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Serum proteomics reveals hemophagocytic lymphohistiocytosis-like phenotype in a subset of patients with multisystem inflammatory syndrome in children

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Abbreviations: ACMG, American college of medical genetics; ALAT, Alanine transaminase; AP3B1, Adaptor related protein complex 3 subunit beta 1; ASAT, Aspartate transaminase; CD, Cluster of differentiation; COPP, Clinical features of COVID-19 in pediatric patients; CRP, C-reactive protein; CXCL10, C-X-C motif chemokine ligand 10; DNA, Deoxyribonucleic acid; HLH, Hemophagocytic lymphohistiocytosis; ICU, Intensive care unit; IFN-γ, Interferon-gamma; MIS-C, Multi-system inflammatory syndrome in children; NT pro-BNP, N-terminal prohormone of brain natriuretic peptide; PD-L1, Programmed cell death ligand 1; sIL-2RA, Soluble interleukin-2 receptor alpha; TNF- α, Tumor necrosis factor-alpha; TNFRSF1A, Tumor necrosis factor receptor super family 1A; VCF, Variant calling format; VUS, Variant of unknown significance; WHO, World health organization.

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A B S T R A C T

Children with Multisystem Inflammatory Syndrome in Children (MIS-C) can present with thrombocytopenia, which is a key feature of hemophagocytic lymphohistiocytosis (HLH). We hypothesized that thrombocytopenic MIS-C patients have more features of HLH. Clinical characteristics and routine laboratory parameters were collected from 228 MIS-C patients, of whom 85 (37%) were thrombocytopenic. Thrombocytopenic patients had increased ferritin levels; reduced leukocyte subsets; and elevated levels of ASAT and ALAT. Soluble IL-2RA was higher in thrombocytopenic children than in non-thrombocytopenic children. T-cell activation, TNF-alpha and IFN-gamma signaling markers were inversely correlated with thrombocyte levels, consistent with a more pronounced cytokine storm syndrome. Thrombocytopenia was not associated with severity of MIS-C and no pathogenic variants were identified in HLH-related genes. This suggests that thrombocytopenia in MIS-C is not a feature of a more severe disease phenotype, but the consequence of a distinct hyperinflammatory immunopathological process in a subset of children.

1. Introduction

The severe hyperinflammatory post-infectious condition Multisystem Inflammatory Syndrome in Children (MIS-C) is a rare and potentially life-threatening complication of SARS-CoV-2 infection in children [1,2]. The proposed etiology of this hyperinflammatory syndrome includes superantigen exposure [3–5], prolonged intestinal SARS-CoV-2 shedding [6], exaggerated innate immune response [4], and the presence of autoreactive antibodies [7]. The clinical presentation of MIS-C has overlapping features with Kawasaki Disease [8,9] and Toxic Shock Syndrome [10,11]. There are also similarities to cytokine storm syndromes like hemophagocytic lymphohistiocytosis (HLH) [12,13]. However, there are very few patients that meet the diagnostic criteria for HLH (2004-HLH criteria) [14,15].

In HLH, a genetic or acquired inability of immune cells to adequately respond to presented antigens results in uncontrolled proliferation of immune cells, organ immune cell infiltration, cytopenia (including thrombocytopenia), macrophage activation with hemophagocytosis, and excessive secretion of cytokines [16]. Hallmark cytokines in this pathophysiological process include the T-cell activation marker soluble interleukin-2 receptor alpha (sIL-2RA), also known as soluble CD25, tumor necrosis factor-alpha (TNF-α), and interferon-gamma (IFN-γ) [17–19].

Similar to HLH, MIS-C patients can present with thrombocytopenia. This is in contrast to the thrombocytosis often seen in Kawasaki Disease and systemic juvenile idiopathic arthritis [20]. The etiology of thrombocytopenia in MIS-C is currently unresolved. We hypothesized that thrombocytopenic children with MIS-C more often had features of HLH than non-thrombocytopenic MIS-C patients.

2. Materials and methods

2.1. Study design, setting, and participants

All studies were approved by the local medical regional ethics committee (METC-LDD: N20.043, NL76177.058.21 and P22.020, NL80853.058.22; METC-GHENT: BC-07574) and local Biobank Review Committee (RP22.003). Written informed consent was obtained from all patients and/or legal guardians. A total of 278 MIS-C patients were screened from two independent cohorts: a multicenter prospective cohort study of severe pediatric COVID-19 patients in the Netherlands (COPP study [21]) and a multicenter cohort of MIS-C patients hospitalized in Belgium (GHENT study [5]). The COPP study included patients from March-2020 to December-2022, and the GHENT study from March-2020 to April-2023. Patients below the age of 18 years presenting to hospital and fulfilling the World Health Organization (WHO) criteria for MIS-C were included [22]. Exclusion criteria for the current analysis were patients without information on thrombocyte count at presentation, and no clinical information on disease severity or treatment regimen. In total, 228 patients met the inclusion criteria for this study (174 from the COPP study and 54 from the GHENT study). Biobanked sera from 20 healthy age-matched stem cell donors were used as controls. For the genetic analysis of HLH-related genes, we approached MIS-C patients that met the WHO criteria for MIS-C and participated in the COPP study (GRIP study; NCT05722717). Sixty-two MIS-C patients from the COPP-study were included, as well as 79 unrelated pediatric healthy SARS-CoV-2 exposed controls. Of the genotyped MIS-C patients, 19 (31%) were thrombocytopenic, 31 (50%) had normal/high thrombocyte counts, and 12 (19%) had an unknown thrombocyte level upon admission.

2.2. Clinical and laboratory data collection

Collected clinical data included general patient characteristics (age, sex, height), data on organ involvement, treatment, and diastolic and systolic blood pressure upon admission. Laboratory markers include quantification of hematopoietic cell lineages in blood (hemoglobin concentration, thrombocyte, leukocyte, neutrophil, and lymphocyte counts), markers for liver injury and function (aspartate transaminase (ASAT), alanine transaminase (ALAT), and total bilirubin), renal function (estimated glomerular filtration rate [23]), hemostasis (D-dimer), cardiac involvement (N-terminal prohormone of brain natriuretic peptide (NT Pro-BNP) and troponin T), and general inflammatory markers (ferritin, lactate dehydrogenase, and C-reactive protein (CRP)). Severe disease was defined as the requirement for inotropic drug administration and/or mechanical ventilatory support during hospital admission (there were no deaths in this cohort).

2.3. Serum proteomics assays (Luminex and Olink)

Serum inflammatory protein levels were quantified in the serum of 43 patients from 9 treatment centers in the COPP study, all during the acute phase of MIS-C, together with 20 healthy age-matched controls. Thawed serum samples were analyzed using a custom 60-plex Luminex panel according to the manufacturer's instructions. Analytes were excluded from all analyses if 1) $>25\%$ of the samples were out of range on the lower bound ($n = 15$) or 2) $>25\%$ of the samples had a very low mean fluorescence intensity <50, resulting in unreliable values based on extrapolation of the reference curve ($n = 9$). This resulted in the inclusion of 39 analytes (Table S1). From the included patients in the GHENT cohort, protein quantification was performed on 17 MIS-C patient samples using a 3072-plex Olink panel on thawed serum samples according to the manufacturer's protocol. Only analytes associated with thrombocytopenia in the COPP cohort Luminex panel were extracted from the Olink dataset of the GHENT cohort, to validate the findings from the Dutch cohort.

2.4. Whole-exome-sequencing

We collected saliva samples from 62 MIS-C patients and 79 unrelated

pediatric healthy SARS-CoV-2 exposed controls. DNA isolation, quality control, exome enrichment (Agilent SureSelect Human All Exon V7), and sequencing (Illumina NovaSeq 6000) were performed. Raw sequencing files were aligned to the human reference genome (GRCh38) [24], variants were called and annotated using the VIP pipeline [25]. The variant calling format (VCF) files were subset using *bcftools* based on genomic positions corresponding to 15 genes involved in the processing of cytotoxic granules causal for primary HLH and curated by the International Union of Immunological Societies to be involved in the development of HLH (Table S2) [26].

Variants in exonic coding regions or splice site regions were selected. We applied two distinct filtering strategies. The first strategy aimed to identify MIS-C patients with a genetic diagnosis of HLH. For this purpose, we analyzed rare variants with a minor allele frequency < 0.005 (GnomAD v3.1.2) [27] and applied the American college of medical genetics (ACMG) guidelines to classify these variants [28]. A genetic diagnosis was established based on the inheritance pattern of the disorder (e.g. for autosomal recessive disorders two (likely) pathogenic variants). Variants inside low complexity regions and with low quality score, were deemed artifacts and were excluded. In the second strategy, we wished to establish if there was an enrichment of rare potentially functional variants in patients with MIS-C versus healthy unrelated exposed controls. Here, variants were included when they met all of the following inclusion criteria 1) minor allele frequency < 0.005 (GnomAD v3.1.2) [27] 2) non-synonymous variants in an exonic coding region; or a splice site region and were predicted to have a moderate to high probability of having any effect on splicing (maximum Splice AI score > 0.1) [29] 3) high in silico prediction score > 20 (CADD v1.6) [30]. For the enrichment analysis, we evaluated the number of individuals carrying at least one or at least two qualifying variants in the MIS-C group versus the control group.

2.5. Statistical analysis

Descriptive values are reported by their numerator and denominator for categorical values, and median and interquartile ranges for continuous values. Statistical analyses were performed using R software (version 4.2.1). To determine differences between patient groups Fisher's Exact tests, Chi-squared test, and Wilcoxon rank-sum tests were performed. In case of linear modeling, all routine laboratory and Luminex values were log-transformed, except for hemoglobin and thrombocyte count. Two-stage linear regression was performed by first correcting for the effect of disease severity, and next to determine the effect between thrombocyte count and the marker of interest on the calculated residuals. A heatmap was constructed by log-transforming the analytes and scaling the values to z-scores based on the mean and standard deviation of 20 healthy pediatric controls. Clustering was performed with complete Euclidean hierarchical clustering, and displayed using a dendrogram. Normalized protein expression values from the Olink platform were used for calculations and plotting. All *p*-values were corrected using the Benjamini-Hochberg procedure. Only corrected p-values <0.05 were considered statistically significant.

3. Results

3.1. Thrombocytopenia in MIS-C correlates with HLH-associated laboratory markers, but not disease severity

Eighty-five out of 228 (37%) included patients were thrombocytopenic at first presentation (platelet count <150 10^9/L). One-hundred-two (45%) patients had severe disease, as defined by the requirement of inotropic drug administration or mechanical ventilatory support. Thrombocytopenic patients had significantly lower counts of leukocytes, neutrophils, and lymphocytes (Table 1). Hemoglobin levels did not differ between thrombocytopenic and non-thrombocytopenic children. In thrombocytopenic patients, ferritin was significantly elevated as compared to non-thrombocytopenic patients (728 vs. 467 μg/L, *p*-value: 0.003; Table 1). In addition, thrombocytopenic patients had significantly higher levels of liver markers (ASAT, ALAT, and total bilirubin). Thrombocytopenic patients did not have a more severe clinical phenotype: there were no significant differences in disease severity, cardiac involvement, cardiac injury markers (NT pro-BNP, troponin T), or CRP

Table 1
Clinical and laboratory characteristics of thrombocytopenic and non-thrombocytopenic patients with MIS-C. Numeric values are presented as medians and interquartile ranges. Fisher's exact test, Chi-squared test, and Wilcoxon rank-sum test were used for statistical testing, and a *p*-value correction was performed following the Benjamini-Hochberg procedure. *Abbreviations*: ICU: Intensive Care Unit, ASAT: Aspartate transaminase.

Characteristic	Normal/high thrombocytes, N = 143	Thrombocytopenia, N = 85	p-value	Adjusted p-value
Age (years)	9.0 (6.2, 12.5)	9.9 (6.9, 11.8)	0.8	0.8
Sex			0.9	>0.9
Male	90/143 (63%)	54/84 (64%)		
Female	53/143 (37%)	30/84 (36%)		
ICU admission			0.10	0.2
Yes	75/142 (53%)	55/85 (65%)		
No	67/142 (47%)	29/85 (35%)		
ICU stay (days)	3.00 (2.00, 5.00)	4.00 (3.00, 6.00)	0.082	0.2
Disease severity			0.13	0.2
Mild	85/143 (59%)	41/85 (48%)		
Severe	58/143 (41%)	44/85 (52%)		
Thrombocyte level (*10^9/L)				
> 450	7/143 (4.9%)	NA		
150–450	136/143 (95%)	NA		
100–150	NA	49/85 (58%)		
50–100	NA	31/85 (36%)		
<50	NA	5/85 (5.9%)		
Echo-cardiac abnormalities			0.2	0.3
Yes	67/114 (59%)	33/69 (48%)		
No	47/114 (41%)	36/69 (52%)		
Hemoglobin (mmol/L)	7.20 (6.60, 7.76)	7.00 (6.52, 7.62)	0.4	0.5
Lymphocyte count (*10^9/L)	0.91 (0.70, 1.40)	0.63 (0.40, 0.90)	<0.001	<0.001
Leukocyte count (*10^9/L)	11.9 (8.5, 15.6)	7.1 (5.2, 11.3)	<0.001	<0.001
Neutrophile count (*10^9/L)	9.4 (6.7, 12.8)	6.0 (4.1, 8.2)	<0.001	<0.001
ASAT (U/L)	30 (23, 47)	50 (36, 79)	<0.001	<0.001
Ferritin (ug/L)	467 (264, 780)	728 (425, 1412)	<0.001	0.003
Troponin T (ng/L)	43 (7, 134)	32 (8, 86)	0.3	0.4

(Table 1, Table S3) compared to non-thrombocytopenic patients. Next, we used a two-stage linear model to correct for the potential impact of disease severity on the variable of interest. This analysis confirmed that, corrected for disease severity, thrombocyte count (as a continuous covariate) was inversely associated with lower leukocyte subsets and elevated levels of ASAT, ALAT, bilirubin, and ferritin (Fig. 1, Fig. S1).

3.2. Thrombocytopenic MIS-C patients have a distinct HLH-like serum proteomic profile

Since thrombocytopenic MIS-C patients had routine laboratory results also seen in HLH, we evaluated whether the cytokine profile in these patients was also consistent with an HLH-like phenotype. All 43 MIS-C patients for whom Luminex data were available had very high levels of inflammatory cytokines as compared to healthy age-matched controls (Fig. 2A). Unsupervised hierarchical clustering of the protein quantification data revealed a distinct MIS-C patient cluster with an even more pronounced hyperinflammatory phenotype. Patients in this hyperinflammatory cluster ($n = 14$) had higher levels of procalcitonin, Growth-differentiation factor 15 (GDF-15), Hepatocyte Growth Factor (HGF), sIL-2RA, Granulocyte colony-Stimulating Factor (G-CSF), Interleukin 1 receptor-like 1 (ST2), Chitinase 3-like 1, Galectin-9, and Chemokine C-C motif ligand 8 (CCL-8) than the other MIS-C patients (Fig. 2A, Table S4). The proportion of thrombocytopenic MIS-C patients

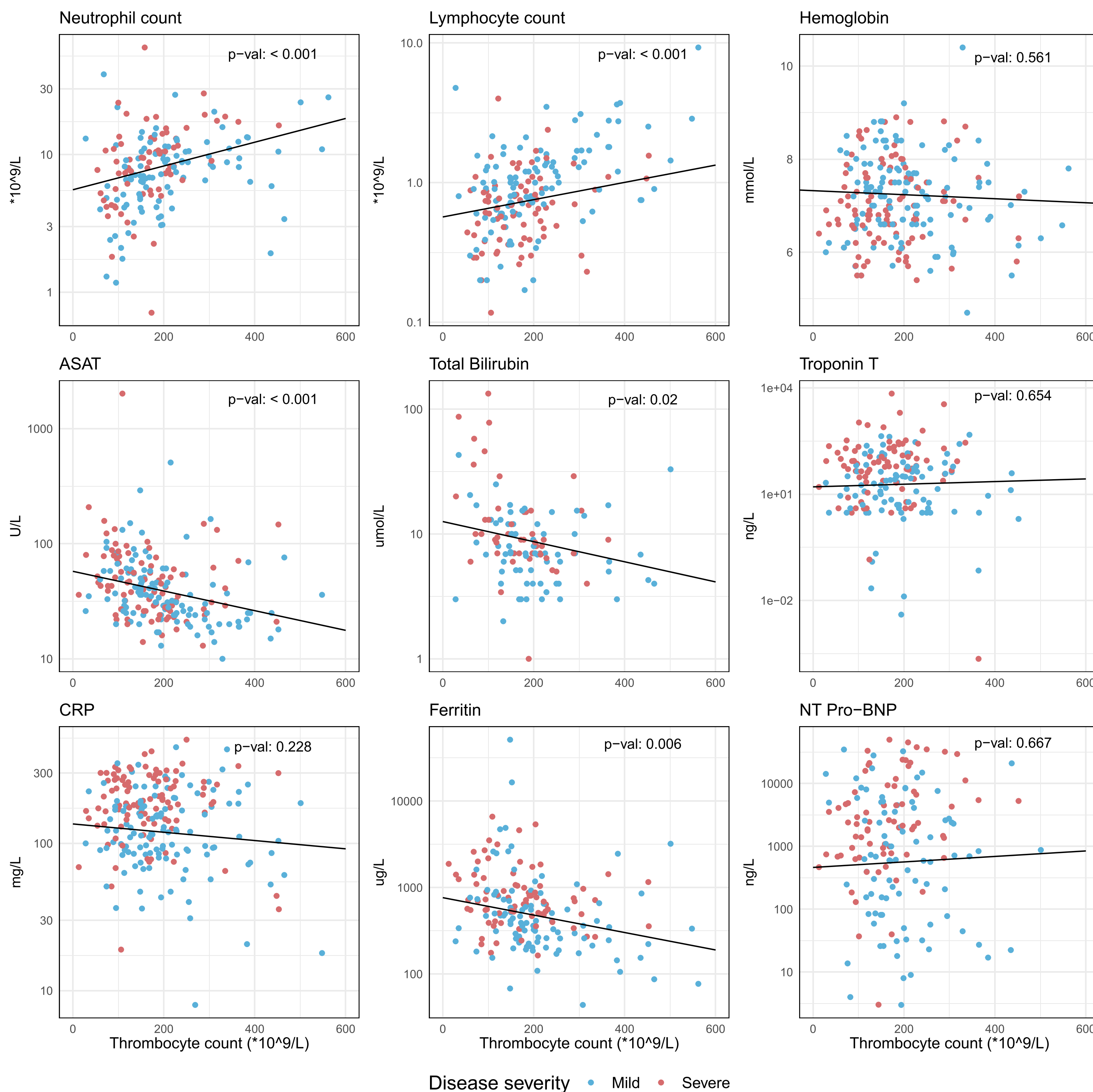

Fig. 1. Routine laboratory parameters of patients with MIS-C. Linear regression model depicting the association between thrombocyte count upon admission and the variable of interest. The model was first corrected for disease severity. Red dot: severe MIS-C, blue dot: mild MIS-C. *Abbreviations*: ASAT: Aspartate transaminase; CRP: C-reactive protein. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

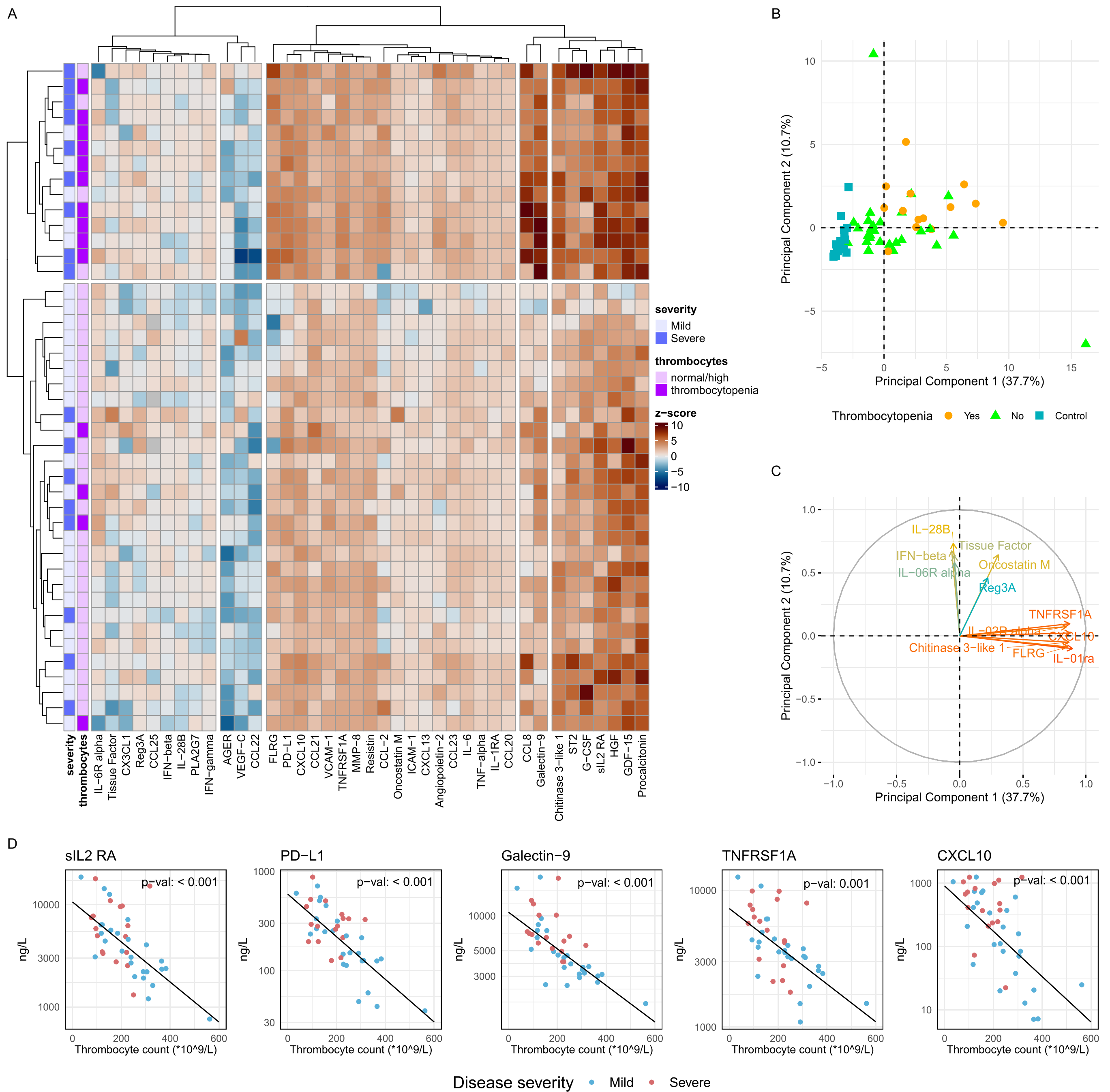

Fig. 2. Protein quantification data of patients with MIS-C. A: Heatmap of all Luminex values of analytes that passed the quality control. *Z*-scores were calculated using the mean and standard deviation of 20 healthy pediatric donors. Unsupervised complete Euclidean hierarchical clustering was used to create groups in the data, using an unbiased approach. B: Principal component analysis (PCA) of the data used to construct the heatmap. C: The six most important parameters for PCA components 1 and 2 are shown. D: Linear regression model depicting the association between thrombocyte count upon admission and the variable of interest. The model was first corrected for disease severity. Red dot: severe MIS-C, blue dot: mild MIS-C *Abbreviations*: ASAT: Aspartate transaminase; CRP: C-reactive protein; sIL-2RA: Interleukin-2 Receptor Alpha; PD-L1: Programmed Cell Death Ligand 1; TNFRSF1A, tumor necrosis factor receptor superfamily 1A; CXCL 10, C-X-C Motif Chemokine Ligand 10. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

was 71% versus 14% in the hyperinflammatory vs. inflammatory cluster, respectively (*p-value* < 0.001). The proportion of severe MIS-C cases was also higher in the hyperinflammatory cluster, albeit slightly less pronounced (64% vs. 28% had severe disease, *p-value* $= 0.04$, Table S4). There was no significant difference in the proportion of therapy naïve patients in the hyperinflammatory vs. inflammatory MIS-C clusters (Table S4). Using principal component analysis, thrombocytopenic MIS-C patients separated from the other MIS-C patients, which was primarily driven by HLH-associated markers (Fig. 2B-C).

After correction for disease severity using a two-stage linear model, low thrombocyte level at admission was strongly correlated with increased levels of HLH-associated markers. Specifically, sIL-2RA, TNF-α, and the IFN-gamma-induced chemokine C-X-C motif chemokine ligand-10 (CXCL-10) were inversely correlated to thrombocyte counts (Fig. 2D, Fig. S1). TNF receptor superfamily member 1A (TNFRSF1A), a key receptor for TNF-α, was also upregulated in patients with

thrombocytopenia (Fig. 2D). Another notable finding in patients with thrombocytopenia was a high concentration of T-cell inhibitory ligands (programmed cell death ligand-1 (PD-L1) and Galectin-9), which bind to PD-1 and T-cell immunoglobulin and mucin-domain containing-3 (TIM3) on the T-cell surface, respectively. External validation of these serum proteomic findings was performed in an independent cohort of 17 MIS-C patients analyzed using Olink technology. This analysis confirmed our key findings (Fig. S2).

3.3. Genetic screening of HLH-associated genes did not identify any causative variants in patients with MIS-C, nor an enrichment of these variants

Our first filtering strategy had the aim to identify MIS-C patients with a diagnosis of primary HLH. Here, we identified one homozygous variant in a thrombocytopenic MIS-C patient without a medical history prior to disease development. This patient harbored a homozygous missense variant (NP_003655.3:p.Arg730Trp) in the Adaptor related protein complex 3 subunit beta 1 (AP3B1) gene, which we annotated as a variant of unknown significance (VUS) based on the ACMG guidelines. Therefore, we concluded that this patient did not have a genetic diagnosis of primary HLH. In our second filtering strategy, we evaluated whether there was an enrichment of genetic variants in patients with MIS-C. We identified 14 unique heterozygous variants in 5 HLH-related genes, which were selected for further analysis (Table S5). There was no significant difference between the proportion of MIS-C patients and healthy SARS-CoV-2 exposed children carrying at least one qualifying genetic variant in HLH-related genes (8/62 (13%) versus 6/79 (7.6%); *p-value* = 0.9) (Table 2). No patient or control harbored two variants in the same (homozygous or compound heterozygous) or two different HLH-related genes (digenic mode of inheritance).

4. Discussion

Our study identifies a distinct subset of patients with MIS-C with features of HLH, independent of disease severity. This phenotype is characterized by thrombocytopenia, reduced leukocyte subsets, increased ferritin levels, elevated liver enzymes, and a profound hyperinflammatory cytokine profile. We assessed routine laboratory parameters, serum proteomics, and the genomic profile of MIS-C patients with or without thrombocytopenia at hospital admission to further elucidate this HLH-like phenotype in a subset of MIS-C patients.

Consistent with findings from other studies, all MIS-C patients had high concentrations of inflammatory biomarkers in serum compared to healthy age matched donors [31,32]. However, patients exhibiting this HLH-like phenotype developed an even more pronounced hyper-inflammatory state, primarily driven by hallmark HLH-related serum biomarkers. Specifically, cytokines associated with T-cell activation, as well as markers associated with TNF-α and IFN-γ signaling were upregulated. Interestingly, in parallel to T-cell activation markers (sIL-2RA), T-cell inhibitory signals were upregulated (PD-L1 and galectin-9). This can be a directed attempt to suppress the exaggerated T-cell activation, or a consequence of heightened IFN-γ stimulation, leading to upregulation of PD-L1 in myeloid, lymphoid, and endothelial cells [33]. For galectin-9, the interaction with IFN-γ stimulation is less clear. However, patients with acute severe COVID-19 that developed cytokine release syndrome also displayed high levels of galectin-9 [34]. Although the measured serum samples were not exclusively from therapy-naïve patients, there was no significant difference in the proportion of therapy naïve patients in the hyperinflammatory versus inflammatory MIS-C clusters.

We did not identify MIS-C patients with a genetic diagnosis of primary HLH. This was not surprising, since children with primary HLH are often diagnosed before the age of one year, and MIS-C patients have a median age of nine or ten years of age [35]. We found one patient with a homozygous VUS in AP3B1. However, this patient did not have a medical history prior to MIS-C development and was not reported to have any signs phenotypical of Hermansky-Pudlak syndrome (i.e. oculocutaneous albinism and congenital neutropenia). Interestingly, this variant has been identified in a heterogenous cohort of patients with a platelet defect [36]. Altogether, in these 62 analyzed MIS-C patients, no confident deleterious or (likely) pathogenic variants in HLH-associated genes were identified, in either the patients with or without thrombocytopenia.

Due to the fact a digenic mode of inheritance is reported to play a role in some patients with HLH [37]; and there is growing evidence of a genetic predisposition in secondary HLH [38], we evaluated if heterozygous or polygenic variants in HLH-related genes are associated with developing an HLH-like phenotype upon SARS-CoV-2 infection. In contrast to one study reporting a high number of variants in primary HLH genes in children with MIS-C [13] and a different study reporting numerous rare heterozygous missense variants in the LYST gene in MIS-C patients [39], we found no significant enrichment of variants in these genes in comparison with pediatric SARS-CoV-2 exposed controls. We did find a higher proportion of genetic variants in HLH-genes in MIS-C patients than in controls, but this was not statistically significant. Since our sample size is relatively small, this does not completely rule out the possibility of a genetic predisposition to an HLH-like inflammatory response to SARS-CoV-2 infection in a subset of children. Also, while we did not identify any directly causative deleterious variants in known HLH genes, this does not rule out the possibility of aberrations in other genes, or copy-number-variants or intronic non-coding variants in the studied HLH genes, that may explain the genetic predisposition to MIS-C.

Altogether, this study provides relevant insights into the pathophysiological manifestations of MIS-C and its distinct link to HLH. Combining routine clinical parameters, serum proteomics, and DNA sequencing, we managed to establish a detailed picture of immunologically divergent MIS-C phenotypes. These findings not only relate to new cases of MIS-C [40], but may have implications for other hyper-inflammatory conditions presenting with thrombocytopenia as well.

Table 2
Count and corresponding proportions of controls and MIS-C patients harboring a qualifying variant in a HLH-associated gene. Fisher's exact test was used for statistical testing and p-values were corrected for multiple testing using the Benjamini-Hochberg procedure. *Abbreviations*: MIS-C: Multisystem Inflammatory Syndrome in Children.

	CONTROL, *N* = 79	MIS-C, *N* = 62	p-value	Adjusted p-value
LYST	3 (3.8%)	5 (8.1%)	0.3	0.9
RAB27A	1 (1.3%)	1 (1.6%)	>0.9	>0.9
UNC13D	1 (1.3%)	1 (1.6%)	>0.9	>0.9
FAAP24	0 (0%)	1 (1.6%)	0.4	0.9
AP3B1	1 (1.3%)	0 (0%)	>0.9	>0.9
One or more variants	6 (7.6%)	8 (13%)	0.4	0.9
Two or more variants	0 (0%)	0 (0%)		

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CRediT authorship contribution statement

Adam J. Tulling: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data

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Declaration of competing interest

The authors declare no conflict of interests.

Data availability

The genetic data for the regions corresponding to the genes analyzed in this study (VCF format) can be obtained on reasonable request to the corresponding author.

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Appendix A. Supplementary data

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