective vs. non-infective, and early vs. late onset) (Figure 3d and Supplementary figure 1). This showed that CHI3L1 was elevated in patients who developed post-HCT severe lung disease compared to mild lung disease (unadjusted $P = 0.027$). When comparing patients with infective versus non-infective lung complications one cytokine, CCL3 (MIP1α) ($P = 0.019$) was elevated compared to five cytokines which were reduced: CCL4 (MIP-1β) ($P = 0.016$), Osteocalcin ($P = 0.019$), CCL5 (RANTES) ($P = 0.026$), TNF-β ($P = 0.028$) and IL-9 ($P = 0.034$). There was no difference in pre-HCT cytokine signature related to the timing of onset of the post-HCT lung disease (Figure 3d).

Cytokines associated with post-HCT lung disease in children with a non-malignant indication

In the group of patients who underwent HCT for a non-malignant indication ($n = 20$), 45% ($n = 9$) developed a lung complication post-HCT. When evaluating our primary aim in this group, pre-HCT plasma cytokine levels in patients who developed a post-HCT lung complication, we found significantly elevated TNF-α, CCL11 (Eotaxin) and IL-1β (Figure 4a). The strongest association was for elevated TNF-α ($P = 0.014$) and CCL11 (Eotaxin) ($P = 0.019$) shown in the box plot in Figure 4b. When exploring the characteristics of the lung complications in the non-malignant group (Figure 4c and d and Supplementary figure 2), we found that pre-HCT plasma CCL5 (RANTES) was higher in those who developed severe disease

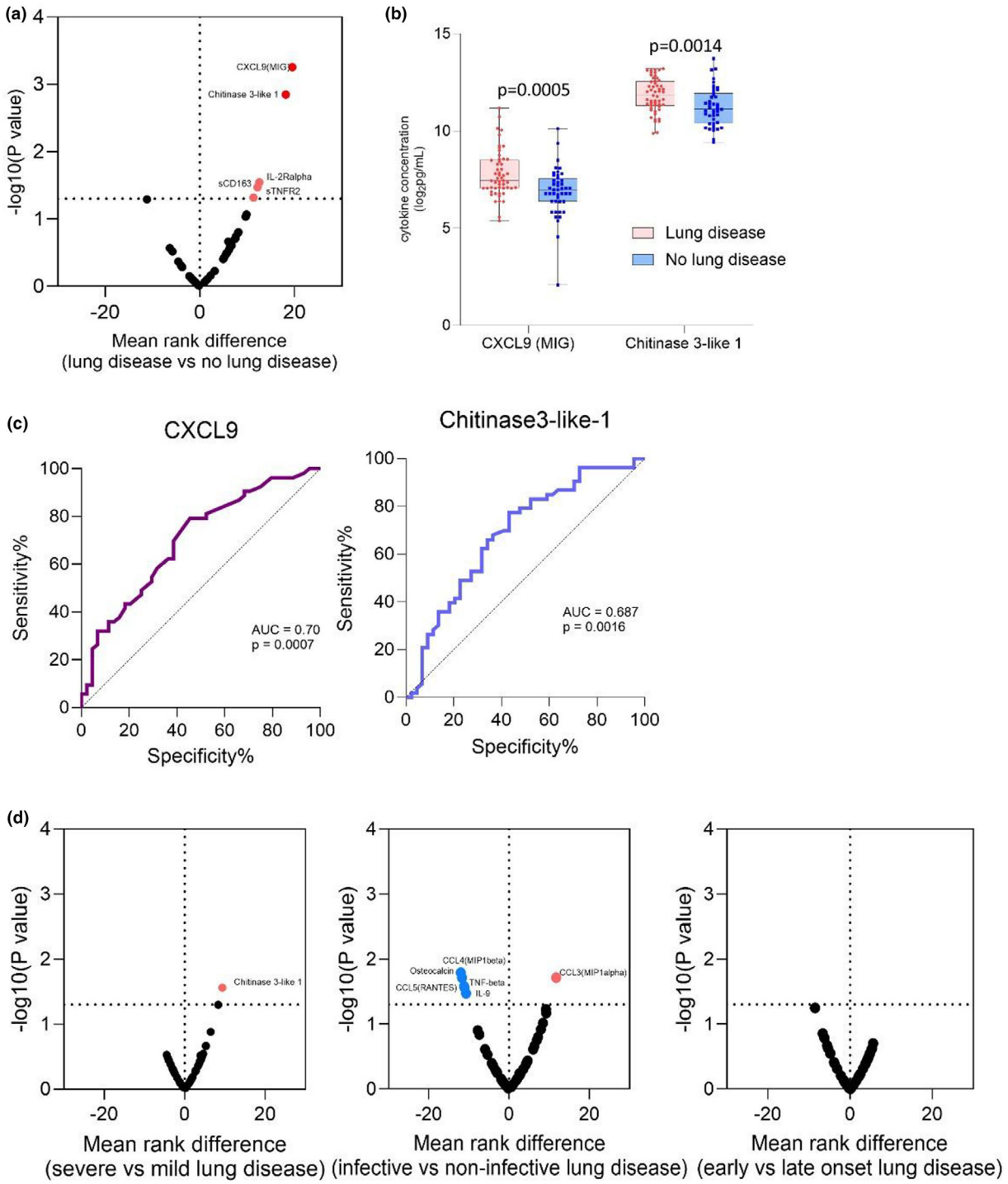

Figure 3. Pre-HCT plasma analytes that are associated with post-HCT lung disease in children with a malignant indication. **(a)** Volcano plot depicting statistical analysis of pre-HCT plasma analytes in children with a malignant indication who developed a lung disease (n = 53) compared to those who did not (n = 44). **(b)** Box plots depicting the top two most significant analytes associated with lung disease outcomes: CXCL9 (MIG) and chitinase 3-like 1 (CHI3L1). **(c)** Receiver operating characteristic curve (ROC) analyses for pre-HCT plasma CXCL9 and CHI3L1 to predict the development of post-HCT lung complications. **(d)** Volcano plots depicting statistical analysis of pre-HCT plasma analytes in children with a malignant indication who developed a severe vs mild lung disease, infective vs non-infective lung disease, and an early (100 days post-HCT) lung disease. Unadjusted *P*-values are shown; light red/light blue dots represent analytes that passed an unadjusted *P*. HCT, haematopoietic stem cell transplant.

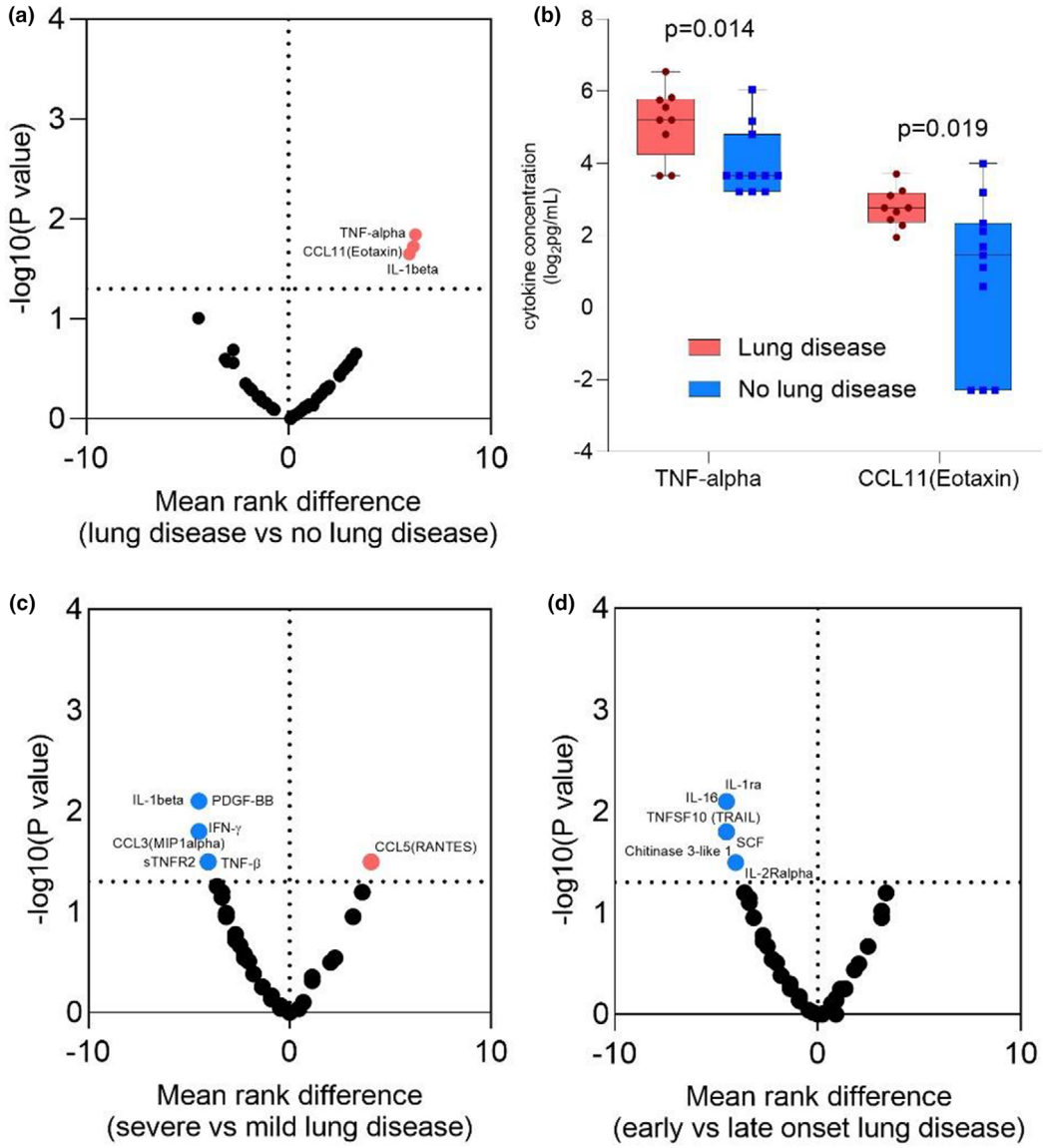

Figure 4. Pre-HCT plasma analytes that are associated with post-HCT lung disease in children with a non-malignant indication. **(a)** Volcano plot depicting statistical analysis of pre-HCT plasma analytes in children with a non-malignant indication who developed a lung complication (n = 9) compared to those who did not (n = 11). **(b)** Box plot depicting the top two most significant analytes associated with lung disease outcomes: TNF-alpha and CCL11 (Eotaxin). **(c)** Volcano plot depicting statistical analysis of pre-HCT plasma analytes in children with a non-malignant indication who developed severe vs mild lung disease. **(d)** Volcano plot depicting statistical analysis of pre-HCT plasma analytes in children with a non-malignant indication who developed early (100 days post-HCT) lung disease. Unadjusted *P*-values are shown, light red/light blue dots represent analytes that passed an unadjusted *P*. HCT, haematopoietic stem cell transplant.

compared to mild disease (P = 0.032). The following six cytokines were significantly reduced in patients who developed severe lung disease compared to mild lung disease: PDGF-BB, IL-1β, CCL3 (MIP1α), sTNFR2 and TNF-β. Unlike the patients with a malignant indication, there was no difference seen in pre-HCT levels of plasma CHI3L1 (median 1606.8 pg/mL versus 1795.28 pg/mL) in patients who developed post-HCT lung disease. By comparison, similar to the finding in patients with a malignant indication, pre-HCT levels of plasma CXCL9 were 1.4 times higher in patients who developed lung disease in the non-malignant group (median 133.4 pg/mL, vs

median 95.0 pg/mL). This difference was however not statistically significant.

DISCUSSION

This study has identified candidate biomarkers in the plasma of children pre-HCT that may predict the development of post-HCT lung complications. The strongest associations were elevated pre-HCT plasma levels of CXCL9 and CHI3L1 in children with a malignant indication who developed post-HCT lung complications. There was a trend towards an increase of CXCL9 in the same direction for patients with a non-malignant indication but not for CHI3L1.

Our study also demonstrates that cytokine concentrations in paediatric patients pre-HCT have a high degree of variability. These differences are driven primarily by age and indication for HCT (malignant or non-malignant). Infants in the malignant cohort showed upregulation of growth factors (G-CSF and S-CGF-β), interferon/TNF superfamily factors (TNF-α and IFN-γ) and interleukins(IL-16 and IL-7) compared to all other age groups. This finding of an elevated pro-inflammatory signature in infants has also been demonstrated in healthy infants compared to older children.[20]

The cohort of pre-HCT patients in this study was predominantly made up of children who had a malignant indication for HCT (> 80%) and had frequently received prior chemotherapy (prior to conditioning). It is hence not surprising that the largest cytokine differences detected in this study were found in the patients with a malignant indication for HCT as opposed to patients with a non-malignant indication. When focusing on the findings in patients with a malignant indication, pre-HCT upregulation of two cytokines was consistently associated with the development of post-HCT lung complications: CXCL9 and Chitinase 3-like 1 (CHI3L1). Both cytokines are mediated through upregulation of the IFN-γ pathway.

CXCL9 also known as monocyte induced by interferon-γ (IFN-γ) (MIG) mediates its downstream functions through the chemokine receptor CXCR3 present on T-helper-1 and CD8+ T cells.[21,22] CXCL9 has been described as having roles in the recruitment of effector cells to sites of inflammation, regulating eosinophil accumulation in asthma[21] and contributing to chronic obstructive lung disease (COPD) in adults.[23] CXCL9 has also been described as having a role in tumor progression, which is important to note in this cohort of patients undergoing HCT because of a history of malignancy. Specifically in the HCT setting CXCL9 has been reported to be a biomarker for the development of chronic GVHD (cGVHD) and also for the prediction of cGVHD severity in adult HCT patients.[24,25] A large study, involving two separate validation cohorts by Kitko *et al*.[26] also reported the validity of CXCL9 as a biomarker for cGVHD but also showed the impact of concurrent immunosuppression on plasma levels.

This study showed that in patients with a malignant indication for HCT, CXCL9 was predictive of the development of post-HCT lung disease. It is important to highlight that patients in this study only proceeded to transplant if they were in remission from their leukaemia (majority of the population), that is < 1 MRD, coinciding with the time of plasma sample collection pre-HCT. This suggests our finding of CXCL9 upregulation to be unlikely to be due simply to the presence of active malignancy. It is unclear however whether CXCL9 continues to be elevated in patients earlier pre-HCT and indeed post-HCT. This requires further longitudinal serial monitoring of plasma cytokines to determine whether these levels are predictive of post-HCT lung disease when measured at other timepoints.

Chitinase 3-like 1 (CHI3L1) is a protein secreted by neutrophils, macrophages, fibroblast-like cells, stem cells and tumor cells.[27] CHI3L1 has been found to have a role in inflammation, tissue repair and Th1/Th2 inflammatory balance.[27] CHI3L1 is induced by several upstream pathways, including (Th1) IFN-γ, IL-1β and suppressed by Th2 cytokine IL-4 in activated macrophages.[27] Specific to lung disease, overexpression of CHI3L1 has been linked to asthma severity in adults, particularly related to distinct genetic variants of CHI3L1.[28] Mechanistically, CHI3L1 stimulates bronchial smooth muscle growth and proliferation.[28] CHI3L1 may also function synergistically with IGF-1 to promote fibroblast differentiation and fibrosis.[27,29] This finding is not specific to the lung[30] and has been shown in other organs such as the liver and skin in conditions such as systemic sclerosis.[27,29,31] Murine models have shown severe respiratory syncytial virus (RSV) infection is linked to upregulation of the CHI3L1 pathway that leads to airway hyperresponsiveness.[32] Relevant to this study, CHI3L1 has also been shown to have a role

in oncogenesis but not related specifically to HCT.[27] Several *ex-vivo* studies exploring a neutralising antibody therapy against CHI3L1 are underway for patients with solid tumors.[33] Overall, overexpression of CHI3L1 has established roles in infective, inflammatory and malignant conditions. The finding of CHI3L1 elevation in the pre-HCT cohort in this study suggests a plausible role in the pathogenesis of post-HCT lung disease, given the significant overlap between infective and non-infective lung disease seen in these patients.

Whilst elevated CXCL9 or CHI3L1 pre-HCT was associated with lung disease post-HCT, only CHI3L1 was predictive of the characteristics of the post-HCT lung disease. Elevated CHI3L1 was seen in those who developed a severe lung complication compared to a mild lung complication. The hypothesis had originally been that the pre-HCT cytokine signature may predict not only the development of a lung complication, but also the type, timing and severity of this lung complication. Not seeing a clear pre-transplant signature for this suggests that many other factors influence the characteristics of what lung disease develops post-HCT such as the conditioning regimen, donor HLA matching and exposure to other factors such as viral reactivation and graft versus host disease. It is also potentially reflective of the finding that lung complications are often heterogeneous and can include both infectious and non-infectious diseases. To better understand the characteristics of lung complications and potential variation in cytokine patterns, longitudinal sampling is required in patients post-HCT, including at the time of developing a lung complication. For example, exploring whether the same cytokines CXCL9 and CHI3L1 remain increased post-HCT at the time of lung complication or whether there is an alternate pattern of soluble analytes increased, still mediated by the IFN-γ pathway. This has been performed in other specific post-HCT lung diagnoses, most notably for the diagnoses of idiopathic pneumonia syndrome, leading to the use of, TNF-α targeted therapy with etanercept.[17] A prospective observational study sampling peripheral blood pre- and post-HCT for patients who develop lung complications and comparing this to patients who maintain lung health would assist in validating our findings.

The limitations of this study include the relatively small sample size for patients undergoing HCT for a non-malignant indication, which limits statistical power to draw firm conclusions in this cohort. Specifically, because of the small non-malignant cohort it was not meaningful to separate this group further based on diagnosis, for example inborn error of immunity compared to patients with bone marrow failure for example. This would be an important distinction to make in future studies with an appropriate sample size. The advantages however, were the inclusion of only paediatric patients, comparison with HCT patients not impacted by lung complications, and analyses of a comprehensive set of immunomodulatory cytokines.

In summary, post-HCT lung complications are a significant cause of post-HCT morbidity and mortality for paediatric patients, highlighting the urgent need for strategies to better understand the mechanisms driving these complications. Aside from a history of past lung disease, lung imaging and lung function testing, there are few tools available to identify patients at risk of these complications *prior* to HCT. As a result, strategies which shed light on molecules and pathways that can predict pulmonary dysfunction or associate with its development are urgently required. This study identifies the elevation of *pre-HCT* plasma CXCL9 and CHI3L1 in patients with a malignant indication for HCT which require further investigation as potential tools to identify patients at risk of lung complications *post-HCT*.

METHODS

Cohort

This was a retrospective study of children undergoing HCT. Patients (n = 203) were included if they received an allogeneic HCT between 2016 and 2022 at the Royal Children's Hospital, Melbourne Australia and had a plasma blood sample collected prior to HCT (n = 117/203). Patients were excluded if they did not have a stored plasma sample available or if they underwent an autologous HCT. Plasma samples were collected at a median of 26 days prior to HCT Day 0 (interquartile range 16–62 days) and accessed from the Children's Cancer Centre (CCC) Biobank at The Royal Children's Hospital. Biobank samples were collected when a patient was undergoing a pre-HCT bone marrow disease assessment and therefore represented patients in remission from a malignant disease [minimal residual disease (MRD)-negative]. The patient episodes of transplant were identified using the Australian Bone Marrow Database Registry (ABMDR). Relevant clinical and

outcome data were retrospectively collected from the electronic medical record (EMR). Clinical data on patients were collected for a minimum of 8 months or until transitioned to an adult centre or until the end of 2022 whichever occurred latest.

Definitions

Pulmonary complications were defined as signs and symptoms of pulmonary disease (including tachypnoea, respiratory distress, fever, hypoxia or haemoptysis) and newly developed pulmonary imaging changes [chest x-ray, (CXR) or computed tomography (CT)] or changes in lung function following allogeneic HCT. Complications were classified as occurring early (first 100 days post-transplant) or late (after 100 days post-transplant). Lung complications were included if they required admission to either the day oncology unit or inpatient ward setting for management. Pulmonary complications were then further classified as either infectious, non-infectious complications or a combination of both. Infectious complications were classified as either a microbiologically defined infection (MDI) or clinically defined infection (CDI).[34] A MDI was defined as an infection that is clinically detectable and microbiologically proven. Causes of bacterial pneumonia were identified on direct BAL sampling or peripheral blood cultures. A CDI was defined as a site of infection that is diagnosed but its microbiological pathogenesis cannot be proven or is inaccessible to examination. Non-infectious complications were defined using definitions from The American Thoracic Society, National Institutes of Health (NIH)[3,7,35] and expert consensus definitions and included in a previous publication in this journal.[4] Pulmonary complications were classified as severe based on the following outcome data: if a patient required oxygen therapy, intensive care admission, developed chronic lung disease or died because of the pulmonary complication.

General transplant outcomes including rates of acute GVHD (Seattle criteria[36]), chronic GVHD (NIH criteria[37]), veno-occlusive disease (VOD) (EBMT Paediatric Criteria[38]) and transplant-associated thrombotic microangiopathy (TA-TMA) (Modified Jodele criteria[39]) were also collected.

Ethics statement

Ethics approval was obtained by The Royal Children's Hospital Human Research Ethics Committee (HREC/91777/RCHM-2023 and CCC Biobank HREC 33207).

Collection and processing of samples for cytokine analysis

Blood samples were kept at room temperature and processed immediately after collection. EDTA blood was centrifuged at 700 × g for 7 min at room temperature and the supernatant (plasma) was stored at −80°C. Plasma samples were thawed, and cytokines were measured using the 48-plex Bio-Plex Pro™ Human Cytokine Screening Panel (Bio-Rad, California, USA) and the 37-plex Bio-Plex Pro™ Human Inflammation panel (Bio-Rad, California, USA) according to the manufacturer's instructions. Plasma samples were diluted 1:5 in phosphate-buffered saline (PBS) as per the manufacturer's recommendations. Data were acquired on the Bio-Plex 200 system and QC was done using the Bio-Plex Manager™ 6.1 software (Bio-Rad). For data QC, standard curve outliers were removed, and individual standards were adjusted to achieve a recovery rate of 100 ± 5% (observed concentration/expected concentration). A fit probability > 0.9 was achieved for each standard curve. The concentration values of each analyte control were then compared to the expected concentration range specified on the assay data sheet for the control lot. Analytes that were not detected in > 50% of the samples were excluded from further analysis. For each analyte included in the analysis, samples that fell below the detection range were reported as half the lower limit of detection, previously described as recommended for this assay.[40] For each analyte included in the analysis, samples that fell above the detection range were reported as double the upper limit of detection, also as recommended for this assay.

Following the QC, 50 out of the possible 78 cytokines were included in the analysis presented herein.

Statistical analysis

Principal components analysis (PCA) was performed on log transformed analyte data to identify potential sources of variation in the data. This was performed using the FactoMineRpackage, including plate (batch), age, sex, lung complication outcome (yes or no) and indication for HCT (malignant or non-malignant) as supplementary variables. As plate (batch) contributed significantly to the variation in our dataset, we used the default settings of the ComBat package to adjust for batch effects. As underlying indication and age were most associated with PC1 in the batch-corrected data, we compared cytokine levels between children with malignant and non-malignant indications, children of different ages (infant, preschool, childhood and adolescent), as well as between children who did or did not develop a lung complication post-HCT. Mann–Whitney *U*-tests were used to compare the concentration of analytes between groups. For all analyses, an unadjusted $P < 0.05$ was considered significant, however analytes that reached an false discovery rate (FDR) < 0.1 threshold are also highlighted throughout the manuscript. Prism version 11 was used for these analyses. FDR adjustment was done using the Benjamini and Hochberg approach.[41] Area under the curve (AUC) analysis was performed for cytokines that were identified as significantly different between the lung complication and no lung complication groups; $P < 0.05$ was considered significant and sensitivity and specificity are reported.

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AUTHOR CONTRIBUTIONS

HW, GMH, TC, SS, DH and MRN obtained the funding, designed the study and interpreted the data. HW and LG conducted the experiments. HW and MRN conducted the data analysis. HW wrote the first draft of the manuscript with MRN. All authors contributed to the manuscript and approved the final version of the manuscript for publication.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.