# Definition and validation of serum biomarkers for optimal differentiation of hyperferritinaemic cytokine storm conditions in children: a retrospective cohort study

*Christoph Kessel, Ndate Fall, Alexei Grom, Wilco de Jager, Sebastiaan Vastert, Raffaele Strippoli, Claudia Bracaglia, Erik Sundberg, AnnaCarin Horne, Stephan Ehl, Sandra Ammann, Carine Wouters, Kai Lehmberg, Fabrizio De Benedetti, Carolin Park, Claas Hinze, Helmut Wittkowski, Katharina Kessel, Karin Beutel, Dirk Foell, Dirk Holzinger*

## Summary

**Background** Cytokine storm syndromes are life-threatening complications that can occur in children with rheumatic conditions (macrophage activation syndrome [MAS]), inherited cytotoxicity defects (ie, primary haemophagocytic lymphohistiocytosis [HLH]), or as a result of infection or malignancies (ie, secondary HLH). To adequately steer treatment, an early and clear discrimination of these entities is essential. We aimed to define and validate serum biomarker profiles that can differentiate between primary HLH, secondary HLH (predominantly infection-associated), and MAS associated with systemic juvenile idiopathic arthritis (systemic JIA-MAS).

**Methods** In this multicentre, retrospective, cohort study, serum samples from patients (0–18 years) with a clinical diagnosis of primary HLH, secondary HLH, or systemic JIA-MAS were analysed by immunoassays for 55 cytokines and chemokines. Serum samples were collected from patients treated at seven clinical centres in Europe and North America. 15 serum biomarkers were validated using an independent commercial assay, and the diagnostic accuracy of the best performing biomarkers was tested in an independent validation cohort.

**Findings** Serum samples were collected between Dec 7, 2010, and Jan 26, 2018. In the discovery cohort of 43 patients (24 girls and 19 boys) multi-marker analyses revealed distinct serum biomarker profiles associated with primary or secondary HLH versus systemic JIA-MAS. Ten biomarkers were identified that were differentially elevated in either HLH or systemic JIA-MAS and distinguished between these clinical entities, six of which were tested in an independent validation cohort of 79 patients (34 girls and 45 boys). Serum concentrations of S100A12 and interleukin-18, as well as ratios of both S100A12 and IL-18 with chemokine (C-X-C motif) ligand (CXCL)9 and CXCL10 were identified as the most promising candidates for differential diagnostics.

**Interpretation** At initial presentation, when it is unclear whether a patient with excessive hyperferritinaemic inflammation has primary HLH, infection-associated secondary HLH, or MAS, high serum concentrations of S100A12 indicate an initial differential diagnosis of systemic JIA-MAS, thus helping to guide subsequent treatment decisions. We therefore suggest the inclusion of serum S100A12 and IL-18 in the diagnostic investigations for hyperferritinaemic syndromes; however, the definition and introduction of universally applicable cutoff values are still required.

**Funding** German Research Foundation, the Center for Interdisciplinary Clinical Research at University Hospital Muenster, the EU's Horizon 2020 research and innovation programme, and the Deutsche Kinderkrebsstiftung.



## Introduction

Hyperinflammatory conditions are life-threatening complications resulting from a systemic proinflammatory response triggered by infection, injury, or chronic inflammation.[1] They can occur at any age, but children are especially susceptible. Current data suggest a substantial immunophenotypic overlap among conditions characterised by hyperferritinaemia, including macrophage activation syndrome (MAS) associated with rheumatic diseases, and haemophagocytic lymphohistiocytosis (HLH).[2,3] Primary HLH results from mutations that affect the lytic function of cytotoxic T and NK cells and thus impair clearance of infections and activated T cells.[3,4] Before the introduction of aggressive chemotherapy and immunosuppressive treatment protocols (eg, the HLH-2004 protocol), including etoposide, only about 5% of patients with primary HLH survived the first year after diagnosis. Therefore, the prompt initiation of the HLH-2004 protocol is highly recommended.[5] However, most patients with primary HLH still require haematopoietic cell transplantation as a definitive treatment.[6]

Secondary HLH is triggered by infection, trauma, metabolic disease, malignancy, or autoimmunity and autoinflammation.[7] The term MAS is frequently used when secondary HLH presents in the context of rheumatic diseases,[3] including systemic juvenile idiopathic arthritis

*Lancet Rheumatol* 2021; 3: 563–73

Published Online June 8, 2021 https://doi.org/10.1016/S2665-9913(21)00115-6

See Comment page 535

**Department of Pediatric Rheumatology and Immunology, University Children's Hospital Muenster, Muenster, Germany** (C Kessel PhD, C Park, C Hinze MD, H Wittkowski MD, K Kessel PhD, Prof D Foell MD); **Division of Rheumatology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA** (N Fall MS, Prof A Grom PhD); **Center for Translational Immunology** (W de Jager PhD) **and Department of Pediatric Rheumatology and Immunology** (S Vastert PhD), **University Medical Center Utrecht, Utrecht, Netherlands; Luminex Corporation, Austin, TX, USA** (W de Jager); **Department of Molecular Medicine, Sapienza University, Rome, Italy** (R Strippoli, PhD); **Division of Rheumatology, Ospedale Pediatrico Bambino Gesù, IRCCS, Rome, Italy** (R Strippoli, C Bracaglia MD, Prof F De Benedetti PhD); **Paediatric Rheumatology Unit** (E Sundberg PhD) **and Childhood Cancer Research Unit** (Prof A Horne PhD), **Karolinska University Hospital Solna, Stockholm, Sweden; Institute for Immunodeficiency, Center for Chronic Immunodeficiency, Faculty of Medicine, University of Freiburg, Freiburg, Germany** (Prof S Ehl MD, S Ammann PhD); **Department of Microbiology and Immunology, Laboratory of Adaptive Immunology and Immunobiology** (Prof C Wouters PhD) **and Department of Pediatric Rheumatology** (Prof C Wouters),

University of Leuven, Leuven, Belgium; Department of Pediatric Hematology and Oncology, University Medical Center Hamburg Eppendorf, Hamburg, Germany (K Lehmberg MD); Children's Hospital München-Schwabing, München Klinik and Klinikum München Rechts der Isar, Technical University Munich, Munich, Germany (K Beutel MD); Department of Pediatric Hematology-Oncology, University of Duisburg-Essen, Essen, Germany (D Holzinger MD)

Correspondence to: Dr Christoph Kessel, Department of Pediatric Rheumatology and Immunology, University Children's Hospital Muenster, 48149 Muenster, Germany
christoph.kessel@uni-muenster.de

## Research in context

### Evidence before this study

Before analysing discovery cohort samples (Jan 1, 2015), we searched PubMed using the search terms "macrophage activation syndrome", "sJIA MAS", "hemophagocytic lymphohistiocytosis", "hyperferritin", and "biomarker" OR "serum marker" OR "differential diagnosis" in publication titles and abstracts. This search for articles in English was run on a regular basis. Macrophage activation syndrome (MAS) and haemophagocytic lymphohistiocytosis (HLH) are life-threatening cytokine storm conditions with substantial immunophenotypic overlap. Elevated concentrations of interleukin (IL)-18 have already been suggested to differentiate MAS from HLH based on inherited cytotoxicity defects (primary HLH).

### Added value of this study

In samples collected from children at multiple centres, using separate discovery and validation assay platforms, we identified and validated serum biomarkers differentiating MAS from both infection-associated secondary HLH and primary HLH. From an initial panel of 55 serum markers, we identified granulocytic S100A12 to best discriminate MAS from either primary or secondary HLH.

### Implications of all the available evidence

In excessive hyperferritinaemic inflammation, quantification of serum S100A12 and IL-18 can both support early differential diagnosis and guide treatment decisions.

(systemic JIA), systemic lupus erythematosus, Kawasaki disease, and juvenile dermatomyositis.[3] Therapy for patients with MAS and other forms of secondary HLH consists of suppression of hyperinflammation and treatment of the underlying disease.

Importantly, MAS shows substantial clinical and immunophenotypic overlap with other forms of secondary HLH and with primary HLH; overlapping clinical symptoms include persistent fever, organomegaly, cytopenia, hyperferritinaemia, and coagulation disorders. Because HLH and MAS arise most often in individuals with infection, autoimmune diseases, and malignancy, these underlying conditions can easily obscure the evolving cytokine storm.[2,3,8] MAS can also occur as the first symptom of as-yet undiagnosed autoimmune or autoinflammatory diseases.[9] Therefore, the identification of biomarkers that can distinguish HLH from MAS as early as possible is important to decide whether a cell-directed therapy (etoposide or alemtuzumab) and a rapid donor search is required (ie, for primary HLH), or whether steroids, cyclosporin, or cytokine-directed therapies are sufficient to control disease (ie, for secondary HLH or MAS).

Studies from the past 5 years suggest that elevated serum concentrations of IFNγ and chemokine (C-X-C motif) ligand (CXCL)9[10] and increased activity of adenosine deaminase 2 (ADA2)[11] are biomarkers of MAS and that elevated serum titres of interleukin (IL)-18 (formerly known as IFNγ-inducing factor) reflect MAS disease activity and development[12] and discriminate MAS from primary HLH.[13] Further, high concentrations of IL-18 and an increased ratio of soluble tumour necrosis factor (TNF) receptor II to soluble TNF receptor I have been suggested to distinguish MAS from Epstein-Barr virus infection-associated secondary HLH.[14]

In contrast to previous reports that focused on a restricted panel of biomarkers,[13,14] in the present study, we analysed a broad spectrum of serum biomarkers in patients with primary and infection-associated secondary HLH compared with systemic JIA-associated MAS (systemic JIA-MAS), inactive and active systemic JIA, and healthy controls, with the aim to identify a set of biomarkers that can clearly distinguish primary and secondary HLH from systemic JIA-MAS. For discovery analyses, we built a custom multiplex bead array assay based on published MAS and HLH biomarker data, and we quantified S100A12 by ELISA. For validation, we first assessed the discriminatory performance of biomarkers selected from discovery analyses using a commercially available bead array platform; we then used this commercial assay, along with S100A12 measurements, to test candidate biomarkers in an independent validation cohort.

## Methods

### Study design and participants

In this multicentre, retrospective, cohort study, serum samples of patients (aged 0–18 years) with a clinical diagnosis of primary HLH (n=28), secondary HLH (n=44), or systemic JIA-MAS (n=40) based on established clinical criteria[5,15] were collected from patients treated at seven different centres in Europe and North America (University Hospital Freiburg, Freiburg, Germany; University Medical Center Utrecht, Utrecht, Netherlands; Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA; Istituto di Ricovero e Cura a Carattere Scientifico Ospedale Pediatrico Bambino Gesù, Rome, Italy; Karolinska Institute, Stockholm, Sweden; University Children's Hospital Muenster, Muenster, Germany; and University Hospital Leuven, Leuven, Belgium). Samples from patients with primary HLH, secondary HLH, or systemic JIA-MAS were split into a discovery cohort (primary HLH [n=10]; secondary HLH [n=12], systemic JIA-MAS [n=11]) and a validation cohort (primary HLH [n=18]; secondary HLH [n=32]; systemic JIA-MAS [n=29]). The discovery cohort also included ten healthy controls (appendix p 7). Details on the number of samples collected in the various patient groups from different centres are detailed in the appendix (p 1).

Disease activity in patients with HLH was defined according to the HLH-2004 diagnostic guidelines.[5] In

See Online for appendix

this study, the primary HLH cohort included patients with genetically proven defects in cytotoxicity (*FHL2* [n=12]; *FHL3* [n=5], *FHL4* [n=1]; *FHL5* [n=8]), and Griscelli syndrome (n=2), and all samples were collected during active disease and before patients commenced treatment with specific cell-directed or cytokine-directed therapies (some patients had received steroids or intravenous immunoglobulin). The secondary HLH cohort included 44 patients with infections (Epstein-Barr virus [n=15]; cytomegalovirus [n=5]; or other [n=17; appendix p 4), metabolic syndrome (n=1), or unknown cause (n=6). In the 38 patients with secondary HLH enrolled at University Hospital Freiburg, primary HLH was excluded according to a previously reported algorithm.[16]

MAS was defined according to the 2016 ACR/EULAR criteria,[15] and only patients with MAS secondary to systemic JIA were included in this study. Inactive disease in systemic JIA was defined as the absence of fever, rash, and arthritis, and a C-reactive protein concentration or ESR in the normal range.[17]

All parents or care givers of the study participants provided written informed consent. The study was approved by the respective local ethics committees (Cincinnati [P01 and P60]; Freiburg [610/15]; Leuven [S58814]; Muenster [2015-670-f-S]; Rome [494_11]; Stockholm [Dnr 2009/1139-31/4 and Dnr 2010/165-31/2]; and Utrecht [11-499]).

### Procedures

Discovery cohort samples were tested on a custom-made multiplexed immunoassay for quantification of 54 serum analytes, and concentrations of S100A12 were measured separately by in-house ELISA. We also analysed serum samples from separate patients with active (n=11) and inactive (n=11) systemic JIA-MAS, and from patients with active (n=10) and inactive (n=11) systemic JIA compared with healthy controls, as a means of internal assay validation. All biomarkers quantified in discovery cohort samples were individually analysed for their ability to distinguish between systemic JIA-MAS and primary HLH or secondary HLH, or both using multiple comparison statistics.

Biomarkers that were able to distinguish between these clinical entities were re-tested using a commercially available multiplex bead array assay (ProcartaPlex multiplex immunoassay platform, Thermo Fisher Scientific, Waltham, MA, USA), and correlation between data generated from the custom multiplex assay and the commercially available multiplex assay was assessed. Biomarkers with a between-assay Spearman's correlation coefficient of $r>0·7$ were selected for testing in the validation cohort (along with S100A12), with cutoff values determined according to the Youden index. Validation cohort samples were analysed in a blinded manner using the commercial multiplex assay and S100A12 ELISA, and samples were designated as either HLH or systemic JIA-MAS based on the previously determined cutoff values. Following unblinding, the performance of biomarkers in distinguishing systemic JIA-MAS from primary HLH, or secondary HLH, or both, was evaluated by receiver operating characteristic analyses. The study complied with the Standards for Reporting of Diagnostic Accuracy guidelines (appendix pp 2–3).[18]

Detailed methods for serum biomarker quantification and sample distribution over discovery and validation cohorts are detailed in the appendix (p1).

### Statistical analysis

Serum biomarker data were analysed for unsupervised clustering using correlation distance and ward.D2 linkage by the pheatmap R package and RStudio, 2015. Principle component analyses were done using the ggfortify and autoplot R packages and RStudio. Multiple Spearman's rank correlation analyses of serum analytes were done and plotted using the corrplot R package and RStudio or Graphpad Prism software, version 8.0.

For **RStudio** see http://www.rstudio.com/

Data for individual serum markers were analysed for normality distribution by D'Agostino and Pearson normality test using Graphpad Prism software. Most data did not pass this test and were therefore subjected to non-parametric multiple comparison analyses by Kruskal Wallis followed by Dunn's multiple comparison test (Graphpad Prism). Receiver operating characteristic curve analyses were also done using Graphpad Prism software. $p<0·05$ was considered to indicate a statistically significant difference.

### Role of the funding source

The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

## Results

Serum samples analysed in the study were collected between Dec 7, 2010, and Jan 26, 2018 (table 1). In the discovery phase, we analysed serum samples of 43 children (24 girls and 19 boys), including patients with active primary HLH (n=10), secondary HLH (n=12), systemic JIA-MAS (n=11), and healthy controls (n=10). Biomarkers were analysed using a custom-made multiplexed bead array assay comprising 54 analytes, including those with a general role in inflammation (eg, IL-6 or TNF), those previously identified in MAS or HLH (eg, soluble IL-2R, CXCL10, IL-18, or soluble Fas ligand [FASL]), and those anecdotally reported in case studies or murine models. S100A12 serum concentrations were established separately using an in-house ELISA.

Among discovery cohort samples, unsupervised hierarchical clustering of biomarker expression profiles identified three distinct clusters (figure 1A). Biomarkers selectively increased in serum from patients with secondary HLH and primary HLH constituted cluster 1. Biomarkers with elevated concentrations in both healthy

controls and systemic JIA-MAS were assigned to cluster 2; this cluster included several biomarkers that were not differentially expressed between patients with systemic JIA-MAS and those with HLH. Cluster 3 comprised markers selectively elevated in systemic JIA-MAS or healthy controls (figure 1A). Corresponding principal component analyses separated samples from patients with HLH from those with systemic JIA-MAS, despite some overlap with healthy controls (figure 1B). When analysing all markers quantified in discovery cohort samples individually for their ability to discriminate between HLH and systemic JIA-MAS using multiple-comparison statistics, we identified a set of serum proteins that were selectively elevated in secondary HLH, primary HLH, or systemic JIA-MAS (figure 2A, appendix pp 5–6, 8). A subset of serum proteins was selectively elevated in patients with HLH compared with systemic JIA-MAS (ie, monocyte chemotactic protein [MCP]-2 [also known as CCL8], macrophage colony stimulating factor [MCSF], cathepsin B, and soluble FASL; figure 2B); others were overexpressed during episodes of systemic JIA-MAS compared with HLH (eg, IL-18 and S100A12; figure 2C). Ratios of IL-18 to CXCL9, and IL-18 to CXCL10—considered to be surrogates of IFNγ signalling—were significantly elevated in samples from patients with systemic JIA-MAS compared to those with primary or secondary HLH and could distinguish between disease entities (figure 2C). Ratios of S100A12 to CXCL9 and of S100A12 to CXCL10 were also higher in samples from patients with systemic JIA-MAS and distinguished between these disease entities (figure 2C). Multiple correlation analyses of markers that showed significantly different expression in HLH or systemic JIA-MAS compared with concentrations of C-reactive protein, ferritin, fibrinogen, or thrombocyte counts showed distinct patterns in systemic JIA-MAS compared with secondary HLH or primary HLH (appendix p 9). For example, although we observed a significant positive correlation between ferritin concentration and IL-18 (p=0·020) and between ferritin and S100A12 (p=0·0058) in systemic JIA-MAS, these associations were weak or absent in secondary HLH and were inversely correlated in primary HLH (appendix p 9). Similarly, thrombocyte counts were inversely correlated with both S100A12 (p=0·0003) and IL-18 (p=0·031) in systemic JIA-MAS, whereas this association was weaker or absent in primary and secondary HLH.

| | Healthy controls (n=10) | Inactive systemic JIA (n=11) | Active systemic JIA (n=10) | Inactive systemic JIA-MAS (n=11) | Active systemic JIA-MAS (n=40) | Primary HLH (n=28) | Secondary HLH (n=44) |
|---|---|---|---|---|---|---|---|
| **Demographics** | | | | | | | |
| Age, years | 11·0 (6·0–18·0) | 2·6 (0·1–14·2) | 6·7 (2·6–12·9) | 4·7 (2·8–15·7) | 10·7 (2·3–17·8) | 1·0 (0·1–4·2) | 8·9 (0·7–17·8) |
| Sex | | | | | | | |
| Girls | 6 (60%) | 8 (73%) | 8 (80%) | 2 (18%) | 15 (38%) | 14 (50%) | 23 (52%) |
| Boys | 4 (40%) | 3 (27%) | 2 (20%) | 9 (82%) | 25 (63%) | 14 (50%) | 21 (48%) |
| **Clinical manifestations** | | | | | | | |
| Fever | 0 | 0 | 9 (90%) | 0 | 39 (98%) | 22 (79%) | 39 (89%) |
| Hepatomegaly | 0 | 0 | 2 (20%) | 0 | 17 (43%) | 26 (93%) | 34 (77%) |
| Splenomegaly | 0 | 0 | 2 (20%) | 1 (9%) | 23 (58%) | 24 (86%) | 28 (64%) |
| Neurological symptoms | 0 | ND | ND | 0 | 3 (8%); ND in 10 (25%) | 9 (32%) | 10 (23%) |
| Rash | 0 | 0 | 5 (50%) | 0 | 20 (50%) | 6 (21%) | 7 (16%) |
| Arthritis | 0 | 0 | 9 (90%) | 4 (36%) | 18 (45%) | 0 ND in 5 (18%) | 2 (5%) |
| Serositis | 0 | 0 | 0 | 0 | 11 (28%) | 1 (4%) | 10 (23%) |
| **Laboratory parameters** | | | | | | | |
| Haemoglobin, g/dL | ND | 12·6 (10·7–14·1) | 10·5 (9·0–12·3) | 12·8 (11·7–15·4) | 10·1 (6·5–12·8) | 6·6 (3·0–9·0) | 7·6 (5·6–10·9) |
| Neutrophil count, ×10³/mL | ND | 3·5 (1·8–6·2) | 9·0 (6·0–13·1) | 3·0 (1·9–5·5) | 2·3 (0·4–13·9) | 0·8 (0·0–23·0) | 0·7 (0·0–15·3) |
| Platelet count, ×10³/mL | ND | 288 (175–324) | 437 (310–550) | 288 (122–371) | 124 (65–446) | 65 (18–402) | 78 (35–362) |
| Aspartate aminotransferase, units per L | ND | 20 (15–30) | 75 (40–95) | 29 (15–116) | 98 (29–873) | 120 (12–3699) | 100 (20–8078) |
| Triglycerides, mg/dL | ND | 110 (90–165); ND in 7 (64%) | 112 (100–125); ND in 8 (80%) | 122 (115–169); ND in 8 (73%) | 175 (45–785) | 229 (2–330) | 272 (93–803) |
| Fibrinogen, mg/dL | ND | 290 (220–320); ND in 7 (64%) | 300 (270–410); ND in 7 (70%) | 220 (170–355); ND in 7 (64%) | 228 (50–711) | 76 (40–294) | 153 (50–771) |
| Ferritin, ng/mL | ND | 95 (50–170) | 386 (104–4237) | 36 (12–477) | 2985 (562–121937) | 6046 (550–65000) | 1500 (223–30406) |
| C-reactive protein, mg/dL | ND | <0·5 | 9·3 (4·0–30·0) | <0·5 | 7·2 (2·7–21·1) | 5·4 (0·9–22·8) | 5·6 (0·9–35·3) |
| Haemophagocytosis, present/absent/ND | 0/0/10 | 0/0/11 | 0/0/10 | 0/0/11 | 6/4/30 | 21/7/0 | 28/11/5 |

Data are n (%), or median (range) unless specified. ND=not determined. MAS=macrophage activation syndrome. HLH=haemophagocytic lymphohistiocytosis. JIA=juvenile idiopathic arthritis.

*Table 1:* **Study population characteristics**

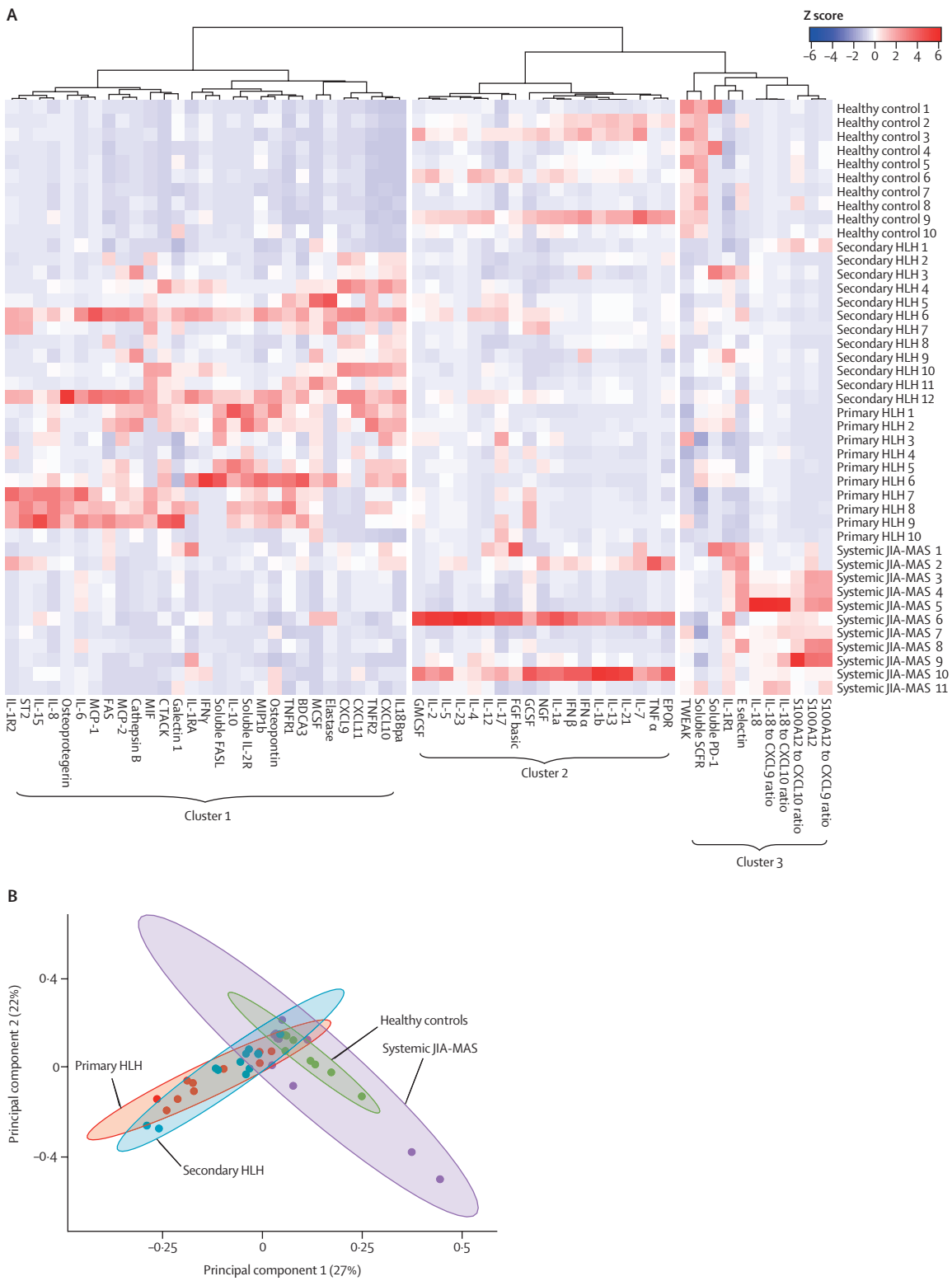


***Figure 1:*** **Serum biomarker profiles in HLH and systemic JIA-MAS among discovery cohort samples**
(A) Discovery cohort serum samples of patients with active primary HLH (n=10), secondary HLH (n=12), systemic JIA-MAS (n=11), and healthy controls (n=10) were analysed in a custom-made multiplexed bead array assay comprising 54 analytes. Serum S100A12 concentrations were determined by in-house ELISA. Data including ratios for IL-18 or S100A12 with CXCL9 or CXCL10 are presented as a heat map after unsupervised hierarchical clustering of biomarker expression profiles according to correlation distance and ward.D2 linkage. Colours indicate column Z score. (B) Principal component analysis of serum biomarkers as presented in A. IFN=interferon. IL=interleukin. HLH=haemophagocytic lymphohistiocytosis. JIA=juvenile idiopathic arthritis. MAS=macrophage activation syndrome. MCP=monocyte chemotactic protein. FAS=Fas cell surface death receptor. MIF=macrophage inhibitory factor. CTACK=cutaneous T cell-attracting chemokine. MIP1b=macrophage inflammatory protein 1 β. BDCA3=thrombomodulin. MCSF=macrophage colony stimulating factor. GMCSF=granulocyte macrophage colony stimulating factor. FGF=fibroblast growth factor. GCSF=granulocyte colony stimulating factor. NGF=nerve growth factor. EPOR=erythropoietin receptor. TWEAK=tumour necrosis factor-like weak inducer of apoptosis. SCFR=stem cell growth factor receptor. TNF=tumour necrosis factor.

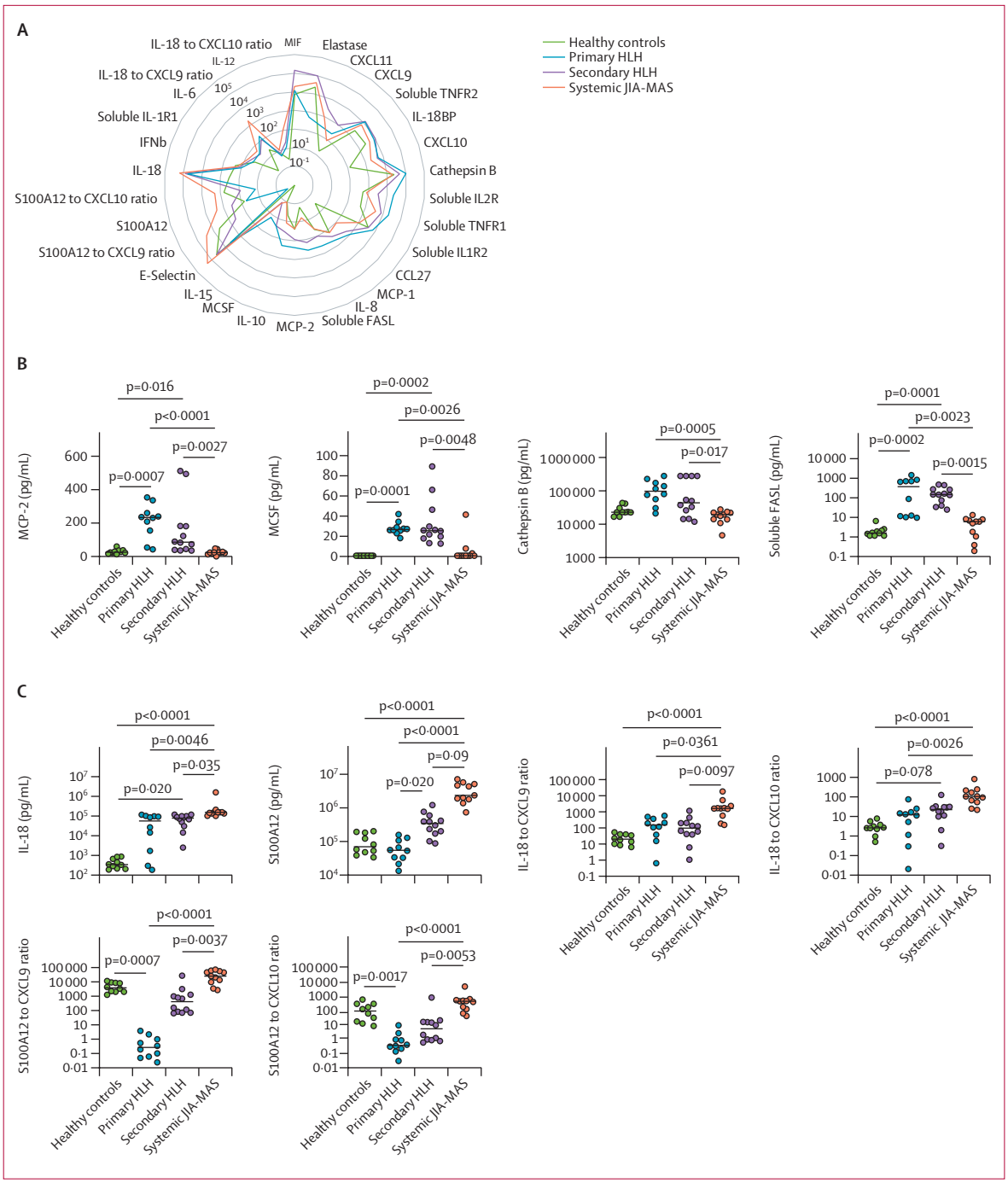


***Figure 2:*** **Serum biomarkers with differential expression in HLH and systemic JIA-MAS among discovery cohort samples**
(A) Radar plot depicts median serum marker concentrations and related ratios with significant or almost significant (p≥0·05-0·08) differences between healthy controls, primary HLH or secondary HLH, and systemic JIA-MAS discovery cohort samples. Serum biomarkers that discriminated between systemic JIA-MAS and HLH and were elevated in HLH (B) or systemic JIA-MAS (C) were analysed by multiple comparison statistics. Data points represent individual patient samples, bars represent medians. IL=interleukin. HLH=haemophagocytic lymphohistiocytosis. JIA=juvenile idiopathic arthritis. MAS=macrophage activation syndrome. MCP=monocyte chemotactic protein. MIF=macrophage inhibitory factor. FASL=Fas ligand. TNF=tumour necrosis factor. MCSF=macrophage colony stimulating factor. CXCL=chemokine (C-X-C motif) ligand.

To internally validate the initial biomarker quantification, we analysed serum biomarker profiles in samples from patients with active systemic JIA-MAS (n=11 from the discovery cohort; appendix p 7), inactive systemic JIA-MAS (n=11), active systemic JIA (n=10), inactive systemic JIA (n=11), and healthy controls (n=10).

Although unsupervised hierarchical clustering showed some differential expression related to underlying disease activity, we did not detect distinct clusters (appendix p 10). However, using multiple comparison analyses of individual biomarkers, we identified specific biomarkers that were significantly elevated in samples from patients with active systemic JIA compared to inactive systemic JIA-MAS (appendix p 11), and biomarkers that were selectively elevated during active compared with inactive systemic JIA (appendix p 11). Although expression of most biomarkers was higher in samples from patients with active disease, expression of some biomarkers was elevated in samples from patients with inactivate disease and in healthy controls (ie, soluble stem cell factor receptor and the TNF family member TWEAK; appendix p 11).

To validate biomarkers that were found in the discovery cohort to be significantly elevated in systemic JIA-MAS and that distinguished between systemic JIA-MAS and primary or secondary HLH, we first tested these markers using a commercially available bead array platform (Thermo Fisher Scientific, Waltham, MA, USA). The commercial platform included biomarkers that significantly discriminated between systemic JIA-MAS and primary or secondary HLH, as well as those that significantly discriminated between systemic JIA-MAS and primary HLH only (eg, soluble IL-2R, IL-8, IL-10, CCL27, TNF-R1, and E-selectin; appendix pp 5–6). We also included biomarkers that discriminated between systemic JIA-MAS and primary HLH at near significance (p close to 0·05; eg, IL-15 and macrophage migration inhibitory factor [MIF]; appendix pp 5–6), and biomarkers with a documented role in MAS pathogenesis (eg, IL-6). We did not include some biomarkers (eg, cathepsin B), because detection reagents for serum quantification of cathepsin B were not available at the time of the analyses.

Correlation analyses of biomarker concentrations determined by the custom-made discovery panel and commercial assay revealed good association between the assays; eg, MCP-2 ($r$=0·72; p<0·0001), soluble FASL ($r$=0·83; p<0·0001), IL-18 ($r$=0·91; p<0·0001), CXCL9 ($r$=0·73; p<0·0001), and CXCL10 ($r$=0·86; p<0·0001). By contrast, concentrations of MCSF quantified by the commercial panel correlated poorly with those measured by the custom bead array (appendix p 12). Other analytes included in the commercial bead array panel (ie, soluble IL-2R, IL-6, IL-8, IL-10, CCL27, TNF-R1, IL-15, MIF, and E-selectin) revealed spearman correlation coefficients between the commercial and custom-made discovery assay ranging from 0·47 (TNF-R1) to 0·90 (IL-10) (appendix p 12). We further analysed the association of serum biomarkers among each other when quantified by either assay (appendix p 12). In general, we observed lower inter-marker associations when using the

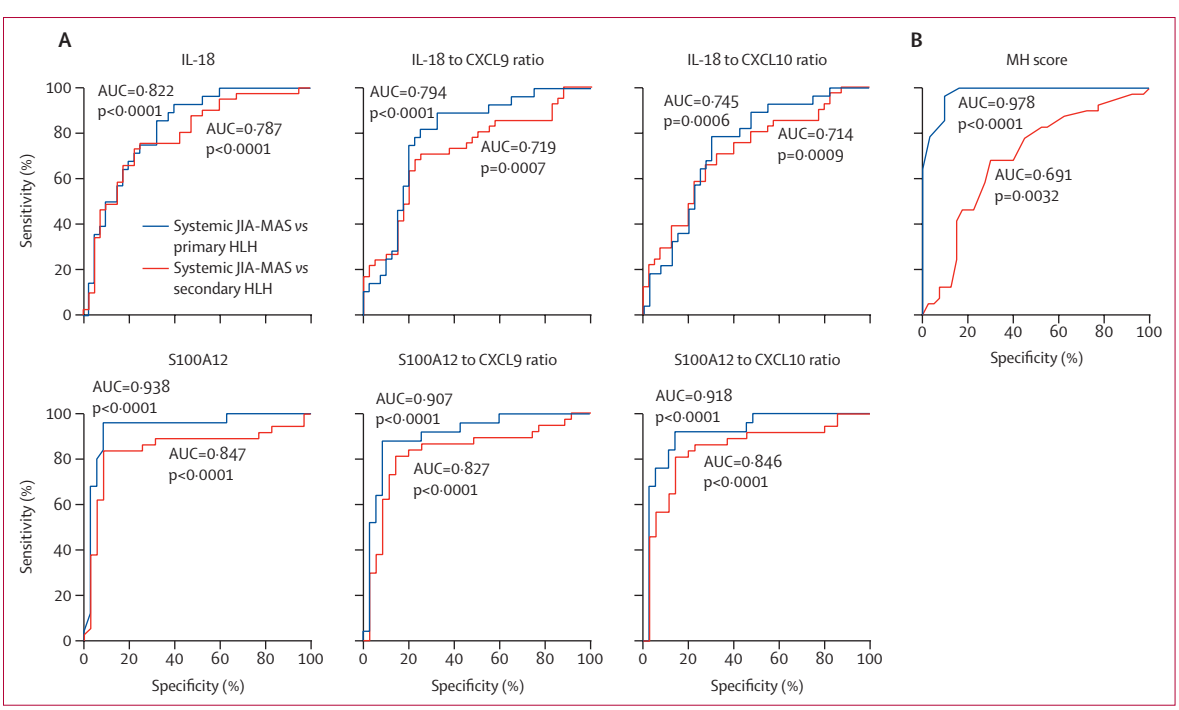


***Figure 3:*** **Validation of selected serum markers in an independent cohort**
(A) Indicated serum biomarker concentrations or MH score (B) were quantified in an independent patient cohort (systemic JIA-MAS [n=29]; primary HLH [n=18]; secondary HLH [n=32]) in a blinded manner. Based on previously defined cutoffs for serum markers and respective ratios (appendix p 13) and MH score,[19] samples were assigned to either primary HLH, secondary HLH, or systemic JIA-MAS. Receiver operating characteristic curve analyses and respective AUC indicate performance in correctly predicting either primary HLH (blue) or secondary HLH (red) versus systemic JIA-MAS. IL=interleukin. HLH=haemophagocytic lymphohistiocytosis. JIA=juvenile idiopathic arthritis. MAS=macrophage activation syndrome. AUC=area under the curve. CXCL=chemokine (C-X-C motif) ligand.

| | Systemic JIA-MAS versus primary HLH | | | Systemic JIA-MAS versus secondary HLH | | |
|---|---|---|---|---|---|---|
| | AUC | 95% CI | Cutoff (sensitivity/specificity) | AUC | 95% CI | Cutoff (sensitivity/specificity) |
| IL-18 | 0·822 | 0·724–0·920 | 1157 pg/mL (85·7%/67·5%) | 0·787 | 0·687–0·888 | 1022 pg/mL (73·2%/77·5%) |
| S100A12 | 0·938 | 0·867–1·000 | 535 ng/mL (96·0%/91·4%) | 0·847 | 0·744–0·949 | 635 ng/mL (83·8%/91·4%) |
| IL-18 to CXCL9 ratio | 0·794 | 0·653–0·967 | 4·5 (82·1%/75·0%) | 0·719 | 0·606–0·832 | 4·4 (68·3%/77·5%) |
| IL-18 to CXCL10 ratio | 0·745 | 0·627–0·862 | 14·8 (78·6%/70·0%) | 0·714 | 0·602–0·826 | 10·6 (65·9%/72·5%) |
| S100A12 to CXCL9 ratio | 0·907 | 0·827–0·988 | 1593·0 (88·0%/91·4%) | 0·827 | 0·723–0·931 | 2231·0 (81·1%/85·7%) |
| S100A12 to CXCL10 ratio | 0·918 | 0·842–0·994 | 9293·0 (92·0%/85·7%) | 0·846 | 0·749–0·943 | 10 959·0 (81·1%/85·7%) |

JIA=juvenile idiopathic arthritis. MAS=macrophage activation syndrome. HLH=haemophagocytic lymphohistiocytosis. AUC=area under the curve. IL=interleukin. CXCL=chemokine (C-X-C motif) ligand.

***Table 2:*** **Best performing parameters in the differentiation of systemic JIA-MAS from primary HLH and secondary HLH**

commercial compared with the custom-made assay; however, except for biomarkers that correlated poorly between the two assays (eg, TNF-R1, IL-15, and MCSF), the overall pattern between the two platforms was similar.

We then tested whether MCP-2, soluble FASL, IL-18, and S100A12 (by ELISA), as well as ratios of IL-18 to CXCL9, IL-18 to CXCL10, S100A12 to CXCL9, and S100A12 to CXCL10 distinguished between samples from patients with HLH or systemic JIA-MAS in an independent patient cohort, using cutoff values defined in the discovery cohort according to Youden's method (appendix p 13). Samples in the validation cohort (primary HLH [n=18]; secondary HLH [n=32]; systemic JIA-MAS [n=29]) were tested in a blinded manner using the commercial bead array assay. The biomarkers that best distinguished between systemic JIA-MAS and HLH were S100A12 (primary HLH *vs* systemic JIA-MAS: area under the curve [AUC]=0·938; secondary HLH *vs* systemic JIA-MAS: AUC=0·847), and IL-18 (primary HLH *vs* systemic JIA-MAS: AUC=0·822; secondary HLH *vs* systemic JIA-MAS: AUC=0·787) (figure 3A, table 2). Given that the validation cohort was larger than the discovery cohort, we refined respective cutoff values for discriminating systemic JIA-MAS from HLH and found that, with the exception of the IL-18 to CXCL10 ratio, these values were comparable to those determined in the discovery cohort analyses (appendix p 14).

S100A12 and IL-18, as well as ratios of S100A12 and IL-18 to CXCL9 and CXCL10 out-performed the previously described MAS to MH score[19] in distinguishing secondary HLH from systemic JIA-MAS (AUC=0·691; figure 3B). However, the MH score outperformed our biomarker analyses in differentiating between primary HLH and systemic JIA-MAS (AUC=0·978; figure 3B). In the validation analysis, MCP-2 and soluble FASL were poor at discriminating systemic JIA-MAS from primary or secondary HLH, and these biomarkers were only able to differentiate between primary HLH and systemic JIA-MAS at a significant level (appendix p 14). Calculating ratios of either S100A12 or IL-18 with CXCL9 or CXCL10 did not result in improved discrimination between disease entities compared with S100A12 or IL-18 alone (figure 3A, appendix p 14).

In multiple correlation analyses among validation cohort samples, we again observed significant correlations between IL-18 and S100A12 serum concentrations with ferritin concentrations in systemic JIA-MAS. However, this correlation was not evident in secondary HLH or primary HLH, despite markedly elevated ferritin concentrations in these patients (table 1, appendix p 15). In contrast to the discovery cohort, the correlation between circulating thrombocytes and serum S100A12 and IL-18 concentrations was weak in systemic JIA-MAS samples in the validation cohort (appendix p 15).

## Discussion

In this study, we report on a broad approach to identify serum biomarker profiles in systemic JIA-MAS compared with primary and secondary HLH. Of all analysed markers, S100A12 followed by IL-18, and ratios of IL-18 to CXCL9, IL-18 to CXCL10, S100A12 to CXCL9, and S100A12 to CXCL10 proved most powerful in differential diagnosis. To our knowledge, this is the first study describing and validating granulocytic S100A12 as a serum biomarker that best differentiates systemic JIA-MAS from both primary and secondary HLH.

S100A12 and IL-18 are both well-established biomarkers of systemic JIA, and our study supports previous findings.[12,20,21] Concentrations of these markers are highly elevated in patients with JIA and correlate with disease activity.[12,20,21] Our data validate previous studies showing that IL-18 concentrations distinguish MAS from primary HLH[13] and that the IL-18 to CXCL9 ratio distinguishes between MAS and infection-associated secondary HLH.[22] However, in our study, IL-18 concentrations measured by the commercial bead array assay were lower than those detected using our custom bead array as well as those quantified by ELISA in other studies.[12] Further, IL-18 concentrations in the present study do not differentiate between free (ie, circulating bioactive IL-18) and total IL-18 (ie, free IL-18 and IL-18 complexed with IL-18BP).[13] Serum concentrations of IFNγ and the IFNγ-induced chemokines CXCL9 and CXCL10 have also been associated with severity of disease in systemic JIA-MAS.[10]

Our study emphasises the crucial role of the IL-18-IFNγ axis in MAS pathophysiology.[23] Free IL-18 has been shown to be a key driver of hyperinflammation,[24] which can be controlled by therapeutic application of its natural inhibitor, IL-18 binding protein.[25,26] Similarly, serum concentrations of granulocytic S100A12 strongly correlate with systemic JIA disease activity[21] and peripheral blood neutrophil counts,[27] and neutrophils from patients with systemic JIA have elevated inflammatory gene expression, including *S100A12*.[27,28] Once released from cells, S100A12 is thought to perpetuate and amplify inflammation as an endogenous ligand to Toll-like receptor 4.[29]

The specific reason for the pronounced overexpression of S100A12 and IL-18 in both systemic JIA and JIA-MAS remains unclear, but our results in systemic JIA-MAS versus primary HLH in particular show that expression of these biomarkers is not simply related to elevations in blood ferritin. Overexpression of IL-18 and S100A12 might be partly linked to the prominent role of the IL-1β pathway in underlying disease pathophysiology. IL-1β and S100 are thought to form a positive feedback loop in systemic JIA, and S100 depletion from patient serum was shown to abrogate IL-1β expression.[30] Similarly, IL-18 expression can be induced by inflammatory stimuli, but recent evidence suggests type I interferon signalling to have a prominent role in regulating IL-18 expression.[31,32] Although this finding might be relevant for excessive IL-18 expression in MAS, in which viral infections in particular are thought to be prominent triggers,[23] it does not readily explain the overexpression of IL-18 in patients with systemic JIA devoid of hyperinflammation.

Expression of MCP-2, MCSF, cathepsin B, and soluble FASL was also detected in our discovery cohort samples. These markers were elevated in the serum of patients with primary HLH and clustered within the primary or secondary HLH biomarker profile signature; they were not elevated in samples from patients with systemic JIA-MAS or healthy controls. As with S100A12 and IL-18, differential expression of these biomarkers in secondary HLH and systemic JIA-MAS further underscores potential differences in the underlying disease pathophysiology, despite large or even complete clinical phenotypic overlap between these entities.

Nonetheless, the findings of our study need to be discussed in view of three main limitations. First, although the multiple-marker panel used for discovery cohort screening included a wide range of markers with a general role in inflammation, as well as those with established or anecdotally reported roles in MAS and HLH pathophysiology, we might still have missed important parameters. In 2019, ADA2 activity was reported as a promising indicator of MAS,[11] tracking with MAS disease intensity and strongly correlating with ferritin, IL-18, and CXCL9 concentrations. However, as ADA2 expression can be induced by IL-18 and IFNγ,[11] it is not yet clear whether ADA2 has a unique, previously unknown role in MAS pathophysiology or whether it is simply a surrogate for dysregulated IL-18 or IFNγ expression. For similar reasons, we did not include S100A8/A9 in our study. S100A8/A9 serum titres are highly elevated in systemic JIA (even more so than S100A12),[30,33,34] but expression strongly correlates with S100A12.[35] To reduce redundancy, we decided to include only one in our study.

Second, while our initial analyses revealed a wider panel of promising molecules, only a few could be successfully validated using commercially available detection reagents. Candidate biomarkers such as cathepsin B or MCSF could not be included in the validation panel either because reagents were not available commercially (cathepsin B) or showed poor performance (MCSF). Further, the number of biomarkers that we found to differentiate between disease entities might also have been limited by sample quality, particularly among validation cohort samples. These samples were collected at different centres throughout the world, and we cannot completely exclude a centre bias. Furthermore, samples were collected under conditions reflecting clinical reality, rather than those meeting rigid proteomic study standards such as rapid sample processing and prompt storage at –20°C or –80°C. These conditions might favour detection of more thermostable analytes such as IL-18 and S100A12.[36] However, we consider this feature to be an advantage, as the results should be reproducible and amendable to further validation at any clinical centre with access to detection reagents and instrumentation or that is included in HLH registries that offer similar functional analyses for initial evaluation.

As a third study limitation, it is important to emphasise that in many clinical centres, assays for quantification of S100A12, IL-18, CXCL9, and CXCL10 are not yet available or are run in differing formats, which limits the comparability of results. At the time of writing, the MAS working party of the Pediatric Rheumatology European Society are developing a project to support wide introduction of respective biomarker analyses in the universal diagnostic tests of hyperferritinaemic syndromes.

Identifying evolving MAS in a patient with an underlying inflammatory disorder is difficult. The 2016 ACR/EULAR criteria[15] were designed for classification of MAS in clinical trials and were not validated for diagnostic purposes in the clinical setting. Therefore, different approaches have been used to discriminate MAS from active systemic JIA (eg, the MS score[37] and ferritin to ESR ratio[38]) and to discriminate between primary HLH and MAS (eg, MH score sensitivity: 91%, specificity 93%[19]). However, as also evident from our analyses, these criteria and scores are of limited use, particularly in discriminating MAS from secondary HLH. Thus, we suggest that biomarker analysis be included in diagnostic tests in addition to clinical evaluation and standard laboratory and functional tests to distinguish MAS from secondary HLH at an early stage, and thus to enable introduction of the appropriate anti-inflammatory treatment.[23]

Recently, a multidisciplinary approach to HLH and MAS management was introduced, in which paediatric

rheumatologists act as gatekeepers charged with overseeing the diagnostic evaluation.[39] The diagnostic algorithm put forward in this approach included IL-18 and CXCL9 as biomarkers for MAS.[39] Our data provide additional support for the ability of IL-18, CXCL9, and particularly S100A12 in distinguishing between different disease entities that are associated with a life-threatening cytokine storm. These findings can support an early differential diagnosis between systemic JIA-MAS and HLH and thus can help to guide treatment decisions.

#### Contributors

CK, WdJ, and CP were responsible for serum marker quantification. CK, WdJ, SV, CP, KK, CH, HW, DF, and DH were responsible for data analyses. NF, AG, SV, RS, CB, SE, ACH, SE, SA, CW, KL, FDB, CH, HW, KB, DF, and DH were responsible for collection of clinical data and material. CK, CH, HW, DF, and DH prepared the first manuscript draft. All authors had full access to all the data in the study and had final responsibility for the decision to submit for publication. DF and DH verified the data. All authors revised and approved the final draft.

#### Declaration of interests

CK served as a consultant for Novartis. AG served as a consultant for AB2Bio, SOBI, and Novartis, and received research support from Novimmune and AB2Bio. WdJ is an employee of Luminex Corp. SV served as a consultant for SOBI and Novartis and received non-restricted research support from SOBI. ACH received consulting fees from Novartis and SOBI. SE has received research funding from UCB outside the submitted work. SA, KL, and KB have served as consultants for SOBI. CW served as a consultant for SOBI, Novimmune, Novartis, and Roche, and has received unrestricted research grants from GSK, Roche, and Pfizer. FDB served as a consultant for AbbVie, SOBI, Novimmune, Novartis, Roche, and Sanofi, and has received unrestricted research grants from SOBI, Novartis, Novimmune, Sanofi, Roche, and Pfizer. CH has received honoraria (lecture fees) from Novartis. HW has received honoraria (lecture fees) from Novartis and Takeda, and travel support from Octapharma and CSL-Behring. DF received honoraria (speaker fees) from Chugai-Roche, Novartis, and SOBI, and research support from Novartis, Pfizer, and SOBI. DH received honoraria (lecture fees) from Novartis and SOBI. All other authors declare no competing interests.

#### Data sharing

All data required to evaluate the conclusions in the paper are present in the manuscript or its appendix. Further information on the study protocol or de-identified datasets generated and analysed within this publication are available from the corresponding authors on reasonable request.

#### Acknowledgments

Multiplex immunoassays were in-house developed, validated, and performed at the Multiplex core facility of the Laboratory for Translational Immunology of the University Medical Center Utrecht. The authors thank Susanne Schleifenbaum, Melanie Saers, and Sabrina Fuehner for excellent technical support. This study was supported by the German Research Foundation (FO 354/14-1 and SFB1160/2 TP A1), the Center for Interdisciplinary Clinical Research at University Hospital Muenster (IZKF, Fö2/018/20), the EU's Horizon 2020 research and innovation programme (grant agreement number 779295; ImmunAID), and the Deutsche Kinderkrebsstiftung (2016.04 and DKS 2018.11).

#### References

1 Weaver LK, Behrens EM. Hyperinflammation, rather than hemophagocytosis, is the common link between macrophage activation syndrome and hemophagocytic lymphohistiocytosis. *Curr Opin Rheumatol* 2014; **26:** 562–69.

2 Carcillo JA, Simon DW, Podd BS. How we manage hyperferritinemic sepsis-related multiple organ dysfunction syndrome/macrophage activation syndrome/secondary hemophagocytic lymphohistiocytosis histiocytosis. *Pediatr Crit Care Med* 2015; **16:** 598–600.

3 Crayne CB, Albeituni S, Nichols KE, Cron RQ. The immunology of macrophage activation syndrome. *Front Immunol* 2019; **10:** 119.

4 Stepp SE, Dufourcq-Lagelouse R, Le Deist F, et al. Perforin gene defects in familial hemophagocytic lymphohistiocytosis. *Science* 1999; **286:** 1957–59.

5 Henter JI, Horne A, Aricó M, et al. HLH-2004: diagnostic and therapeutic guidelines for hemophagocytic lymphohistiocytosis. *Pediatr Blood Cancer* 2007; **48:** 124–31.

6 Grom AA. Primary hemophagocytic lymphohistiocytosis and macrophage activation syndrome: the importance of timely clinical differentiation. *J Pediatr* 2017; **189:** 19–21.e1.

7 Janka GE. Familial and acquired hemophagocytic lymphohistiocytosis. *Annu Rev Med* 2012; **63:** 233–46.

8 Castillo L, Carcillo J. Secondary hemophagocytic lymphohistiocytosis and severe sepsis/ systemic inflammatory response syndrome/ multiorgan dysfunction syndrome/macrophage activation syndrome share common intermediate phenotypes on a spectrum of inflammation. *Pediatr Crit Care Med* 2009; **10:** 387–92.

9 Jordan MB, Allen CE, Greenberg J, et al. Challenges in the diagnosis of hemophagocytic lymphohistiocytosis: recommendations from the North American Consortium for Histiocytosis (NACHO). *Pediatr Blood Cancer* 2019; **66:** e27929.

10 Bracaglia C, de Graaf K, Pires Marafon D, et al. Elevated circulating levels of interferon-γ and interferon-γ-induced chemokines characterise patients with macrophage activation syndrome complicating systemic juvenile idiopathic arthritis. *Ann Rheum Dis* 2017; **76:** 166–72.

11 Lee P, Schulert G, Canna SW, et al. Adenosine deaminase 2 as a biomarker of macrophage activation syndrome in systemic juvenile idiopathic arthritis. *Ann Rheum Dis* 2020; **79:** 225–31.

12 Yasin S, Fall N, Brown RA, et al. IL-18 as a biomarker linking systemic juvenile idiopathic arthritis and macrophage activation syndrome. *Rheumatology* 2020; **59:** 361–66.

13 Weiss ES, Girard-Guyonvarc'h C, Holzinger D, et al. Interleukin-18 diagnostically distinguishes and pathogenically promotes human and murine macrophage activation syndrome. *Blood* 2018; **131:** 1442–55.

14 Shimizu M, Inoue N, Mizuta M, Nakagishi Y, Yachie A. Characteristic elevation of soluble TNF receptor II : I ratio in macrophage activation syndrome with systemic juvenile idiopathic arthritis. *Clin Exp Immunol* 2018; **191:** 349–55.

15 Ravelli A, Minoia F, Davì S, et al. 2016 classification criteria for macrophage activation syndrome complicating systemic juvenile idiopathic arthritis: a European League Against Rheumatism/ American College of Rheumatology/Paediatric Rheumatology International Trials Organisation Collaborative Initiative. *Arthritis Rheumatol* 2016; **68:** 566–76.

16 Ammann S, Lehmberg K, Zur Stadt U, et al. Effective immunological guidance of genetic analyses including exome sequencing in patients evaluated for hemophagocytic lymphohistiocytosis. *J Clin Immunol* 2017; **37:** 770–80.

17 Wallace CA, Ruperto N, Giannini E, Grp C, Grp P, Grp P. Preliminary criteria for clinical remission for select categories of juvenile idiopathic arthritis. *J Rheumatol* 2004; **31:** 2290–94.

18 Bossuyt PM, Reitsma JB, Bruns DE, et al. STARD 2015: an updated list of essential items for reporting diagnostic accuracy studies. *BMJ* 2015; **351:** h5527.

19 Minoia F, Bovis F, Davì S, et al. Development and Initial Validation of the macrophage activation syndrome/primary hemophagocytic lymphohistiocytosis score, a diagnostic tool that differentiates primary hemophagocytic lymphohistiocytosis from macrophage activation syndrome. *J Pediatr* 2017; **189:** 72–78.e3.

20 de Jager W, Hoppenreijs EP, Wulffraat NM, Wedderburn LR, Kuis W, Prakken BJ. Blood and synovial fluid cytokine signatures in patients with juvenile idiopathic arthritis: a cross-sectional study. *Ann Rheum Dis* 2007; **66:** 589–98.

21 Wittkowski H, Frosch M, Wulffraat N, et al. S100A12 is a novel molecular marker differentiating systemic-onset juvenile idiopathic arthritis from other causes of fever of unknown origin. *Arthritis Rheum* 2008; **58:** 3924–31.

22 Put K, Avau A, Brisse E, et al. Cytokines in systemic juvenile idiopathic arthritis and haemophagocytic lymphohistiocytosis: tipping the balance between interleukin-18 and interferon-γ. *Rheumatology* 2015; **54:** 1507–17.

23 Grom AA, Horne A, De Benedetti F. Macrophage activation syndrome in the era of biologic therapy. *Nat Rev Rheumatol* 2016; **12:** 259–68.

24 Girard-Guyonvarc'h C, Palomo J, Martin P, et al. Unopposed IL-18 signaling leads to severe TLR9-induced macrophage activation syndrome in mice. *Blood* 2018; **131:** 1430–41.

25 Canna SW, Girard C, Malle L, et al. Life-threatening NLRC4-associated hyperinflammation successfully treated with IL-18 inhibition. *J Allergy Clin Immunol* 2017; **139:** 1698–701.

26 Yasin S, Solomon K, Canna SW, et al. IL-18 as therapeutic target in a patient with resistant systemic juvenile idiopathic arthritis and recurrent macrophage activation syndrome. *Rheumatol* 2020; **59:** 442–45.

27 Ter Haar NM, Tak T, Mokry M, et al. Reversal of sepsis-like features of neutrophils by interleukin-1 blockade in patients with systemic-onset juvenile idiopathic arthritis. *Arthritis Rheumatol* 2018; **70:** 943–56.

28 Brown RA, Henderlight M, Do T, et al. Neutrophils from children with systemic juvenile idiopathic arthritis exhibit persistent proinflammatory activation despite long-standing clinically inactive disease. *Front Immunol* 2018; **9:** 2995.

29 Kessel C, Fuehner S, Zell J, et al. Calcium and zinc tune autoinflammatory Toll-like receptor 4 signaling by S100A12. *J Allergy Clin Immunol* 2018; **142:** 1370–73.e8.

30 Frosch M, Ahlmann M, Vogl T, et al. The myeloid-related proteins 8 and 14 complex, a novel ligand of toll-like receptor 4, and interleukin-1beta form a positive feedback mechanism in systemic-onset juvenile idiopathic arthritis. *Arthritis Rheum* 2009; **60:** 883–91.

31 Zhu Q, Kanneganti TD. Cutting Edge: Distinct regulatory mechanisms control proinflammatory cytokines IL-18 and IL-1β. *J Immunol* 2017; **198:** 4210–15.

32 Verweyen E, Holzinger D, Weinhage T, et al. Synergistic signaling of TLR and IFNα/β facilitates escape of IL-18 expression from endotoxin tolerance. *Am J Respir Crit Care Med* 2020; **201:** 526–39.

33 Holzinger D, Frosch M, Kastrup A, et al. The Toll-like receptor 4 agonist MRP8/14 protein complex is a sensitive indicator for disease activity and predicts relapses in systemic-onset juvenile idiopathic arthritis. *Ann Rheum Dis* 2012; **71:** 974–80.

34 Aljaberi N, Tronconi E, Schulert G, et al. The use of S100 proteins testing in juvenile idiopathic arthritis and autoinflammatory diseases in a pediatric clinical setting: a retrospective analysis. *Pediatr Rheumatol* 2020; **18:** 7.

35 Hinze CH, Foell D, Johnson AL, et al. Serum S100A8/A9 and S100A12 levels in children with polyarticular forms of juvenile idiopathic arthritis: relationship to maintenance of clinically inactive disease during anti-tumor necrosis factor therapy and occurrence of disease flare after discontinuation of therapy. *Arthritis Rheumatol* 2019; **71:** 451–59.

36 de Jager W, Bourcier K, Rijkers GT, Prakken BJ, Seyfert-Margolis V. Prerequisites for cytokine measurements in clinical trials with multiplex immunoassays. *BMC Immunol* 2009; **10:** 52.

37 Minoia F, Bovis F, Davì S, et al. Development and initial validation of the MS score for diagnosis of macrophage activation syndrome in systemic juvenile idiopathic arthritis. *Ann Rheum Dis* 2019; **78:** 1357–62.

38 Eloseily EMA, Minoia F, Crayne CB, Beukelman T, Ravelli A, Cron RQ. Ferritin to erythrocyte sedimentation rate ratio: simple measure to identify macrophage activation syndrome in systemic juvenile idiopathic arthritis. *ACR Open Rheumatol* 2019; **1:** 345–49.

39 Halyabar O, Chang MH, Schoettler ML, et al. Calm in the midst of cytokine storm: a collaborative approach to the diagnosis and treatment of hemophagocytic lymphohistiocytosis and macrophage activation syndrome. *Pediatr Rheumatol Online J* 2019; **17:** 7.