Transient anti-cytokine autoantibodies superimpose the hyperinflammatory response in Kawasaki disease and multisystem inflammatory syndrome in children: a comparative cohort study on correlates of disease

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Summary

Background Children with SARS-CoV-2 related Multisystem Inflammatory Syndrome in Children (MIS-C) often present with clinical features that resemble Kawasaki disease (KD). Disease severity in adult COVID-19 is associated to the presence of anti-cytokine autoantibodies (ACAAs) against type I interferons. Similarly, ACAAs may be implicated in KD and MIS-C. Therefore, we explored the immunological response, presence of ACAAs and disease correlates in both disorders.

Methods Eighteen inflammatory plasma protein levels and seven ACAAs were measured in KD (n = 216) and MIS-C (n = 56) longitudinally by Luminex and/or ELISA. Levels (up to 1 year post-onset) of these proteins were related to clinical data and compared with healthy paediatric controls.

Findings ACAAs were found in both patient groups. The presence of ACAAs lagged behind the inflammatory plasma proteins and peaked in the subacute phase. ACAAs were mostly directed against IFN-γ (>80%) and were partially neutralising at best. KD presented with a higher variety of ACAAs than MIS-C. Increased levels of anti-IL-17A (P = 0·02) and anti-IL-22 (P = 0·01) were inversely associated with ICU admission in MIS-C. Except for CXCL10 in MIS-C (P = 0·002), inflammatory plasma proteins were elevated in both KD and MIS-C. Endothelial angiopoietin-2 levels were associated with coronary artery aneurysms in KD (P = 0·02); and sCD25 (P = 0·009), angiopoietin-2 (P = 0·001), soluble IL-33-receptor (ST2, P = 0·01) and CXCL10 (P = 0·02) with ICU admission in MIS-C.

Interpretation Markers of endothelial activation (E-selectin, angiopoietin-2), and innate and adaptive immune responses (macrophages [CD163, G-CSF], neutrophils [lipocalin-2], and T cells [IFN-γ, CXCL10, IL-6, IL-17]), are upregulated in KD and MIS-C. ACAAs were detected in both diseases and, although only partly

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neutralising, their transient presence and increased levels in non-ICU patients may suggest a dampening role on inflammation.

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Keywords: Inflammation; Cytokines; Autoantibodies; Kawasaki disease; MIS-C

Research in context

Evidence before this study

A systematic search in PubMed/Medline was performed on February 2nd 2022 to identify all studies that focused on anti-cytokine autoantibodies in Kawasaki disease (KD) and Multisystem Inflammatory Syndrome in Children (MIS-C). Search terms included terms related to "Kawasaki disease", "MIS-C", "antibodies" and "cytokines". Two studies were identified. One study by Pfeifer et al., showed the presence of anti-IL-1RA autoantibodies in about two thirds of MIS-C patients, while not detecting such antibodies in KD, pediatric COVID-19 and healthy controls. A second study by Bodansky et al., did not detect anti-IFN-α autoantibodies in MIS-C, with the exception of a patient who had a severe COVID-19 presentation, followed by a suspected MIS-C presentation. Other reports have also reported anti-cytokine autoantibodies (ACAA), mostly against type I interferons, in adult COVID-19. These ACAA have been shown to correlate with disease severity and increase with age.

Added value of this study

In the current cohort study we investigated the presence of ACAA in KD and MIS-C over time. We found transient ACAA in both hyperinflammatory disorders that peaked in the subacute phase. Anti-IFN-γ autoantibodies were identified most often and were in some cases partially neutralizing in a STAT1 phosphorylation assay. Higher pre-treatment levels of anti-IL-17A and anti-IL-22 were seen in patients that did not require admission to the ICU.

Implications of all the available evidence

ACAA are potentially involved in the natural response to mitigate the effects of some of the major pro-inflammatory cytokines induced during the acute hyperinflammatory stage of KD and MIS-C.

Introduction

Kawasaki disease (KD) is a paediatric vasculitis with a risk of developing coronary artery aneurysms and is the leading cause of acquired heart disease in Europe and North America.[1] Since it was first described in 1961,[2] its exact aetiology has been widely disputed and remains uncertain. KD is hypothesized to be a post-infectious inflammatory disorder characterized by activation of neutrophils, monocytes and lymphocytes, including high levels of cytokines and a pronounced involvement of the IL-17 pathway.[1,3,4]

Since the start of the COVID-19 pandemic, a new SARS-CoV-2 related paediatric disease emerged, which was later characterized as Multisystem Inflammatory Syndrome in Children (MIS-C).[5] Noticeably, affected children often present with clinical features that resemble Kawasaki disease (Supplementary Table E1).[6,7] Children with MIS-C typically show a higher age of onset compared to KD patients, and more often present with multi-organ dysfunction, including vasoplegic and cardiac shock in about half of the cases.[5,8] Similar to KD, MIS-C is a post-infectious inflammatory disease, typically occurring 3–6 weeks after SARS-CoV-2 infection.[5,8] MIS-C presents with neutrophilia, lymphopenia and an immunophenotypic profile indicating activation of neutrophils, monocytes and T cells.[3,9] It is characterized by elevations in various cytokines and a more pronounced involvement of the IFN-γ pathway (e.g., IFN-γ, CXCL9, CXCL10), when compared to KD.[4,6,10]

In adults acutely infected by SARS-CoV-2, autoreactivity and the formation of autoantibodies have been suggested to contribute to an inflammatory state during COVID-19 infection.[11–16] Several studies have shown the presence of autoantibodies against type I interferons in adult COVID-19,[11–16] even prior to disease contraction. The natural occurrence of these autoantibodies is associated with an increase in age and disease severity. Also, they have complete neutralizing capacity to their corresponding interferon.[11–13]

Considering the association between COVID-infection and MIS-C, we postulated that ACAAs may be implicated in KD and MIS-C as well. A recent study reported anti-IL-1RA autoantibodies in 62% (13/21) of MIS-C patients, while not detecting such antibodies in

KD, paediatric acute COVID-19 and healthy controls.[17] A second study did not detect anti-IFN-α autoantibodies in MIS-C patients prior to treatment.[18] To our knowledge, no other studies investigating presence of ACAAs in KD and MIS-C have been reported.

To clarify the dysregulated immunological pathways in KD and MIS-C, we investigated the presence of a panel of inflammatory biomarkers in addition to standard laboratory parameters (e.g., CRP, ferritin, D-dimers, NT-pro-BNP) that are commonly measured, particularly in MIS-C. Also, we determined the longitudinal patterns of anti-cytokine autoantibodies (ACAAs) against seven distinct cytokines (IFN-α, IFN-γ, IL-6, IL-17A, IL-17F, IL-22, and the antagonistic IL-1RA), in KD and MIS-C, and investigated correlates of disease for both diseases.

Methods

Study population

Patients with KD[1] or MIS-C[19,20] (Supplementary Table E1) were recruited at the Amsterdam UMC, and participating hospitals in the broad Amsterdam region, as part of the long-term observational Kawasaki disease study as approved by the Medical Ethical Board of the AMC (no. NL41023.018.12). Patients were recruited between 01-2000 and 02–2022 (Supplementary Fig. E1). All of our included KD-patients enrolled during the COVID-era (n = 25) were SARS-CoV-2 seronegative. Compared to pre-COVID KD patients, they did not differ significantly regarding clinical aspects (i.e., sex, age, delayed treatment, IVIG resistance, CAA development and ICU admission). Controls were selected from the PERFORM study (Personalised Risk assessment in Febrile illness to Optimise Real-life Management; London-Central Research Ethics Committee, 16/LO/1684) and included afebrile children with blood tests for reasons other than investigation of infectious or inflammatory illness and without fever or vaccination ≤3 weeks (Steels S, Van Elslande J, Leuven C et al. [2023, manuscript submitted for publication]). A graphical representation of the study methods is shown in Fig. 1.

Data collection

Clinical and laboratory information from the acute disease episode and outpatient clinic was collected using electronic health records processed in a combined database (Castor) (Supplementary Box E1).

Blood plasma EDTA samples from KD and MIS-C patients (<2 years old 2 mL, >2 years old 4.5 mL) were collected during admission and at the OPD and categorized in the following time-points: pre-IVIG, ≤2 days post-treatment, >2 to ≤7 days post-treatment, >1 to ≤2 weeks post-treatment, >2 to ≤4 weeks post-treatment, ±1–3 months post-treatment, ±3–9 months post-treatment and ≥9 months post-treatment. After collection, plasma samples were aliquoted and stored at –20° for further analyses. The samples were not subjected to multiple freeze thaw cycles.

Analysis of blood-derived proteins

A total of eighteen inflammatory markers previously selected to study the host response during acute bacterial and viral infections (Steels S, Van Elslande J, Leuven C et al. [2023, manuscript submitted for publication]), including: angiopoietin-2, E-selectin, ST2 (soluble interleukin 1 receptor-like 1), IL-18, C-X-C motif chemokine ligand 10 (CXCL10), IFN-γ, granulocyte colony-stimulating factor (G-CSF), CD163, galectin-3 binding protein, lipocalin-2, soluble CD25 [sCD25], tumour necrosis factor alpha (TNF-α), IL-17A, IL-6, IL-10, IL-8, chemokine ligand (CCL)4, CCL5. These biomarkers were measured in plasma samples of all patients with at least one sample ≤2 days post-IVIG (Supplementary Fig. E1) using a Customized Luminex® human cytokine multiplex panel according to the manufacturer's protocol (R&D Systems, Inc, Bio-Techne, Minneapolis MN, USA) (Supplementary Box E1).

Analysis of anti-cytokine autoantibodies

Levels of antibodies against seven cytokines (IL-17A, IL-17F, IL-22, IFN-γ, IFN-α, IL-6, IL-1 receptor antagonist [RA]) were measured in plasma samples from the acute, subacute and convalescent timeframes of all patients with at least one sample ≤28 days post-IVIG (Supplementary Fig. E1). Autoantibodies against the cytokines IL-17A, IL-17F, IL-22, IFN-γ, IFN-α and IL-6 were measured in 1:100 plasma dilutions using a bead-based assay (Supplementary Box E1). Autoantibodies against anti-IL-1RA IgG were measured 1:20 and 1:40 plasma dilutions using a newly developed ELISA (cat# HK3028, Hycult Biotech, Uden, The Netherlands, not yet commercially available) (Supplementary Box E1).

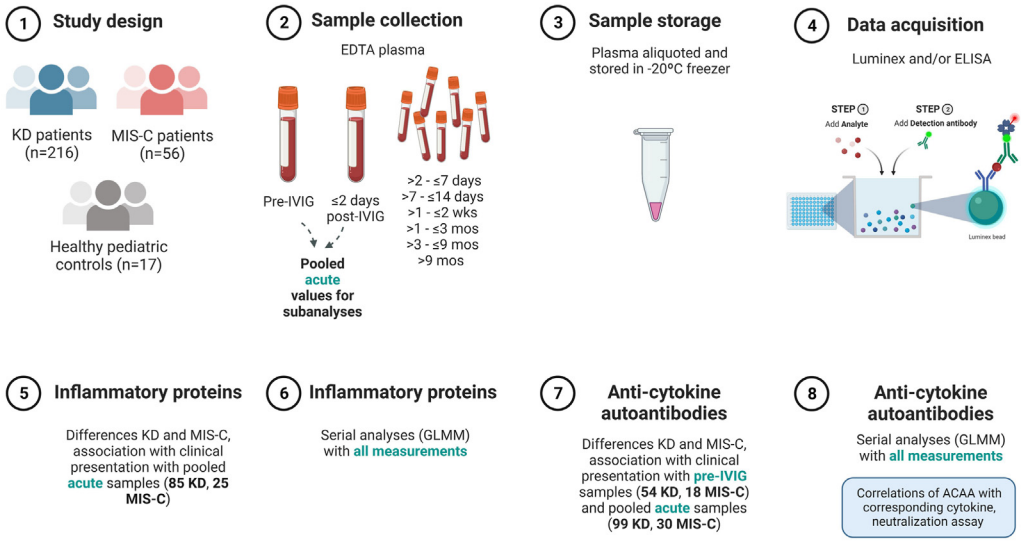
Neutralization assay autoantibodies

To investigate whether ACAA against IFN-γ where neutralizing, inhibition of IFN-γ-induced STAT1 phosphorylation in monocytes was assessed using plasma from a subset of seventeen samples with elevated anti-IFN-γ in the acute phase of KD [n = 9] and MIS-C [n = 8]. Plasma of a patient with high pathologic anti-IFN-γ antibody titers suffering from disseminated infections was used as a positive control sample (Supplementary Box E1).

To investigate the presence of ACAA in therapeutic IVIG (Nanogam), IVIG was diluted 10, 50, 500 and 1000 times, ACAA were measured and neutralizing capacity was investigated using a IFN-γ-induced STAT1 phosphorylation assay (Supplementary Box E1).

Statistics

Because of a non-normal distribution, characteristics of our study population were summarized using



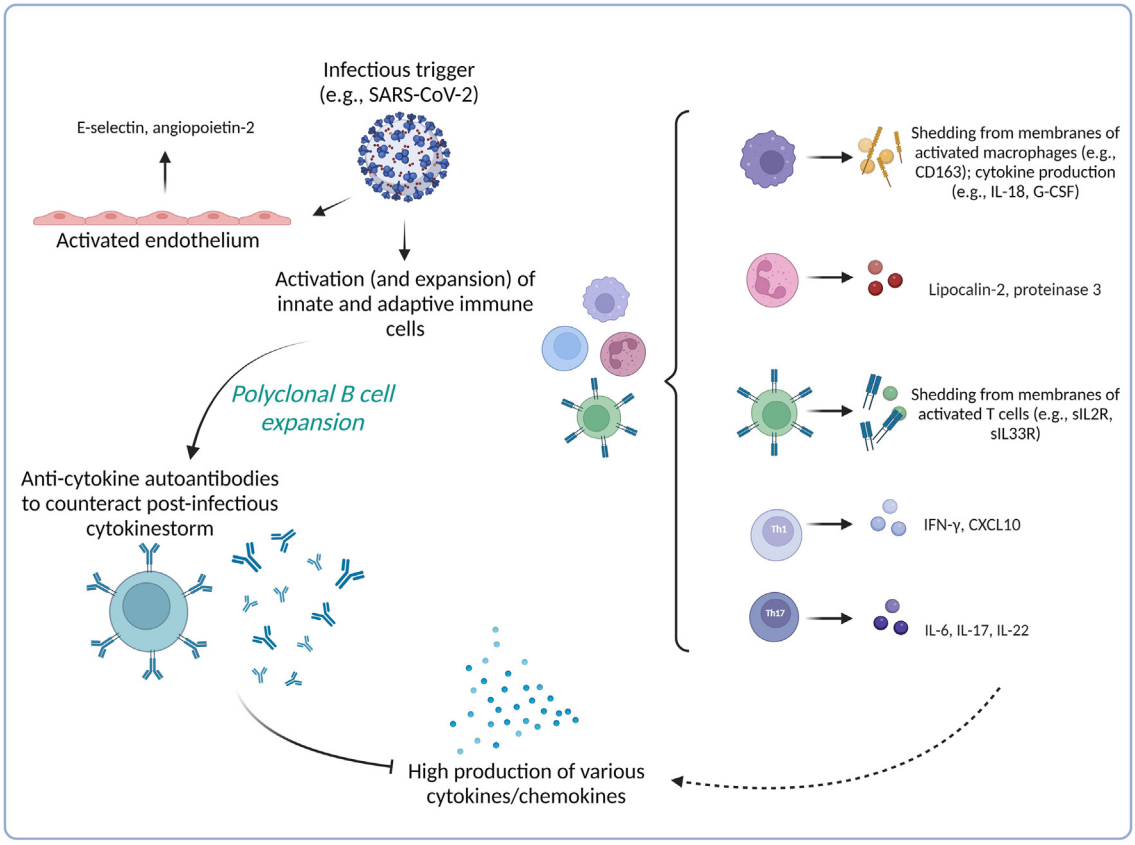


Fig. 1: Graphical abstract of study methods and hypothesis. Created with BioRender.com.

frequencies (proportions) and median (interquartile range) values. The distribution of the study characteristics within the KD and MIS-C group were compared (i.e., Fisher's Exact test, Mann–Whitney U and Kruskal Wallis [with post-hoc Dunn's adjusted for multiple comparisons by statistical hypothesis testing]).

To investigate differences between acute levels of inflammatory proteins in KD and MIS-C and

associations with clinical outcomes, we pooled all acute measurements ≤2 days post-IVIG, since we did not have pre-IVIG samples of all patients. For the ACAA sample series, we also performed these same analyses solely with the pre-IVIG samples, to exclude possible bias caused by IVIG treatment.

Longitudinal patterns of the inflammatory protein levels within the different disease stages of KD and MIS-C were assessed and compared to a group of healthy paediatric controls (and an additional group of adult controls in case of the ACAA). To assess over-time patterns of the serial measurements we used mixed effects models with splines and generalized linear mixed models (GLMM) (Supplementary Box E1).

To postulate the proportion of patients with an elevated ACAA level within each time-frame, high levels of ACAA were defined as patients with a peaking ACAA value exceeding the mean (of disease controls) plus three times the standard deviation of disease controls (similar to previous reports).[17]

Further statistical analyses are described in Supplementary Box E1.

Study approval

The Medical Research Ethics Committee of the Amsterdam UMC provided ethical approval for the Kawasaki study with the reference number: 2012_155. Informed consent and written approval were obtained. All patients were included in accordance with study protocol, the International Conference on Harmonization (ICH) Good clinical Practice guidelines and the provisions of the Declaration of Helsinki.

Funding

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We were not paid to write this article by a pharmaceutical company or other agency.

The authors were not precluded from accessing data in the study and they accept responsibility to submit for publication.

Trial registration number: NL41023.018.12.

Results

Patient characteristics

In total, 216 KD patients, 56 MIS-C patients and 15 controls (median age 5.1 years, 60% female) were included in the study (Supplementary Fig. E1). The patients' ethnic backgrounds did not significantly differ between KD and MIS-C patients. In both patient groups, most patients of non-Dutch ancestry were from Suriname, Morocco or Turkey. Detailed patient characteristics are shown in Table 1. Compared to KD patients, MIS-C patients had a significantly higher median age at onset ($P < 0.001$), more frequently presented with incomplete KD features ($P < 0{\cdot}001$) and required ICU admission more often ($P < 0{\cdot}001$). In addition to IVIG treatment, patients with MIS-C were more often treated with corticosteroids, milrinone ($P < 0{\cdot}001$), noradrenaline ($P < 0{\cdot}001$) and anakinra ($P = 0{\cdot}003$). Regarding cardiac outcomes, coronary artery aneurysms were more common in KD patients ($P = 0{\cdot}05$), while MIS-C patients presented with circulatory shock in 42·9% of the cases ($P < 0.001$). Patients recovered quickly after treatment, irrespective of the final diagnosis (KD or MIS-C). MIS-C patients were admitted to the ICU primarily due to circulatory failure based on cardiac dysfunction (22/23 [95·6%]), but 9/23 (39·1%) required respiratory support with high flow nasal oxygen therapy and 11/23 (47·8%) had acute kidney disease. Patients with cardiac dysfunction regained adequate function within two weeks with the exception of three patients, who recovered more slowly within six months (left ventricle ejection fraction between 46 and 49%, normal fractional shortening, no cardiac medication).

Blood-derived proteins as biomarkers over time

In both KD and MIS-C patients elevated levels of a wide range of proteins (e.g., angiopoietin-2, E-selectin, IL-6, IL-18, IFN-γ, CXCL10, sCD25) were measured in the acute phase of disease. The exact numbers of samples measured in the acute phase are shown in Supplementary Table E2. With the exception of CXCL10 (only elevated in MIS-C, $P = 0{\cdot}006$) and CCL5 (only elevated in KD, $P = 0{\cdot}04$), all proteins were significantly elevated in the acute disease phase of KD and MIS-C patients compared to controls. Proteins measured are shown in Supplementary Table E3. Ferritin, NT-pro-BNP and D-dimers were only measured in MIS-C patients. Serial analyses of over-time patterns were similar for both KD and MIS-C, revealing peak levels during the acute phase and normalisation over time (Fig. 2).

In the acute phase, KD patients presented with higher levels of IL-8 ($P < 0{\cdot}001$), E-selectin ($P = 0{\cdot}03$) and G-CSF ($P = 0{\cdot}04$) than MIS-C patients (Fig. 3A and Supplementary Table E3). MIS-C patients presented with higher levels of ST2 ($P < 0{\cdot}001$), CXCL10 ($P = 0{\cdot}002$) and IL-10 ($P = 0{\cdot}03$) than KD patients (Fig. 3A and Supplementary Table E3).

Anti-cytokine autoantibody profiles over time

We observed high variability in ACAA levels (against IFN-α, IFN-γ, IL-6, IL-17A, IL-17F, IL-22, and the antagonistic IL-1RA) between patients (Figs. 3B and 4, Supplementary Fig. E2). In KD, the median pre-IVIG levels were significantly elevated for all ACAAs compared to paediatric controls. In MIS-C, median pre-IVIG ACAA levels were only significantly elevated for anti-IL-17A and anti-IFN-γ (Supplementary Table E4), although several patients had elevated levels for some of

	KD[a]-group (n = 216)	MIS-C[b] (n = 56)	Significance (*P* value)[c]
Age at diagnosis (years)	2·8 (1·2–4·7)	11·3 (6·6–14·3)	**<0·001**
Sex			
Female	89 (41·4%)	18 (32·1%)	
Male	126 (58·6%)	38 (67·9%)	0·2
Ethnicity			
White	130 (69·5%)	38 (79·2%)	
Black	20 (10·7%)	7 (14·6%)	
Asian	11 (5·9%)	1 (2·1%)	
Mixed	26 (13·9%)	2 (4·1%)	0·2
Symptoms[a]			
Fever[d]	216 (100%)	56 (100%)	N/A
Rash	176 (86·3%)	36 (62·5%)	**<0·001**
Conjunctivitis	177 (87·2%)	42 (75·0%)	**0·04**
Oral changes	174 (86·1%)	37 (66·1%)	**0·001**
Cervical lymphadenopathy	163 (81·1%)	27 (49·1%)	**<0·001**
Changes of the extremities[a]	154 (77·0%)	19 (34·5%)	**<0·001**
Respiratory symptoms		12 (21·4%)	N/A
Intubated		0 (0%)	N/A
Abdominal symptoms		52 (92·9%)	N/A
Neurological symptoms		29 (51·8%)	N/A
Complete KD	168 (82·4%)	13 (23·2%)	**<0·001**
Complications			
Shock[e]	6 (2·8%)	28 (50·0%)	**<0·001**
Respiratory failure	2 (0·9%)	15 (26·8%)	**<0·001**
Acute kidney injury	2 (0·9%)	12 (21·4%)	**<0·001**
Laboratory findings[f]			
CRP, mg/L	125·0 (58·0–183·5)	181·0 (128·7–268·0)	**<0·001**
Leukocytes, 10^9/L	16·1 (12·0–19·4)	14·1 (9·7–21·2)	0·2
Thrombocytes, 10^9/L	379·0 (378·5–527·8)	327·0 (191·0–500·0)	0·06
Haemoglobin, mmol/L	6·7 (6·3–7·4)	6·6 (5·9–7·2)	0·2
Albumin, g/L	34·0 (27·0–39·0)	29·4 (26·3–36·0)	0·1
Treatment			
IVIG	213 (98·6%)	52 (92·9%)	**0·04**
IVIG <10 days	168 (78·9%)	49 (94·2%)	**0·03**
Corticosteroids	43 (19·9%)	43 (76·8%)	**<0·001**
Milrinone	1 (0·5%)	16 (28·6%)	**<0·001**
Noradrenaline	2 (0·9%)	19 (33·9%)	**<0·001**
Anakinra	3 (1·4%)	6 (10·7%)	**0·003**
ICU admission	6 (2·8%)	23 (41·1%)	**<0·001**
Coronary artery aneurysms			
None	149 (69·0%)	49 (87·5%)	
Z score ≥2·5 to <5·0	27 (12·5%)	5 (8·9%)	
Z score ≥5 to <10	9 (4·2%)	1 (1·8%)	
Z score ≥ 10	13 (6·0%)	0 (0%)	**0·05**
Cardiac dysfunction[g]	N/A	32 (57·1%)	N/A
Second KD-episode	3 (1·2%)	0 (0%)	0·6

Data are n (%) or median (interquartile range). Bold indicates a *P* value below 0.05 was considered statistically significant. Abbreviations: KD = Kawasaki disease, MIS-C = multisystem inflammatory syndrome in children, IVIG = intravenous immune globulin, ICU = intensive care unit. [a]American Heart Association criteria for the definition of Kawasaki disease (KD) is to have persistent fever and 4 of the following 5 mucocutaneous features: erythema and cracking of lips, strawberry tongue, and/or erythema of oral and pharyngeal mucosa; bilateral bulbar conjunctival injection without exudate; rash (maculopapular, diffuse erythroderma); erythema and oedema of the hands and feet in acute phase and/or periungual desquamation in subacute phase; and cervical lymphadenopathy (>1·5 cm diameter). Incomplete KD was defined by at least 2 clinical criteria compatible with KD and additional laboratory or cardiac criteria. [b]MIS-C according to criteria by Centers for Disease Control and Prevention (CDC) and World Health Organization (WHO). [c]Fisher's exact Test for categorical variables, Mann–Whitney U for continuous variables. [d]Fever >38 °C. [e]Shock defined as needing inotropic support or fluid resuscitation >20 mL/kg. [f]When multiple blood results were available, the nadir value (<2 weeks post-onset) was used in the analyses. [g]Cardiac dysfunction was defined as a left ventricle ejection fraction <50% and/or a fractional shortening <28%.

Table 1: **Patient characteristics and clinical presentation.**

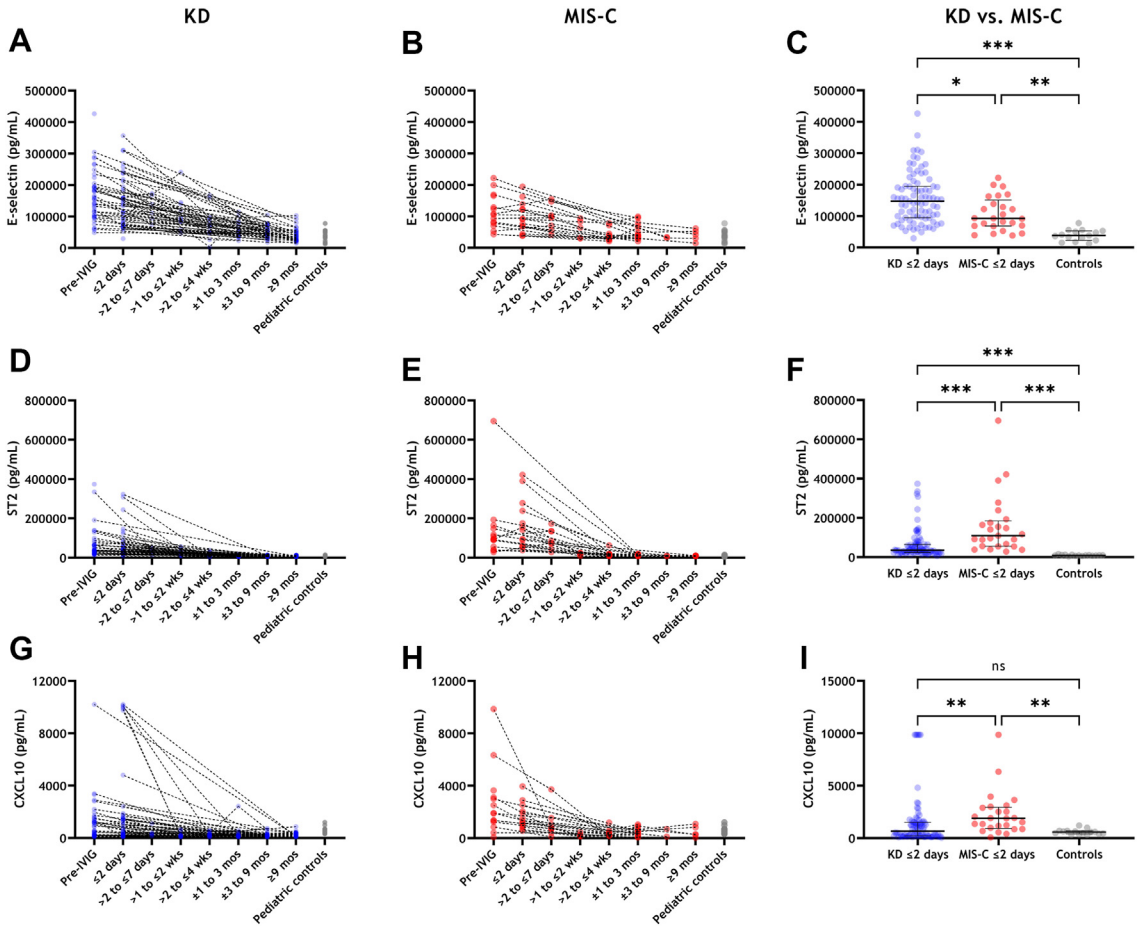

Fig. 2: Over-time trend (A, B, D, E, G, H) and comparison between KD and MIS-C patients during the acute phase (pooled values ≤ 2 days post-IVIG) vs. healthy paediatric controls (C, F, I) of E-selectin (A, B, C), ST2 (D, E, F) and CXCL10 (G, H, I). Abbreviations: KD = Kawasaki disease, MIS-C = Multisystem inflammatory syndrome in Children, IVIG = intravenous immune globulin, ST2 = soluble interleukin 1 receptor-like 1, CXCL10 = C-X-C motif chemokine ligand 10, NS = not significant. *P < 0·05, **P < 0·01, ***P < 0·001 (Kruskal–Wallis with Dunn's multiple comparison).

the other ACAA as well (Supplementary Fig. E2). Pre-IVIG anti-IL-1RA levels were not significantly elevated in any of the KD and MIS-C patients compared to controls.

Within the complete period of follow-up, anti-IFN-γ autoantibodies were most prominently elevated in both diseases (exceeding the predetermined cut-off, see Methods). Being detected in about 30% of patients prior to IVIG (31·5% [17/54] of KD, 28·8% [5/18] of MIS-C), the presence of anti-IFN-γ autoantibodies rose to 80% or more during follow-up (84.6% [44/52] of KD, 80% [12/15] of MIS-C) ≤14 days post-treatment. Importantly, 3 KD patients and 4 MIS-C patients in the study had not been treated with IVIG and of these, all but one had elevated anti-IFN-γ values. Moreover, the levels of ACAAs ≤2 days of IVIG did not significantly differ between patients treated ≤10 days of onset of fever and those that did not, nor between the patient with 1 vs. 2 IVIG infusions. The other ACAA were elevated less often (Supplementary Fig. E2). Pre-IVIG anti-IL-1RA was only elevated in 1 KD patient and in none of the MIS-C patients. In both KD (Fig. 4A) and MIS-C (Fig. 4B), the number of simultaneously elevated ACAA peaked between two days and one week of follow-up, as did the proportion of patients with at least one elevated ACAA level.

Longitudinal patterns of all ACAA differed significantly between KD and MIS-C in a non-linear mixed model with splines (P < 0·001 for all) (Supplementary Table E5), independent of several clinical covariates (i.e., sex, age at onset, timing of the sample, treatment within 10 days, ICU admission and coronary artery aneurysms). Generally, ACAA levels peaked between two days and two weeks and reached a plateau between three and nine months of follow-up. However, in KD patients, ACAA levels peaked between >1 and ≤2 weeks, while in MIS-C patients ACAA levels peaked ≤2 days of follow-up (Supplementary Figs. E2 and E3). This was

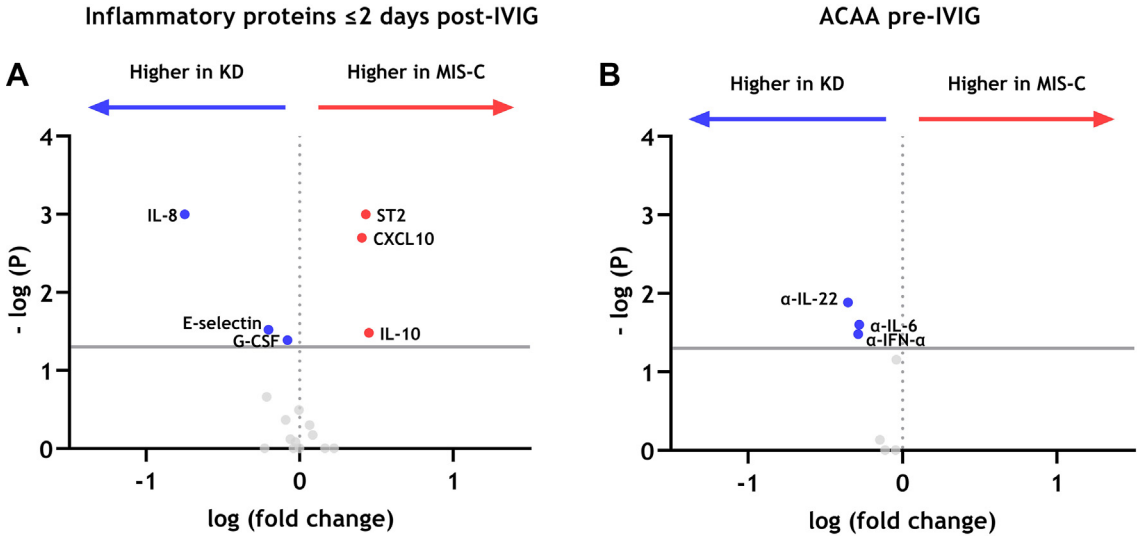

Fig. 3: Volcano plot (*P* values based on Kruskal–Wallis with Dunn's multiple comparisons test) of measured inflammatory protein values measured in the acute phase (pooled values ≤ 2 days post-IVIG) (A) and pre-IVIG levels of ACAA (B) in KD compared to MIS-C patients. Abbreviations: KD = Kawasaki disease, MIS-C = Multisystem inflammatory syndrome in Children, ACAA = anti-cytokine autoantibodies, IVIG = intravenous immune globulin, IL = interleukin, ST2 = soluble interleukin 1 receptor-like 1, G-CSF = granulocyte colony stimulating factor, IFN = interferon, CXCL10 = C-X-C motif chemokine ligand 10.

confirmed in our multivariable GLMM analyses as well (Supplementary Table E6).

Furthermore, KD patients more often had simultaneously elevated ACAAs compared to MIS-C patients (Fig. 4A/B).

In KD, pooled anti-IL-6 levels correlated significantly with IL-6 ($P = 0{\cdot}05$, r = 0·15), as did the anti-IFN-γ levels with IFN-γ ($P = 0{\cdot}002$, r = 0·22). Likewise, higher levels of IL-6, IFN-γ and CXCL10 significantly correlated with several of the other ACAA levels, suggesting that ACAA are produced in response to general hyperinflammation (Supplementary Table E7). None of the ACAA levels correlated with inflammatory protein levels in pooled analyses of the plasma measurements in MIS-C.

IFN-γ inhibition assay

The neutralizing capacity of anti-IFN-γ autoantibodies, the most prevalent ACAA, was measured in a STAT1 phosphorylation assay (Supplementary Fig. E4A and B). Plasma of 5/9 KD patients and 2/8 MIS-C patients partially inhibited IFN-γ-induced STAT1 phosphorylation in monocytes. However, in 10 of the 18 patients no inhibition of IFN-γ-induced STAT1 phosphorylation was observed. Plasma of the healthy control did not inhibit IFN-γ-induced STAT1 phosphorylation (negative control), while plasma of a patient with high anti-IFN-γ antibody titers and a neutralizing mouse anti-human-IFN-γ monoclonal antibody strongly inhibited STAT1 phosphorylation (positive control). Lastly, we did not detect neutralizing activity of IVIG (Nanogam) dilutions against IFN-γ (Supplementary Fig. E4C).

Correlates of disease

First, the association of several clinical factors (i.e., male sex, age at onset <1 year old, ICU admission [as a proxy for circulatory dysfunction], development of coronary artery aneurysms) with the level of acute phase inflammatory protein level (pre-IVIG plus ≤2 days of IVIG treatment) were investigated in multiple regression analyses (Supplementary Tables E8 and E9). The acute levels of angiopoietin-2 were associated with the development of coronary artery aneurysms in KD, independent of sex and age at onset (Supplementary Table E8). Acute levels of angiopoietin-2, sCD25, ST2 (soluble IL-33 decoy receptor) and CXCL10 were significantly associated with ICU admission (as a proxy for circulatory failure) in MIS-C, independent of sex (Supplementary Table E9).

Next, we performed multiple regression analyses for the ACAAs measured pre-IVIG (to exclude bias from IVIG treatment) as well as in the acute phase (pre-IVIG plus ≤2 days of IVIG treatment). None of the pre-IVIG or acute ACAA levels were significantly associated with CAAs in KD patients (Supplementary Table E8). However, the acute values of anti-IL17A ($P = 0{\cdot}02$) and the pre-IVIG ($P = 0{\cdot}02$) and acute values of anti-IL-22 ($P = 0{\cdot}01$) were negatively associated with ICU admission in MIS-C patients (Supplementary Table E9).

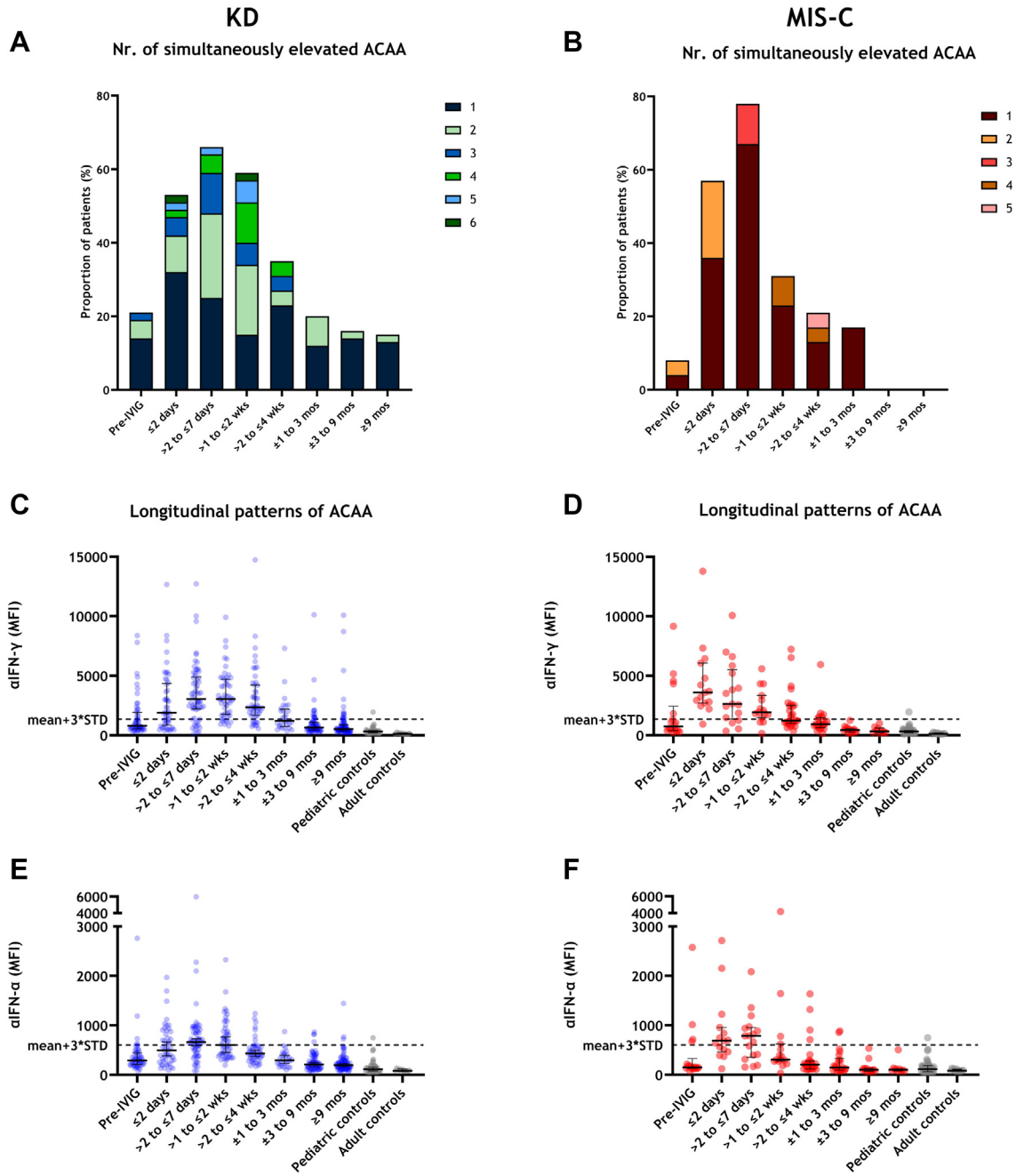

Fig. 4: Proportion of KD (A) and MIS-C (B) patients with simultaneously high levels of ACAA during follow-up; over-time trend (including median and interquartile range) of anti-IFN-α (C, D) and anti-IFN-γ values (E, F) of KD and MIS-C patients with the cut-off for elevated ACAA values (mean plus three times the standard deviation) depicted in the dashed line. Abbreviations: KD = Kawasaki disease, MIS-C = Multisystem inflammatory syndrome in Children, IVIG = intravenous immune globulin, MFI = mean fluorescent intensity, IFN = interferon, STD = standard deviation. *P < 0·05, **P < 0·01, ***P < 0·001 (Kruskal–Wallis with Dunn's multiple comparison).

In our non-linear mixed model with splines, the longitudinal level of anti-IL-17F was negatively associated with ICU admission (P = 0·03). In the GLMM analysis of KD and MIS-C separately, ICU admission was negatively associated with anti-IL22 and anti-IL-6 levels in KD patients, and with anti-IL-17A, anti-IL-17F and anti-IFN-α levels in MIS-C patients (Supplementary Table E6).

Discussion

In the current study we evaluated the presence of ACAAs (i.e., against IL-17A, IL-17F, IFN-α, IFN-γ, IL-6, IL-22, anti-IL1RA) over time, in relation to clinical and immunological responses in an extensive population of KD and MIS-C patients. We detected ACAAs against several of the cytokines in both diseases, although KD patients had more simultaneously elevated ACAAs than MIS-C patients. ACAAs transiently rose during the inflammatory state, with an earlier peak in MIS-C (≤2 days) compared to KD (>1 to ≤2 weeks).

The most frequently detected ACAA was anti-IFN-γ, being detected in ~80% of KD and MIS-C patients. ACAA against other cytokines (in order of magnitude: anti-IL-17, IFN-α, IL-6, IL-22), were detected in part of the patients as well. In contrast to the study of Pfeifer et al. that reported anti-IL-1RA in a large proportion of MIS-C patients pre-treatment (whilst not in KD),[17] we did not detect these autoantibodies, even though the same cut-off definition was adopted. Another study did not detect anti-IFN-α levels (radioligand assay) in MIS-C exceeding levels found in positive disease controls with autoimmune polyglandular syndrome type 1.[18] While we did detect anti-IFN-α in3 5/54 of KD and 4/19 of MIS-C patients prior to treatment, our methods and cut-off differ from the one used in the latter study. These challenges emphasize the importance of elucidating if the different protocols or ELISA's used might explain the observed differences between our studies.

Interestingly, the variety of ACAA positivity was more prominent in KD. Prior to treatment, anti-IL-22 was most clearly lower in MIS-C compared to KD. IL-22 is considered a protective cytokine in the gut mucosa.[21] A lower anti-IL-22 could relate to the more commonly observed gastrointestinal symptoms in MIS-C, as displayed by the 92·9% of our MIS-C patients with such symptoms, but this remains speculative. These complaints could also be related to hypoperfusion due to cardiac dysfunction. It would be interesting to investigate the presence of anti-IL-22 in KDSS, often presenting with concomitant intestinal complaints, to confirm the absence of anti-IL-22 in this disease as well.

All positively detected ACAAs were elevated during the acute and subacute stages of KD and MIS-C and declined thereafter. Patients already showed increased levels of ACAAs prior to the start of treatment, indicating that these ACAAs were not related to IVIG administration. Moreover, the KD and MIS-C patients who were never treated with IVIG also had elevated anti-IFN-γ values, further strengthening the notion that ACAAs were endogenous and not derived from the IVIG preparations. Although we cannot exclude the presence of ACAAs prior to the onset of disease, the only temporary induction of ACAAs during acute disease and decline thereafter, differs from the recent findings of pre-existing autoantibodies against type I interferons in part of adult patients with severe adult COVID-19 infections.[11–13] Conversely, our data are in line with previous data describing transiently increased anti-IFN-y in patients suffering from different viral infections.[22,23]

We measured the neutralizing capacity of the most frequently detected ACAAs, against IFN-γ, and showed it to be limited and partially neutralising. Taken together with the transient presence of ACAAs in MIS-C and KD, this finding may not be completely unexpected, as completely neutralizing ACAA are known to cause significant disease which lead to severe immunodeficiency.[24–26] Instead, we hypothesize that the transient ACAA in KD and MIS-C may reflect polyreactive or autoreactive B cells that are triggered to produce autoantibodies in the context of severe inflammation.[9,27,28] It is known that healthy individuals contain large proportions of polyreactive B cells, which may be beneficial in some cases, for instance during viral infections with low antigen exposure.[29,30] In circumstances in which these polyreactive B cells escape normal suppression, for instance during the temporary decrease in functional regulatory T cell numbers or activity that is seen both KD and MIS-C,[4,9,27,28] these B cells may enable the host to produce increased amounts of antibodies and possibly explain the temporary rise in ACAAs seen during the hyperinflammatory stage of KD and MIS-C. Alternatively, our findings could be similar to an autoimmune phenomenon that has been described in COVID-19 by groups that have demonstrated activation of extrafollicular B cells that form new autoreactive antibodies.[31,32]

Interestingly, lower pre-IVIG levels of anti-IL-17A and anti-IL-22 were seen in MIS-C patients requiring ICU admission. Likewise, ACAAs have been associated with a better prognosis or disease course in several autoimmune diseases, for example in rheumatoid arthritis and SLE (e.g., less severe joint erosions[33,34]; clinical quiescent disease[35–37]), suggesting ACAAs may have a mitigating role under some circumstances. Previous data describing anti-IFN-y in patients suffering from different viral diseases, showed that the antibodies have no inhibitory effect on the antiviral activity of IFN-y, but interfere with the immunomodulating activities of IFN-y.[23] This would also be in support of the use of neutralizing biologics against some of the major cytokines like IL-1, IL-6, or IFN-γ in hyperinflammatory conditions (e.g., Anakinra and Tocilizumab in MIS-C[8,38] and KD[39,40]; or Emapulumab [anti-IFN-γ] in pediatric Hemophagocytic Lymphohistiocytosis[41]). In that context, the clear involvement of the IFN-γ pathway in both MIS-C and KD may hold promise for future therapeutic strategies when standard therapies fail. In line with this, it would be interesting to further characterize hyperinflammatory phenotypes in KD (-like) hyperinflammatory disorders using inflammatory proteins and ACAAs to select the best suited neutralizing biologics.

It has previously been suggested that various inflammatory proteins may be used to differentiate between KD and MIS-C. In that context, the observed hyperinflammatory state seen in KD and MIS-C in our study is in line with previous studies involving cytokines related to the Th1 (e.g., IL-18,[6,10,42,43] IFN-γ,[6,43] CXLCL10,[6,10,43]) and Th17 (e.g., IL-6,[3,4,10,42,43] IL-17,[3,4,43–45]) cytokine cascades. MIS-C and KD immune profiles largely overlapped, although increased concentrations of proteins involved in the IFN-γ immune cascade differentiated MIS-C from KD. Similarly, Esteve-Sole et al., observed an overlap in immune cascades between MIS-C and KD patients, but identified a subgroup of MIS-C patients with more severe inflammation and multisystem involvement with markedly increased levels of IL-18, IFN-γ, and CXCL10.[6]

These data argue that qualitative differences can be detected in MIS-C and KD, with more marked involvement of the IFN-γ pathway in MIS-C than in KD that could potentially be used to differentiate between the two disorders. However, it should be noted that the incidence of MIS-C has diminished concurrent with the rise in (paediatric) SARS-CoV-2 infection and vaccination status and the currently ruling SARS-CoV-2 variant Omicron.[46] Rather than using markers like CXCL10 or ACAAs as differentiating markers, it would be interesting to use them to characterise different phenotypes driven by specific immune cascades (e.g., IFN-γ, IL-1, IL-17) to enable targeted therapy and follow-up.

Early recognition of children at risk of coronary artery aneurysms in KD and circulatory dysfunction in MIS-C is clinically challenging, while early intensification of treatment might be key to prevent giant aneurysms and cardiac sequelae. As of yet there are no clear biomarkers that can guide decision-making. In our study angiopoietin-2, which is an endothelial marker,[47] acted as a promising predictive marker for the subsequent development of coronary artery aneurysms in KD.[48] ST2 did not have a predictive value, but did act as a marker for disease severity in KD and MIS-C, and correlated with ICU admission ($P = 0{\cdot}03$), nearly always due to circulatory failure. We hypothesize that - although ST2 can be expressed by T cells - in MIS-C soluble ST2 is released mostly by the vasculature,[49,50] as suggested by the increased levels in KD patients as well.[51,52] Both proteins hold promise as future predictive biomarkers for cardiac manifestations in these two diseases.

The findings of our study should be reviewed in light of several limitations. We were unable to determine the functional neutralizing capacity of the other ACAAs, although they were less frequently detected. Since blood of the subjects was drawn at different time points (blood draws were always combined with routine blood draws), we were not able to obtain pre-treatment samples of all participants. A major strength of the study was the large patient population and extensive investigation of immunological levels in both KD and MIS-C patients, including the long-term follow-up and the wide range of ACAAs investigated. However, the mechanisms underlying the presence of the ACAAs remain speculative. More studies providing insight in the cellular source of these ACAAs would elucidate the role, persistence and conditions by which these ACAAs are generated. For instance, we did not include a description of the B-/T cell subset frequencies and phenotypes, which may further increase the interpretation of our current findings. We advocate further research into this, for instance with flow cytometry or mass cytometry (i.e., CyTOF). It would also be intriguing to further investigate if the recently reported increase of a polyclonal Vβ21.3-positive subset of T cells in MIS-C patients, absent in KD,[53] may somehow be correlated with the observed ACAAs or CXCL10 levels in our study. In light of a broader epiphenomenon, the presence and role of ACAAs should be investigated in other inflammatory and infectious conditions as well.

In conclusion, our data demonstrate immune dysregulation in both KD and MIS-C with overlapping features and marked hyperinflammation. Angiopoietin-2 and ST2 are associated with cardiac outcomes and we report the temporary presence of ACAAs in both diseases. Although the clinical implications need further investigation, our data suggests they are not present prior to disease onset, but may be generated in context of the dysregulated immune response.

Contributors

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Data sharing statement

The dataset presented will be made available upon request by contacting the corresponding author (s.a.netea@amsterdamumc.nl).

Declaration of interests

BK received a grant from Stichting CJ Vaillant and Innovation Impulse from the Amsterdam UMC. ET is an employee of Hycult Biotech. FLV received a HDM-FUN Horizon 2020 grant. The other authors have declared that no conflict of interest exists.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2023.104736.

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