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Plasma Protein Biomarkers Distinguish Multisystem Inflammatory Syndrome in Children From Other Pediatric Infectious and Inflammatory Diseases

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Background: Multisystem inflammatory syndrome in children (MIS-C) is a rare but serious hyperinflammatory complication following infection with severe acute respiratory syndrome coronavirus 2. The mechanisms underpinning the pathophysiology of MIS-C are poorly understood. Moreover, clinically distinguishing MIS-C from other childhood infectious and inflammatory conditions, such as Kawasaki disease or severe bacterial and viral infections, is challenging due to overlapping clinical and laboratory features. We aimed to determine a set of plasma protein biomarkers that could discriminate MIS-C from those other diseases.

Methods: Seven candidate protein biomarkers for MIS-C were selected based on literature and from whole blood RNA sequencing data from patients with MIS-C and other diseases. Plasma concentrations of ARG1, CCL20, CD163, CORIN, CXCL9, PCSK9 and ADAMTS2 were quantified in MIS-C (n = 22), Kawasaki disease (n = 23), definite bacterial (n = 28) and viral (n = 27) disease and healthy controls (n = 8). Logistic regression models were used to determine the discriminatory ability of individual proteins and protein combinations to identify MIS-C and association with severity of illness.

Results: Plasma levels of CD163, CXCL9 and PCSK9 were significantly elevated in MIS-C with a combined area under the receiver operating characteristic curve of 85.7% (95% confidence interval: 76.6%–94.8%) for discriminating MIS-C from other childhood diseases. Lower ARG1 and CORIN plasma levels were significantly associated with severe MIS-C cases requiring inotropes, pediatric intensive care unit admission or with shock.

Conclusion: Our findings demonstrate the feasibility of a host protein biomarker signature for MIS-C and may provide new insight into its pathophysiology.

Key Words: MIS-C, SARS-CoV-2, Kawasaki, pediatric, biomarker

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Although children have been generally less severely affected than adults by COVID-19 infection, a small proportion of children develop a rare but serious hyperinflammatory condition termed multisystem inflammatory syndrome in children (MIS-C) or pediatric inflammatory multisystem syndrome temporally associated with COVID-19.[1] MIS-C usually develops 2–6 weeks following severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, with children presenting with febrile illness and a multisystem hyperinflammatory state that shows some overlapping clinical characteristics with other infectious and inflammatory childhood disorders including Kawasaki disease (KD) and severe bacterial infections such as toxic shock syndrome (TSS).[2–4]

MIS-C leads to critical illness in ~70% of affected children and, as of October 2023, the Centers for Disease Control and Prevention have reported 9577 cases meeting the full clinical definitions of this novel disorder.[5] While there was no centralized database for reporting MIS-C cases in Europe, 216 cases meeting the full clinical criteria were reported between January and July 2020 in the United Kingdom and Ireland.[3] MIS-C has been reported to have a higher incidence in older children, males and in children of Black, Asian or Hispanic ethnicity.[6–8] Common symptoms of MIS-C include persistent fever, oral mucosal inflammation, conjunctivitis, skin rash, elevated inflammatory markers, gastrointestinal (GI) involvement, cardiac manifestations, multisystem organ dysfunction and shock.[9]

KD is an acute systemic vascular disease of unknown etiology affecting predominately the coronary arteries in infants and children.[10,11] The standard treatment for KD patients is high-dose intravenous immunoglobulin (IVIG), which can quickly alleviate symptoms and reduce the incidence of coronary artery aneurysms.[12] As there is currently no diagnostic test for KD, the diagnosis heavily relies on clinical signs and symptoms, which include high levels of markers of inflammation and mucosal changes. Approximately 40% of patients with MIS-C will meet the diagnostic criteria for KD and, due to the overlapping clinical and laboratory features, IVIG was recommended as a first-line treatment for MIS-C.[5,13] However, recent evidence has shown that treatment of MIS-C with corticosteroids has the same outcome as IVIG treatment, which is important because corticosteroids are cheap and readily available in low- and middle-income countries.[14,15] A diagnostic test to distinguish between these 2 diseases would, therefore, greatly benefit treatment and patient outcome.

Host-derived protein biomarkers have been successfully used to distinguish bacterial from viral infection in children.[16] It has been reported that elevated levels of soluble biomarkers associated with inflammation, vascular endothelial injury, mucosal immune dysregulation, septic shock and cardiac and GI involvement have been observed in MIS-C.[2,6–8,17–20] From a proteomic perspective, it has also been suggested that there are clinical similarities between MIS-C and secondary causes of hemophagocytic lymphohistiocytosis such as macrophage activation syndrome (MAS) and thrombotic microangiopathy, in addition to its clinical similarities with KD and TSS.[19] These studies have singled out potential individual biomarkers of MIS-C, such as cysteine-cysteine motif chemokine ligand 20 (CCL20), C-X-C motif chemokine ligands 9 and 10 (CXCL9, CXCL10), interferon-gamma (IFN-γ), interleukins 6, 7, 8 and 10 (IL-6, IL-7, IL-8 and IL-10), phospholipase A2 group IIA, tumor necrosis factor alpha (TNF-α), nuclear factor kappa B and zonulin. However, the results differ between studies, which may be due to the different comparator groups that were used.

In this study, we aimed to identify a set of proteins that could distinguish MIS-C from other disease groups, including KD, definite bacterial infections (DB) and definite viral infections (DV) by quantifying plasma protein levels of candidate MIS-C biomarkers. Such a protein signature could be used as the basis for a diagnostic test. We also aimed to associate significant changes in individual protein levels with clinical presentation, such association could be further investigated to predict disease severity or inform clinical management.

MATERIALS AND METHODS

Ethics Statement

Informed consent was taken from all patients recruited to the study. Recruitment at all locations took place after ethical permissions were in place for that area. In the DIAMONDS study (Diagnosis and Management of Febrile Illness using RNA Personalized Molecular Signature Diagnosis, https://www.diamonds2020.eu) and the PERFORM study (Personalized Risk assessment in Febrile illness to Optimize Real-life Management, https://www.diamonds2020.eu/our-research-history/perform/), the consortium agreed on a finalized protocol and supporting documents that were translated into local languages. Each participating country took responsibility for gaining ethical approval in their region. For DIAMONDS, the lead site received ethical approval for United Kingdom centers from the London-Dulwich research ethics committee (20/HRA/1714). For the PERFORM study, the lead site received ethical approval for United Kingdom centers from the London-Central research ethics committee (16/LO/1684). The Genetic Determinants of Kawasaki Disease for Susceptibility and Outcome study recruited in the United Kingdom only, and ethical approval was granted by the London-Fulham research ethics committee (13/LO/0026). During the conduct of the study, clinical data and samples were identified only by anonymized study numbers.

Study Population

This study included 108 pediatric patients (≤16 years old) with clinical and laboratory-confirmed MIS-C (n = 22), KD (n = 23), DB (n = 28), DV (n = 27) and healthy controls (HC, n = 8). A breakdown of the DB and DV can be found in Figure, Supplemental Digital Content 1, http://links.lww.com/INF/F417. The World Health Organization (WHO) defines MIS-C as children 0–19 years of age with fever ≥3 days and elevated inflammatory markers [including C-reactive protein (CRP), procalcitonin and erythrocyte sedimentation rate] who have no other obvious microbial cause of inflammation, including bacterial sepsis, staphylococcal or streptococcal shock syndromes and evidence of SARS-CoV-2 infection or probable exposure.[21] They must also exhibit 2 of the most common features of MIS-C including GI symptoms, rash or bilateral conjunctivitis, hypotension, shock or cardiac complications. All MIS-C patients met the WHO criteria, which was the recommended criteria at the time. Plasma samples from all of the other disease groups (KD, DB, DV) were collected before the COVID-19 pandemic (October 2013–January 2019). Plasma samples from KD patients were collected before receiving IVIG treatment in 21 of 23 cases used in this study. All DB and DV patients were phenotyped according to our published algorithm and as previously described.[22–24] In brief, the DB group included patients in whom an appropriate bacterial pathogen was isolated from a normally sterile site and a diagnosis of DV was conditional upon identification of a virus compatible with the clinical syndrome with no evidence of bacterial infection and CRP ≤60 mg/L. No deaths occurred in this study population. Samples were taken at hospital presentation or admission for all disease groups.

Protein Selection

Proteins were selected either through candidate gene biomarkers identified by RNA sequencing (RNA-Seq) or through existing literature at the time of this study design.

Whole blood transcriptome profiling was performed using RNA-Seq on samples obtained from patients with MIS-C, KD, DB, DV and HC. Following preprocessing and normalization, which is described in detail in Jackson et al,[25] differential expression analysis was performed to identify the genes that were significantly differentially expressed (SDE) between MIS-C and KD, DB and DV. Differential expression analysis was performed using DESeq2 with models including age, sex at birth and RNA-Seq batch, as 2 experimental runs were used for sequencing.[26] The following comparisons were made: MIS-C versus KD + DB + DV; MIS-C versus KD; MIS-C versus DB; MIS-C versus DV. *P* values were adjusted for multiple testing using the Benjamini-Hochberg (BH) adjustment and genes with adjusted *P* values <0.05 were considered SDE.[27]

Genes of interest were selected based on the criteria that they were significantly upregulated in MIS-C cases as compared to one or more of the other disease groups. Due to the high number of SDE genes, we selected genes with BH-adjusted *P* values $<10^{-5}$ and a log-fold change >2 and those that encoded for extracellularly secreted proteins for which antibodies were commercially available were considered. The final proteins chosen based on the RNA-Seq data were ADAMTS2, ARG1, CD163, CORIN and PCSK9.

Two additional protein targets, CCL20 and CXCL9, were selected based on existing MIS-C literature at the time of this study because of their reported roles in the GI involvement and hyperinflammatory state observed early in the course of MIS-C disease, respectively.[6,17,28]

Enzyme-linked Immunosorbent Assay (ELISA)

Plasma concentrations of ADAMTS2 were quantified using a commercial enzyme-linked immunosorbent assay kit, human ADAMTS2 (Abbexa, abx519366, Cambridge) according to the manufacturer's protocol. Samples were randomized, diluted 1:10 in sample diluent buffer and run in duplicate. Absorbance was measured at 450 nm on a SpectraMax microplate reader (Molecular Devices) and a standard curve was created using the SoftMax Pro software (version 5.0, San Jose, CA). Samples that fell below the lower limit of quantification were reanalyzed and values that were still below the lower limit of quantification were extrapolated as half of the lowest standard value.

Multiplex Immunoassay

Plasma concentrations of ARG1, CCL20, CD163, CORIN, CXCL9 and PCSK9 were quantified using customized multiplex immunoassay kits from MSD (Meso Scale Discovery, Rockville, MD). CD163 (MSD, FCorreccted21J4) and PCSK9 (MSD, F21ABA) were multiplexed on a 2-assay plate (MSD, K15227N) according to the manufacturer's instructions with samples diluted 1:20 in assay diluent and run in duplicate. ARG1 (MSD, F21Q1), CCL20 (MSD, B21UZ), CORIN (MSD, F210B) and CXCL9 (MSD, F210I) were multiplexed on a 4-assay plate (MSD, K15229N) according to the manufacturer's instructions with samples diluted 1:2 in assay diluent and run in duplicate. Two control plasma samples were included on each plate to account for interplate variation. All plates were analyzed on the Meso Quick Plex SQ120 Instrument (MSD). Data were generated on the Methodological Mind software (version 1.0.36) and analyzed using Discovery Workbench software (version 4.0, MSD).

Statistical Analysis

All statistical analyses were performed using R (version 4.1.1).[29] Individual protein results were analyzed using the nonparametric Kruskal-Wallis test, followed by the Mann-Whitney *U* test to evaluate the differences between each cohort. Principal component (PC) analysis was used to visualize the data. Kruskal-Wallis *P* values were corrected using the Bonferroni procedure, as were Mann-Whitney *U P* values. Associations between each of the protein markers and clinical variables were tested, using the Mann-Whitney *U* test for binary categorical variables [pediatric intensive care unit (PICU) admission: yes or no; oxygen requirement: yes or no; noninvasive ventilation requirement: yes or no; inotrope requirement: yes or no; cardiac involvement: yes or no; GI involvement: yes or no; rash: yes or no; conjunctivitis: yes or no] and linear regression models for continuous variables [body mass index, days of symptoms when sample was taken, CRP levels, white blood cell (WBC) levels, neutrophil levels, lymphocyte levels, monocyte levels and platelet levels]. All *P* values were corrected for multiple testing using the Bonferroni procedure (corrected per clinical/laboratory variable). Clinical and laboratory variables included admission to the PICU, oxygen or ventilation requirement (invasive or noninvasive), inotrope administration, duration of symptoms at the time samples were taken, neutrophils, lymphocytes, platelets, CRP, WBC counts and presentation of symptoms including cardiac and GI involvement, rash, shock and conjunctivitis.

The distinguishing ability of each of the proteins that were significantly differentially abundant between MIS-C and other disease groups was evaluated using the area under the receiver operating characteristic curve (AUC). The performance of the proteins when combined into a multivariate model was also evaluated, with the proteins combined using the simple disease risk score first described in Herberg et al,[22] and calculated as follows:

$$\text{simple} \quad \text{DRS}_i = \sum_{k=1}^{n} \text{value}_k^i - \sum_{l=1}^{m} \text{value}_l^i$$

where *n* and *m* are the proteins that increase and decrease, respectively, in MIS-C versus other disease groups. CRP was added to the protein model to determine whether its inclusion would improve model performance.

RESULTS

Clinical Features

One hundred eight samples were included in this study, with the patient demographic and clinical characteristics summarized in Table 1. All 22 MIS-C patients met the WHO case definitions for inclusion.[21] A higher proportion of cases were male (68.2%, n = 15) and the median age of the patients was higher in MIS-C than in the other disease comparator groups. Over half of MIS-C cases (n = 12, 54.5%) required PICU admission and many cases (n = 18, 81.8%) presented with GI symptoms.

Candidate Protein Biomarkers

Seven protein biomarkers for distinguishing MIS-C from other pediatric infectious and inflammatory diseases were selected through unbiased analyses of host transcriptomic profiles obtained from children with MIS-C[25] as well as from the literature. These included ADAMTS2, ARG1, CD163, CORIN and PCSK9, which were selected from RNA-Seq, and CCL20 and CXCL9, which were selected from the literature (Table, Supplemental Digital Content 2, http://links.lww.com/INF/F418). Genes were selected based on BH-adjusted *P* values $<10^{-5}$, a log-fold change >2 and if they encoded for extracellularly secreted proteins.

All proteins were measured in a cohort of children with MIS-C, KD, DB or DV and HC. When visualized using PC analysis, the disease group was clearly captured by PC 1, with a correlation of 47% (*P* value: 3.3×10^{-7}) between PC1 and whether a patient had MIS-C. Sex and age had a correlation of −1.8% and 7.9%, respectively, with PC1 (Figure, Supplemental Digital Content 3, http://links.lww.com/INF/F419).

CD163, CXCL9 and PCSK9 Are Significantly Differentially Abundant in MIS-C Versus Other Diseases and Healthy Controls

Pediatric plasma samples were analyzed from MIS-C (n = 22), KD (n = 23), DB (n = 28), DV (n = 27) and HC (n = 8). The protein concentrations were compared between MIS-C and other diseases (KD, DB, ad DV combined) and HC to evaluate how well the proteins would be able to discriminate MIS-C from non-MIS-C groups (eg, MIS-C vs. KD, DB, DV, HC). The protein concentrations were then compared between MIS-C against each diagnostic group individually.

When protein abundance levels were compared between MIS-C and all other comparator groups combined (ie, HC, KD, DB, DV), significant differences were observed for PCSK9 (Bonferroni-adjusted *P* value: 2.1×10^{-3}; Fig. 1A), CD163 (Bonferroni-adjusted *P* value: 8.0×10^{-6}; Fig. 1B), CXCL9 (Bonferroni-adjusted *P* value: 1.3×10^{-5}; Fig. 1C) and CORIN (Bonferroni-adjusted *P* value: 4.7×10^{-2}). For CD163, CXCL9 and PCSK9, levels were elevated in MIS-C versus all other comparator groups, with significant *P* values when pairwise comparisons were performed using the Mann-Whitney *U* test, for all comparisons except for MIS-C versus DB for PCSK9 (Table, Supplemental Digital Content 4, http://links.lww.com/INF/F420). When the correlations between symptom duration and protein levels of CD163, CXCL9 and PCSK9 were tested in MIS-C patients, none of the 3 proteins were significantly correlated.

For CORIN, we observed a significant Bonferroni-adjusted *P* value of 0.02 for MIS-C versus DV; however, none of the other pairwise comparisons between MIS-C and the other phenotype groups were significantly different. Although CORIN was significant using the Kruskal-Wallis test, we chose to exclude this protein from our final signature as this difference was only present for MIS-C versus DV. ADAMTS2, ARG1 and CCL20 showed no significant difference (adjusted *P* value >0.05) between MIS-C and both HC and other diseases, nor between MIS-C and each disease group individually.

A 3-protein Signature Can Distinguish MIS-C From Other Pediatric Infectious and Inflammatory Diseases

The performance of PCSK9, CD163 and CXCL9 when combined into a 3-protein signature was evaluated, returning an overall AUC of 85.7% [95% confidence interval (CI): 76.6%–94.8%] for distinguishing between MIS-C and DB, DV and KD (Fig. 2A). When broken down into pairwise comparisons, the 3-protein signature showed the best performance for MIS-C versus DB with an AUC of 88.0% (95% CI: 78.1%–97.9%; Fig. 2B), followed by MIS-C versus DV with an AUC of 85.3% (95% CI: 74.6%–95.9%; Fig. 2C) and then MIS-C versus KD with an AUC of 83.2% (95% CI: 69.9%–96.5%; Fig. 2D).

The performance of smaller combinations of proteins was also evaluated to determine whether all 3 markers (PCSK9, CD163 and CXCL9) were required in the model (Table, Supplemental Digital Content 5, http://links.lww.com/INF/F421). The combination of all 3 proteins was optimal for distinguishing MIS-C from all groups combined (DB, DV, KD), and also for MIS-C versus DB. For MIS-C versus DV and MIS-C versus KD, CD163 + PCSK9 demonstrated the best performance. Despite this improved performance for these comparisons, the 3-protein signature was taken forward for subsequent analyses given the importance in distinguishing MIS-C from severe bacterial infections and the implications for treatment administration.

CRP is a widely measured biomarker, often used for identifying bacterial infections.[30] Given the widespread availability of CRP measurement, we evaluated whether adding CRP to the 3-protein model improved its diagnostic performance (Table, Supplemental Digital Content 5, http://links.lww.com/INF/F421). The 3-protein signature + CRP improved the performance for MIS-C versus KD by 9%; however, a 16% reduction in performance was observed for MIS-C versus DB. CRP could not be accurately assessed for the classification of MIS-C versus DV, as CRP was used in the initial clinical assignment of DV patients (DV patients were required to have CRP <60 mg/L).

Lower ARG1 and CORIN Plasma Levels Are Associated With Severity in MIS-C

Associations between plasma protein concentrations and clinical variables were tested (Table 2). Lower levels of ARG1 were observed in MIS-C patients admitted to PICU (Fig. 3A; Table 2; Bonferroni-adjusted *P* value: 0.014) and requiring inotrope administration (Fig. 3B; Table 2; Bonferroni-adjusted *P* value: 0.027), suggesting that reduced ARG1 concentration is associated with severity of MIS-C. Lower levels of CORIN were observed among patients who experienced shock (Fig. 3C; Table 2; Bonferroni-adjusted *P* value: 4.8×10^{-3}). Where data were available, associations were also tested in DB, DV and KD for ARG1 and inotrope requirement and PICU admission, and for CORIN levels and shock. No significant associations were observed. When evaluating whether the level of clinical

TABLE 1. Demographic and Clinical Characteristics of the Patients With MIS-C, DB, DV and KD used for the Enzyme-linked Immunosorbent Assay and Meso Scale Discovery Validation Assay

	MIS-C (n = 22)	DB (n = 27)	DV (n = 28)	KD (n = 23)	HC (n = 8)
Demographics					
Age	8.0 (6.0–12.2)	3.8 (2.4–8.3)	1.1 (0.4–3.4)	3.0 (1.4–4.0)	12.8 (7.3–14.8)
Sex (male, %)	15 (68%)	15 (56%)	14 (50%)	14 (61%)	6 (75%)
Race (n, %)					
White/European	10 (45%)	22 (82%)	14 (50%)	7 (30%)	8 (100%)
East/West/South Asian	6 (27%)	2 (7%)	8 (29%)	6 (26%)	0 (0%)
Black/African/Caribbean	3 (14%)	1 (4%)	4 (14%)	7 (30%)	0 (0%)
Other	3 (14%)	2 (7%)	2 (7%)	3 (13 %)	0 (0%)
Sample period	August 2020–September 2021	December 2016–January 2019	February 2017–January 2019	October 2013–June 2018	August 2018–March 2020
Treatment					
Antibiotics (n, %)	22 (100%)	18 (67%)	14 (50%)	23 (100%)	0 (0%)
Inotropes (n, %)	6 (27%)	4 (15%)	3 (11%)	0 (0%)	0 (0%)
Oxygen (n, %)	3 (14%)	9 (33%)	14 (50%)	0 (0%)	0 (0%)
Noninvasive ventilation (n, %)	2 (9%)	1 (4%)	10 (36%)	0 (0%)	0 (0%)
Invasive ventilation (n, %)	1 (5%)	7 (26%)	14 (50%)	0 (0%)	0 (0%)
Clinical features					
Days from onset of symptoms	6.5 (5.0–7.0)	3.0 (2.0–5.5)	3.0 (2.0–5.0)	6.0 (5.0–8.0)	N/A
Admitted to PICU (n, %)	12 (55%)	10 (37%)	16 (57%)	1 (4%)	0 (0%)
GI involvement (n, %)	18 (82%)	3 (11%)	2 (7%)	4 (17%)	1 (13%)
Cardiac (n, %)	3 (14%)	0	1 (4%)	9 (39%)	0 (0%)
Pulmonary (n, %)	1 (5%)	2 (7%)	5 (18%)	N/A	0 (0%)
Neurological (n, %)	3 (14%)	2 (7%)	4 (14%)	N/A	0 (0%)
Shock (n, %)	5 (23%)	5 (19%)	2 (7%)	N/A	0 (0%)
Sepsis (n, %)	0	13 (48%)	5 (18%)	N/A	0 (0%)
Comorbidities (n, %)	8 (36%)	7 (26%)	11 (39%)	13 (57%)	0 (0%)
Blood parameters					
Max CRP (mg/L)	241.0 (191.0–301.8)	225.0 (125.7–343.6)	19.2 (4.9–35.2)	89.1 (62.8–136.0)	N/A
Hemoglobin (g/L)	107.0 (95.8–118.8)	104.0 (94.3–115.8)	104.0 (91.0–123.8)	N/A	N/A
Creatinine (μmol/L)	44.5 (35.3–52.8)	44.1 (27.1–59.7)	33.0 (28.0–47.0)	N/A	N/A
ALT (IU/L)	25.5 (21.0–36.0)	11.0 (10.0–23.0)	24.0 (16.5–38.5)	N/A	N/A
Bilirubin (μmol/L)	5.0 (5.0–7.0)	8.5 (4.3–16.5)	5.0 (3.5–12.3)	N/A	N/A
Albumin (g/L)	24.5 (22.3–27.0)	32.5 (29.3–37.3)	30.5 (26.0–34.8)	N/A	N/A
White blood cells (10^9/L)	11.5 (6.0–16.3)	17.4 (13.6–27.1)	7.6 (5.7–10.8)	12.3 (10.6–14.1)	N/A
Neutrophils (10^9/L)	8.4 (5.9–12.2)	13.3 (9.7–19.4)	3.3 (1.6–4.7)	8.3 (6.9–11.2)	N/A
Lymphocytes (10^9/L)	1.3 (0.6–1.8)	2.5 (1.8–3.7)	3.1 (2.1–4.5)	2.5 (1.7–3.0)	N/A
Monocytes (10^9/L)	0.4 (0.2–0.8)	1.2 (0.9–2.3)	0.8 (0.6–1.3)	0.7 (0.5–0.9)	N/A
Platelets (10^9/L)	238.5 (113.3–321.8)	269.5 (221.0–430.0)	272.0 (230.0–321.0)	330.0 (62.8–136.0)	N/A

Values are median (IQR) unless stated.

ALT indicates alanine aminotransferase; DB, bacterial infection; DV, viral infection; IQR, interquartile range; N/A, not available/not applicable.

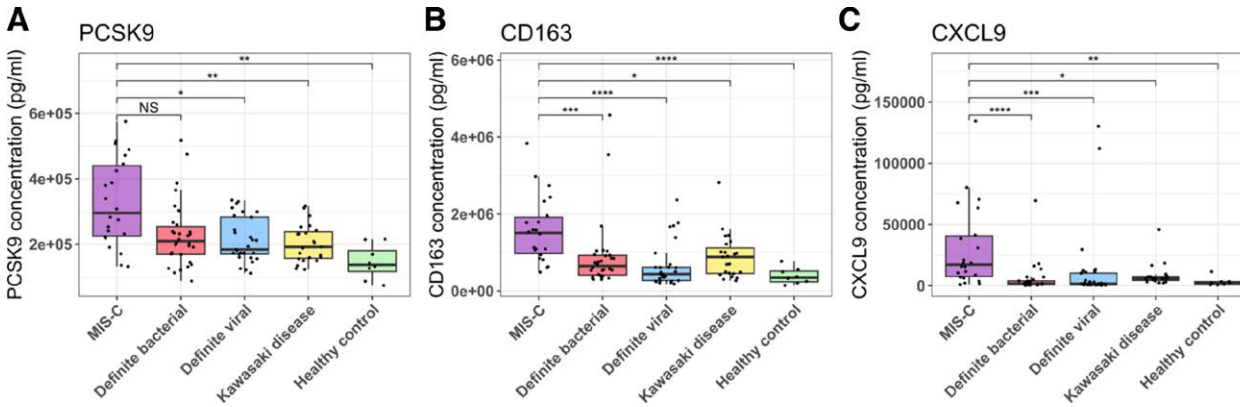

FIGURE 1. Boxplots showing concentrations of proteins with significantly different levels between MIS-C and the comparator groups (KD, DB, DV, HC). Boxplots are shown for PCSK9 (A), CD163 (B) and CXCL9 (C). ns indicates $P > 0.05$. *$P \leq 0.05$; **$P \leq 0.01$, ***$P \leq 0.001$; ****$P \leq 0.0001$. *P* values were generated using the Mann-Whitney *U* test and corrected using the Bonferroni correction.

intervention that MIS-C patients received was uniformly distributed across clinical sites, we found that there was no partiality across sites. This highlights that these associations we observed between severity and protein levels are not driven by differences in clinical interventions between sites (Table, Supplemental Digital Content 6, http://links.lww.com/INF/F422).

DISCUSSION

Weeks after SARS-CoV-2 infection, some children developed a hyperinflammatory condition termed MIS-C. The clinical presentation and laboratory findings in MIS-C are like other inflammatory and infectious diseases such as severe bacterial or viral infection and KD, which makes the appropriate diagnosis and treatment of MIS-C challenging.

Here, we evaluated 7 candidate host protein biomarkers in a cohort of 108 children with MIS-C, KD, DB, DV and HC. All MIS-C cases met the WHO case definitions and over half were admitted to PICU. These patients were compared to KD, DB and DV cases recruited before the COVID-19 pandemic. Five of the candidate biomarkers were selected from RNA-Seq and 2 from the literature because of their reported roles in the GI involvement and hyperinflammatory state observed early in the course of MIS-C disease, respectively.[6,17,28]

The results showed that 3 proteins (PCSK9, CD163 and CXCL9) were significantly higher in MIS-C cases when compared with the other comparator groups. More importantly, the performance of PCSK9, CD163 and CXCL9 when combined into a 3-protein signature could distinguish MIS-C patients from our other disease controls with an AUC of 85.7% (95% CI: 76.6%–94.8%). When we included CRP levels into the 3-protein model, we observed an increase in performance for distinguishing MIS-C from KD of 9%. Given the overlap in clinical features between MIS-C and KD, especially in incomplete KD cases, these findings could be clinically useful due to the importance of timely administration of IVIG to patients with suspected KD to prevent coronary artery aneurysm formation.[31] Similarly, we observed an increase in distinguishing MIS-C from DV when including CRP levels into the 3-protein model; however, this finding needs to be further validated in an independent cohort comprised of patients with viral infections that have been phenotyped independent of CRP, as CRP levels were used in the clinical phenotyping of the DV cases in this study (ie, <60 mg/L).

The proteins included in the 3-protein signature may have biological functions that can provide insight into the pathogenesis of MIS-C. CD163 is a transmembrane macrophage-specific hemoglobin-haptoglobin scavenger receptor that is elevated in MAS and other vasculitic conditions. The biomarker form of this protein is the soluble CD163 (sCD163) found in plasma and produced from increased sCD163 shedding mediated by TNF-α.[32] Increased abundance of sCD163 and marked elevation of macrophage activity have been recognized as markers in several inflammatory diseases.[33] Mostafa and colleagues[34] reported increased levels of sCD163 in children with SAR-CoV-2 infection and MIS-C, compared with HC, which likely reflects the exaggerated proinflammatory host response and suggests a potential therapeutic role for sCD163 antagonists. CD163 levels have also been shown to strongly correlate with the marked neutrophilia observed in MIS-C, suggesting that this is a result of macrophage activation and supporting the observation that the highest levels of this protein are found in the most severe cases of MIS-C.[20,35] CXCL9 is 1 of 3 chemokines that selectively bind to the C-X-C motif chemokine receptor 3 (CXCR3) and its expression is primarily driven by IFN-γ.[36] CXCL9 is commonly expressed by peripheral blood mononuclear cells, but more specifically by macrophages, and is best known for its role in the inflammatory response by mediating immune cell migration and activation.[37] CXCL9 and its mediator, IFN-γ, have been reported to be more abundant in MIS-C cases than in children with mild and severe COVID, confirming the hyperinflammatory state observed in MIS-C.[17,20,28] Our results support these findings and validate CD163 and CXCL9 as viable biomarkers for the diagnosis of MIS-C when compared to KD or other common bacterial and viral childhood infections.

Higher levels of PCSK9 were observed in MIS-C cases when compared to all other disease comparator groups. PCSK9 is vital in the metabolism of plasma cholesterol by regulating the levels of low-density lipoproteins (LDL) receptors that filter out the cholesterol-rich LDL particles from plasma.[38–40] Increased PCSK9 expression has been linked to lower levels of LDL receptors and consequently higher LDL levels. Cholesterol is essential for SARS-CoV-2 infection as the virus binds to cell surface angiotensin converting enzyme 2 fused to cholesterol, causing a signaling cascade and allowing the lipid-enveloped RNA to enter host cells.[41,42] In systemic inflammatory conditions, disruption of the vascular endothelium can lead to dysregulation of lipid transport and metabolism and an increase in circulating LDL and PCSK9. This explains why

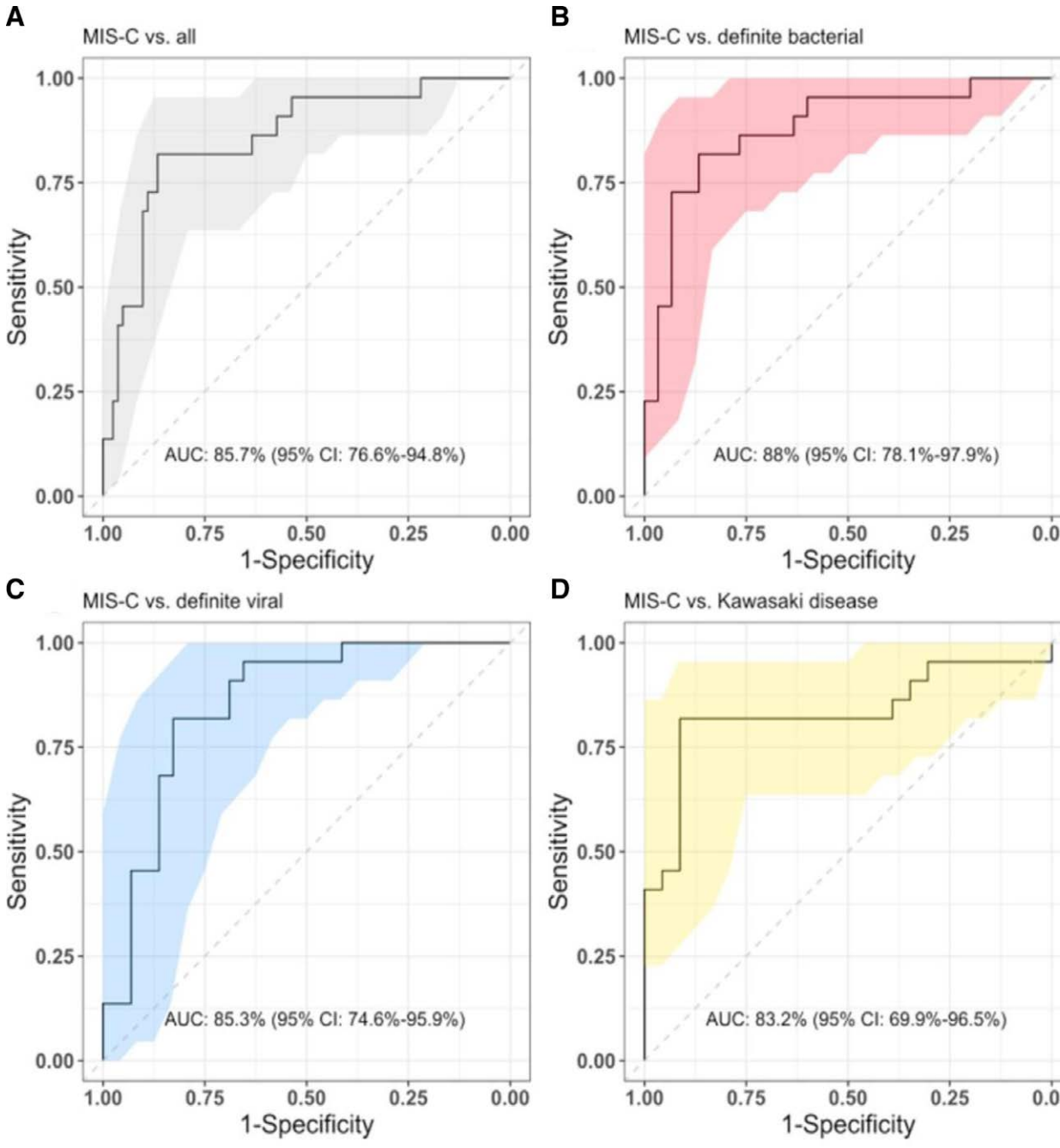

FIGURE 2. Receiver operating characteristic (ROC) curves visualizing the performance of the 3-protein signature (PCSK9, CD163 and CXCL9) for A. MIS-C vs. all; B. MIS-C vs. definite bacterial; C. MIS-C vs. definite viral; D. MIS-C vs. Kawasaki disease. Area under the ROC curve (AUC) and 95% CI are shown on the plots.

higher cholesterol levels are associated with higher susceptibility to SARS-CoV-2.[42] While LDL data was not available from the patients in this study, future studies to explore the effect of MIS-C on LDL levels could provide insight into the pathophysiology of MIS-C and further validate our findings. PCSK9 also exhibits a proinflammatory effect by promoting TNF-α expression while suppressing the anti-inflammatory markers ARG1 and IL-10.[43] The beneficial effect of using PCSK9 inhibitors in adults with severe COVID-19 has been demonstrated[44–46]; however, elevated levels of this protein in MIS-C cases have not, to our knowledge, been previously reported.

ARG1 was not found to be significantly different between our MIS-C cases and other comparator groups; however, lower levels of this protein were associated with cases of MIS-C requiring PICU admission and inotropes. ARG1 is the final enzyme involved in the urea cycle, hydrolyzing arginine to urea, and as such, is highly expressed in the liver.[47,48] It also plays an important role in the immune response where it is released extracellularly under inflammatory conditions to inhibit inflammatory damage and immunity toward intracellular pathogens by reducing T-cell proliferation and cytokine production.[49–51] ARG1 dysregulation has

TABLE 2. Bonferroni-adjusted *P* Values Showing the Association Between Clinical Variables and Each of the Proteins Measured in MIS-C Patients

Proteins	PCSK9	CD163	CXCL9	ARG1	CORIN	CCL20	ADAMTS2
PICU admission	1.000	1.000	1.000	**0.014**	0.212	1.000	1.000
Oxygen requirement	0.609	0.482	1.000	0.609	0.282	1.000	1.000
Inotrope requirement	0.096	0.389	0.638	**0.027**	1.000	1.000	1.000
Noninvasive ventilation requirement	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Cardiac involvement	0.432	0.874	1.000	1.000	1.000	1.000	1.000
GI involvement	0.705	1.000	1.000	1.000	1.000	1.000	1.000
Rash	1.000	1.000	1.000	1.000	0.136	1.000	0.764
Shock	1.000	1.000	1.000	0.908	$\mathbf{4.8\times10^{-3}}$	1.000	1.000
Conjunctivitis	0.860	1.000	1.000	1.000	1.000	0.651	1.000
Days of symptoms	1.000	1.000	1.000	1.000	1.000	1.000	1.000
CRP levels	0.678	0.437	0.437	0.678	0.437	0.229	0.698
WBC levels	1.000	1.000	0.942	1.000	1.000	1.000	1.000
Neutrophil levels	1.000	1.000	0.937	1.000	1.000	1.000	1.000
Lymphocytes	1.000	1.000	1.000	1.000	0.207	1.000	1.000
Monocytes	1.000	1.000	1.000	1.000	0.980	1.000	1.000
Platelets	1.000	1.000	1.000	1.000	0.157	1.000	1.000

Associations between protein levels and binary categorical variables including PICU admission, oxygen requirement, noninvasive ventilation requirement, inotrope requirement, cardiac involvement, GI involvement, rash and conjunctivitis were tested using the Mann-Whitney *U* test. The association between protein levels and continuous variables including body mass index, days of symptoms, CRP, WBC, neutrophil, lymphocytes, monocytes and platelet levels were tested using linear regression models. Bonferroni-adjusted *P* values <0.05 are in bold. *P* values are adjusted for each clinical/laboratory variable.

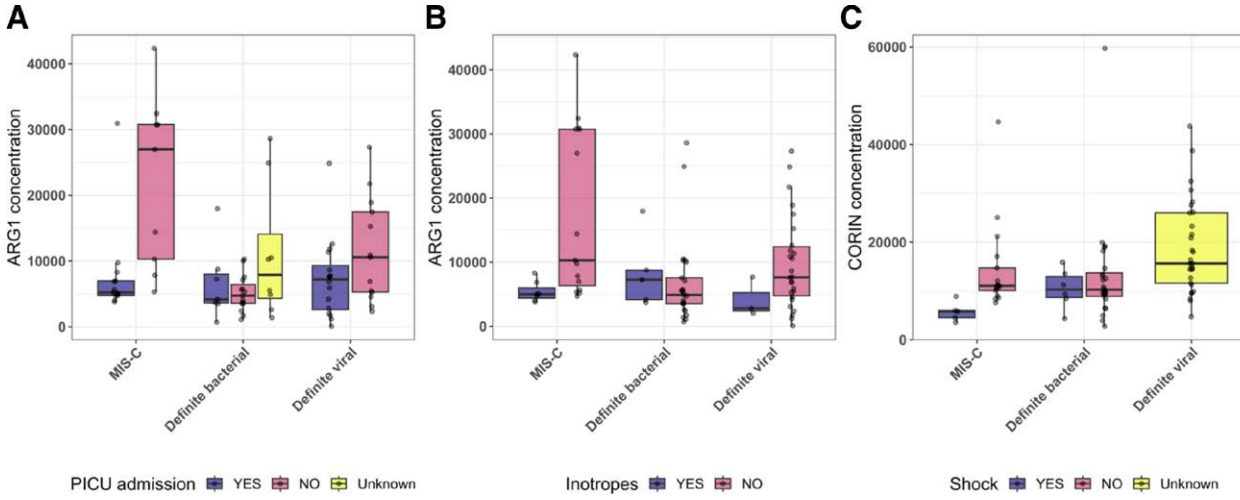

FIGURE 3. Levels of ARG1 and CORIN among patients stratified according to whether they were admitted to PICU (A), required inotropes (B) or presented with shock (C).

become synonymous with various pathological processes involving cardiovascular, immunological, neurodegenerative and tumorigenic disorders.[52] Elevated levels of ARG1 have been reported in COVID-19 cases with high viral loads[51,53] so it is possible that the high levels of PCSK9 observed in MIS-C cases are suppressing ARG1.

Lower levels of CORIN were also associated with severe MIS-C cases with shock. CORIN converts pro-atrial natriuretic peptide (pro-ANP) to biologically active ANP, a cardiac hormone that regulates blood volume and pressure by reducing plasma volume by renal excretion of salt and water, vasodilation and increased vascular permeability.[54] Higher pro-ANP levels, which could result from lower levels of CORIN, are associated with poor survival prognosis in children and adults with severe sepsis and septic shock.[55,56] The lower levels of CORIN we observed could therefore result in higher levels of pro-ANP and lower levels of ANP, which could contribute to the hypertension, inflammation and cardiac manifestations associated with severity and shock in MIS-C. Future studies to measure the ratio of pro-ANP to ANP levels in MIS-C could provide insight into the disease mechanisms and help in validating our findings. While the best performing diagnostic biomarkers (PCSK9, CD163 and CXCL9) were significantly elevated in MIS-C patients, they were not found to be associated with severity of MIS-C. This could reflect the wide spectrum of mild to severe cases in our cohort.

We did not observe significant differences in the levels of ADAMTS2 or CCL20 in the MIS-C cases when compared to our other disease group. ADAMTS2, a biomarker we selected from RNA data, is a metalloprotease that processes procollagen to provide strength and support to many body tissues. Subsequent to our screening of RNA-Seq data, 2 other groups have reported increased gene expression of ADAMTS2 in COVID-19 and MIS-C cases; however, these groups were compared to HC rather than other disease groups.[57,58] Similarly, Gruber et al[6] reported an increased abundance of CCL20 in patients with MIS-C. Our inability to replicate their results could be due to the difference in the control groups

that were used: KD, DB and DV as compared to nonintensive care unit COVID-19-infected children, young adults, adults and convalescent adults.

Although our study provides useful information on potential protein biomarkers for MIS-C, there are several limitations that should be taken into consideration. First, our sample size for MIS-C (n = 22) was restricted by the number of children that had been recruited at the time of this study that met all WHO criteria. Second, these patients were recruited during the period of April 2020–August 2021 and, due to the variants that were circulating during this period, would not include the Omicron variant that was first detected in November 2021. Further investigation of the biomarkers we present in this study is required in a larger independent cohort that includes other relevant DB and DV groups with overlapping clinical features of MIS-C including TSS, MAS and hemophagocytic lymphohistiocytosis. Furthermore, our MIS-C cases were older than our other disease comparator groups and the performance of our signature should be tested in younger children with MIS-C. A limitation of all diagnostic studies comparing MIS-C and KD is the lack of a diagnostic test for either disease; however, we included KD patients that were recruited prepandemic to avoid errors in phenotyping. None of the KD patients in this study required inotropes and were, therefore, less severe than the MIS-C, DB and DV groups. The severity associations that we observed for ARG1 and CORIN require further validation in more severe KD cases or KD shock to confirm if this finding is unique to MIS-C.

Finally, this study reports the first protein-based diagnostic signature to discriminate MIS-C from KD and other common bacterial and viral infections. Our results show that the increase in specific proteins involved in macrophage activation, endothelial dysfunction and lipid metabolism can discriminate MIS-C from other inflammatory diseases in children. We further explored the associations between plasma levels and clinical outcomes to find both ARG1 and CORIN levels are lower in severe MIS-C. Once validated, our results could form the foundation for the development of a point-of-care diagnostic test that could assist pediatricians to diagnose and determine the best course of treatment for MIS-C.

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