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ORIGINAL RESEARCH

Elevated serum levels of interleukin-18 discriminate Still's disease from other autoinflammatory conditions: results from the European ImmunAID cohort

Charlotte Girard-Guyonvarc'h,[1,2] Emiliana Rodriguez,[1,2] Yvonne M Mueller,[3] Assunta Caruso,[1,2] Peter D Katsikis,[3] Cem Gabay ,[1,2] The ImmunAID consortium

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For numbered affiliations see end of article.

Correspondence to
Professor Cem Gabay;
cem.gabay@unige.ch

ABSTRACT

Objectives Systemic autoinflammatory diseases (SAIDs) represent a set of conditions with exaggerated innate immune responses. IL-1β and IL-18 are key cytokines involved in the pathogenesis of some SAID. We aimed to assess the diagnostic value of serum levels of IL-1β, IL-18, their respective inhibitors IL-1Ra and IL-18 binding protein (IL-18BP), and IFN-γ in SAID.

Methods A cohort of patients with active SAID, including monogenic (mSAID) and genetically undiagnosed SAID (guSAID) from different European countries, with active disease at inclusion, was established. Serum levels of cytokines were measured by immunoassays.

Results Sera from 53 mSAID, 220 guSAID and 49 controls without inflammatory disease were analysed. Serum levels of total and free IL-18 were significantly increased in Still's disease in comparison to most SAID and non-inflammatory controls. Levels of total IL-18 were also elevated in patients with familial Mediterranean fever to a comparable extent as in Still's disease. In contrast, free IL-18 levels were selectively higher in Still's disease. Receiver operating characteristic curve analysis showed that total IL-18 was the most sensitive and specific marker for the diagnosis of Still's disease (area under the curve=0.91). There was a positive correlation between IL-18 and ferritin. In 10 patients with Still's disease who had a second blood collection, we found a significant decrease in serum levels of free IL-18 after treatment.

Conclusions Our results show that IL-18 can discriminate Still's disease from other SAID, and free IL-18 levels may be relevant to assess response to therapy in these patients.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Diagnosis of systemic autoinflammatory diseases (SAIDs) can be challenging, especially since reliable biomarkers are lacking. Although IL-1β and IL-18 are described as key players in SAID, it is not known whether these cytokines and their respective inhibitors IL-1 receptor antagonist and IL-18 binding protein can be used as diagnostic biomarkers.

WHAT THIS STUDY ADDS

⇒ Our study shows that elevated serum levels of IL-18 distinguish Still's disease from other SAIDs with good sensitivity and specificity. Free IL-18 levels decreased in patients with Still's disease who responded to anti-inflammatory therapy.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Serum IL-18 levels could be used in clinical practice as a diagnostic biomarker for Still's disease.

INTRODUCTION

Systemic autoinflammatory diseases (SAIDs) comprise a group of conditions marked by recurrent fever episodes and various clinical symptoms resulting from inappropriate activation of the innate immune system.[1] An increasing number of SAIDs, referred to as monogenic SAID (mSAID), have been associated with specific gene mutations. Several distinct pathways are now recognised as being involved in the pathophysiology of mSAID. Mutations in the inflammasome subunits (eg, *MEFV* gene, coding for pyrin, in familial Mediterranean fever (FMF) and in the *NLRP3* gene in cryopyrin-associated periodic syndromes (CAPS)[2]) or inflammasome-interacting proteins (eg, mevalonate kinase deficiency (MKD)[3]) are the most studied mechanisms. Inflammasomes are multiprotein complexes that assemble on binding to danger or pathogen-associated molecular patterns. This triggers the cleavage of pro-caspase 1 into active caspase-1, which subsequently cleaves pro-IL-1β and pro-IL-18, as well as the N-terminal domain of Gasdermin D leading to cell membrane Gasdermin-D pore formation and the release of active forms of IL-1β and IL-18.[4] Additional mSAIDs are consecutive to mutations in genes involved in actin

polymerisation (actinopathies), NF-κB regulation (relopathies), endoplasmic reticulum stress (TRAPS) or IFN type I overexpression (interferonopathies).[1] In contrast, several SAIDs, referred to as genetically undiagnosed SAID (guSAID), remain of unclear origin. Systemic-onset juvenile idiopathic arthritis (sJIA) and adult-onset Still's disease (AOSD) are now widely recognised as the same condition (Still's disease)[5] and, like other guSAID, exhibit overlapping clinical features with mSAID.[1]

Despite significant advancements in the diagnosis and treatment of SAIDs over the past 20 years, these conditions continue to pose substantial challenges, resulting in considerable morbidity and mortality. For instance, Still's disease carries a notable risk of developing macrophage activation syndrome, a life-threatening condition that can be difficult to treat. IL-1 blockade has become a common approach for treating various mSAID and guSAID such as Still's disease.[6] In addition, elevated IL-18 levels are present in the serum of patients with Still's disease.[7] A phase 2 clinical trial in adult patients with refractory Still's disease demonstrated the efficacy of targeting IL-18 using recombinant IL-18 binding protein (IL-18BP),[8] a natural inhibitor of IL-18, indicating that other cytokines can be involved in the pathogenesis of Still's disease and maybe other SAIDs.

The ImmunAID (Immunome project consortium for AutoInflammatory Disorders) consortium is a European initiative aimed at better characterising and classifying SAID by identifying multiomics signatures associated with these diseases. Given the suspected roles of IL-1β and IL-18 in the pathophysiology of some SAID, we measured the levels of IL-1β, IL-18, their respective inhibitors IL-1Ra and IL-18BP, and IFN-γ (an IL-18-induced cytokine) in the serum of patients with active mSAID and guSAID enrolled in the ImmunAID cohort.

METHODS

Patients and controls

Adult and paediatric patients older than 6 months with SAID, and controls without inflammatory diseases (non-inflammatory controls (NICs)) were enrolled in 34 specialised clinical Centres across Europe (UK, France, Spain, Italy, Slovenia, the Netherlands, Germany, Switzerland, Turkey, Belgium and Greece) between December 2019 and April 2023. Eligible diagnoses included mSAID and guSAID, as depicted in online supplemental table 1. Disease had to be active at the time of inclusion, according to predefined specific criteria available in online supplemental table 1. According to recent recommendations, patients with AOSD and sJIA were considered as one disease group (Still's disease).[5] NICs were patients aged from 10 to 60 years old, without personal or familial history of SAID, who attended in one of the participating clinical centres for any reason and were free of inflammatory disorders, as well as healthy individuals. Exclusion criteria for diseases and controls are reported in online supplemental table 1.

Baseline patient information, clinical manifestations, biological values (levels of haemoglobin, aspartate aminotransferase (AST), alanine aminotransferase, neutrophil count and erythrocyte sedimentation rate (ESR), as given by most recent routine blood tests), disease duration and ongoing treatments were collected at inclusion and reported in an online electronic case report form at first visit (V1). GuSAID without ongoing SAID-related treatment at the time of inclusion were invited for a second visit (V2) 3±1 months after V1 for clinical evaluation and blood collection.

Serum and plasma samples

All included patients and controls underwent blood puncture in the recruiting centre at V1. A second blood collection was also performed in eligible patients at V2. Serum and plasma were obtained after 10 min by 1000 g centrifugation of whole blood (collected in pro-coagulation or anti-coagulant-treated tube, respectively) and kept in cryotubes at −80°C, before dispatch to a central biobank. Serum aliquots were sent from the central biobank to the laboratory of Rheumatology at the University of Geneva (Faculty of Medicine Geneva, Switzerland) and to the laboratory of the Infection and Immunity Research Group of Erasmus University (Rotterdam, Netherlands) between June 2022 and October 2023. Plasma samples from the same individuals were similarly sent to the Laboratory of Rheumatology, University of Liège (Belgium). Each shipment was carried out in conditions suitable for preserving sample quality.

Biomarker measurements

Total and free IL-18, IL-18BP, IL-1Ra and IFN-γ ELISAs

Serum levels of IL-18 (total and free), IL-18BP, IL-1β, IL-1Ra and IFN-γ were assessed by using different immunoassays in the laboratory of Rheumatology. Total IL-18, IL-18BP, IL-1Ra and IFN-γ levels were measured using commercial ELISA kits (IL-18 ELISA from MBL (catalogue number 7620) at 5-fold dilution, IL-18BPa DuoSet ELISA from R&D system (catalogue number DY119) at 20-fold dilution, IL-1Ra DuoSet ELISA from R&D system (catalogue number DY280) undiluted, and IFN-γ Quantikine ELISA from R&D system (catalogue number DIF50C), undiluted) according to manufacturer's instructions. When necessary, additional dosages were performed at higher dilutions until obtaining a result within the detection range. Free IL-18 levels were assessed using a previously described homemade ELISA.[9] Plates were read at a wavelength of 450 nm with reference wavelength of 570 nm. Cytokine levels were extracted from optical densities through asymmetric sigmoidal, 5PL standard curve interpolated in GraphPad Prism V.10.2.2 and corrected according to the dilution factor.

IL-1β assay

IL-1β levels were assessed by electrochemiluminescence with the ultra-sensitive S-PLEX Human IL-1β immunoassay from Meso Scale Discovery (Rockville, Maryland,

USA), according to the manufacturer's instructions. Samples were used undiluted, signal read with the MESO QuickPlex SQ 120 plate reader and final IL-1β levels obtained through analysis by the Meso Scale Discovery Workbench Software.

C reactive protein, ferritin levels and serum amyloid A levels

Serum levels of C reactive protein (CRP) and ferritin were measured by the Infection and Immunity Research Group of Erasmus University (Rotterdam, Netherlands). CRP was measured using a turbidimetric assay (Roche Diagnostics, Rotkreuz, Switzerland) on the C502 model of the Cobas 8000 analyser (Roche Diagnostics). Ferritin was measured using an Electro-Chemi-Luminescent-Immuno-Assay (Roche Diagnostics) on the E801 module of the Cobas 8000 analyser (Roche Diagnostics). Plasma levels of serum amyloid A (SAA) were determined in the Laboratory of Rheumatology, University of Liège (Belgium) using a commercially available ELISA kit according to the manufacturer's instructions (ThermoFisher Scientific ref KHA0011 for SAA), as described.[10] Plasma samples were diluted in the range (1:100–1:500 000). Calibration ranges were set between 9.4 and 600 ng/mL. Absorbance data were acquired on a SpectraMax 384Plus spectrophotometric ELISA plate reader using SoftMax Pro software (Molecular Devices, San Jose, USA, V.7.0.2).

Clinical scores

Composite scores were used to evaluate disease activity. In the absence of a validated score enabling disease activity assessment of all types of SAID, a disease activity score was created, combining fatigue, patient and physician global assessment, the total score being the sum of fatigue level, patient global assessment and physician global assessment, each from 0 (absent/inactive disease) to 10 (extreme/extremely active disease). Three other scores were calculated for Still's disease, the sJADAS score, validated for sJIA[11] and the Pouchot and modified Pouchot scores, validated for AOSD.[12 13]

Response to treatment at the second visit was assessed at the discretion of the attending physician, based on clinical and biological outcomes that were not determined in advance. Accordingly, response was considered complete or partial.

Patient and public involvement

Recruited patients were not involved in the project apart from being research subjects.

Murine experiments

In vivo experiments were conducted in mice to explore the potential stabilising effect of IL-18BP on circulating IL-18. Methods are described in detail in online supplemental file 1.

Statistical analyses

Statistical analyses were performed in GraphPad Prism V.10.3.1. Variables were considered non-parametric. Age, disease duration and disease activity were shown as medians with IQR. Levels of cytokines and their inhibitors, CRP and ferritin were represented as individual values (dot plots) and expressed as medians with IQR. The Mann-Whitney test was used to compare two groups, and the Kruskal-Wallis test to compare >2 groups. Correlations were made using the Spearman's rank correlation analysis. Receiver operating characteristic (ROC) curves were drawn to assess the diagnostic sensitivity and specificity for Still's disease of the different tested biomarkers.

RESULTS

Patients, controls and samples

273 adult and paediatric patients with AIDS and 49 controls were included in the study. 53 patients had mSAID and 220 had guSAID. Precise diagnoses are reported in table 1table 1. Still's disease was the most represented AID, followed by inflammation of unknown origin (IUO), chronic recurrent multifocal osteomyelitis (CRMO), FMF and Behçet's disease. 55 patients (20.1%) were 16 or younger. Paediatric patients were twice more represented in guSAID (22.3%) than in mSAID (11.3%), CRMO being the disease group with the highest number of children (20/33 patients (63.6%)). Patients were mainly of Caucasian origin. However, patients with Behçet's disease were mostly of eastern Caucasian or northern African origin, and 40% of neutrophilic dermatitis (ND) patients were from African origin. The FMF group comprised patients from multiple ethnic backgrounds.

Disease duration was significantly longer in mSAID than in guSAID (204 months (IQR: 104.5–408) vs 36 months (7.5–95.5), p<0.0001). Disease activity did not differ between mSAID and guSAID (18 (IQR: 15–21) vs 20,[14–22] p=0.1284) nor between single diseases. Treatments at inclusion are reported in table 1. In total, 322 serum samples from V1 (one sample per patient and control) and 28 serum samples from V2 were received for biomarker level assessment.

Biomarkers of inflammation and cytokine levels in the whole cohort

The serum levels of CRP, ferritin, IL-1β, IL-1Ra, total IL-18 (bound and unbound to IL-18BP), free IL-18 (unbound to IL-18BP), IL-18BP and IFN-γ were measured in all collected sera at V1 (figure 1).

Median CRP concentration in the SAID patients was 44.5 mg/L (IQR: 13.8–83 mg/L). CRP was significantly higher in comparison to controls in all disease groups except CRMO, Takayasu, ND and Kawasaki. CRP levels in CRMO were significantly lower than in patients with Still's, IUO, FMF and recurrent pericarditis (RP), whereas CRP levels in Behçet's disease were significantly lower than in Still's disease. Ferritinaemia was by far the highest in Still's disease, with a median value of 944 µg/L (214–3595) as compared with 155 µg/L (72.5–303.3) in the whole SAID cohort. Ferritin levels were also significantly higher in RP 209 µg/L (166–236.5) than in controls and CRMO.

Table 1 Characteristics of patients at inclusion (V1)

Diseases		Number of patients	Age(median, [IQR])	Number of individuals ≤ 16 yo (%)	% of females	Ethnicity (%)	Disease duration (months, median, [IQR])	Disease activity at inclusion(median, [IQR])	Ongoing treatment at inclusion(patient number (%))
monogenic SAIDs	Familial Mediterranean Fever (FMF)	31	34 [20.5-46.5]	4 (12.9)	64.5	European caucasian: 25.8 Eastern caucasian or Northern African: 22.6Sephardi Jewish: 16.1Mizrahi Jewish: 0African: 19.4Asian: 6.5Mixed: 9.7	180 [79-393]	20 [15.5-23.5]	None: 6 (19.4)Colchicin: 23 (74.2) IL-1 inhibition: 5 (16.1)NSAID: 3 (9.7) Anti-TNF: 1 (3.2)
	TNF-Receptor Associated Periodic Syndrome (TRAPS)	8	49.5 [34-59.5]	1 (12.5)	62.5	European caucasian: 100 Eastern caucasian or Northern African: 0Sephardi Jewish: 0Mizrahi Jewish: 0African: 0Asian: 0Mixed: 0	222 [120-513]	15 [11-16]	None: 4 (50)Corticosteroids: 2 (25) mean dose: 22.5 mg/dayColchicin: 1 (12.5)IL-1 inhibition: 1 (12.5)
	Cryopyrin-Associated Periodic Syndromes (CAPS)	7	35 [30-38]	0 (0)	28.6	European caucasian: 100 Eastern caucasian or Northern African: 0Sephardi Jewish: 0Mizrahi Jewish: 0African: 0Asian: 0Mixed: 0	90 [25.5-306]	13.5 [12.25-17]	None: 5 (71.4)IL-1 inhibition: 2 (28.6) Corticosteroids: 1 (14.3) dose: 60 mg/dayNSAID: 1 (14.3)
	Mevalonate Kinase Deficiency (MKD)	7	28 [19-34.5]	1 (14.3)	42.9	European caucasian: 71.4 Eastern caucasian or Northern African: 0Sephardi Jewish: 0Mizrahi Jewish: 0African: 28.6Asian: 0Mixed: 0	288 [206-396]	19 [17.5-19.5]	None: 4 (57.1)Corticosteroids: 1 (14.3) dose: unknownMethotrexate: 1 (14.3)IL-1 inhibition: 1 (14.3)
genetically undiagnosed SAIDs	Still's disease	61	30 [23-37]	11 (18)	54.1	European caucasian: 72.1Eastern caucasian or Northern African: 4.9Sephardi Jewish: 0 Mizrahi Jewish: 0African: 16.4Asian: 1.6Mixed: 4.9	9.5 [1.4-43.5]	21 [17-24.25]	None: 26 (42.6)Corticosteroids: 25 (41) median dose: 16.25 mg/dayIL-1 inhibition: 9 (14.8)NSAID: 6 (9.8) Methotrexate: 3 (4.9)IL-6 inhibition: 3 (4.9)

Continued

Table 1 Continued

Diseases		Number of patients	Age(median, [IQR])	Number of individuals ≤ 16 yo (%)	% of females	Ethnicity (%)	Disease duration (months, median, [IQR])	Disease activity at inclusion(median, [IQR])	Ongoing treatment at inclusion(patient number (%))
	Inflammation of Unknown Origin (IUO)	47	44 [21-50]	8 (17)	61.7	European caucasian: 66 Eastern caucasian or Northern African: 6.4Sephardi Jewish: 2.1Mizrahi Jewish: 2.1African: 10.6Asian: 4.3Mixed: 8.5	40 [11-222]	21 [18-25]	None: 25 (53.2)Corticosteroids: 8 (17) median dose: 30 mg/dayColchicin: 7 (14.9)NSAID: 5 (10.6)IL-1 inhibition: 5 (10.6) Hydroxychloroquine : 2 (4.2)IL-6 inhibition: 1 (2.1)JAK inhibition: 1 (2.1)Methotrexate: 1 (2.1)
	ChronicRecurrent Multifocal Osteomyelitis (CRMO)	33	14 [12-18]	20 (63.6)	63.6	European caucasian: 75.8 Eastern caucasian or Northern African: 3Sephardi Jewish: 0Mizrahi Jewish: 0African: 15.1Asian: 0Mixed: 6.1	36 [8-60]	18 [10.5-20.5]	None: 6 (18.2)NSAID: 17 (51.5) Anti-TNF: 9 (27.3)Bisphosphonate: 8 (24.2)Calcium antagonist: 4 (12.1)Corticosteroids: 3 (9.1) mean dose: 10 mg/dayColchicin: 2 (6.1) Methotrexate: 2 (6.1)IL-6 inhibition: 1 (3)Anti-IL-17: 1 (3)
	Behçet's vasculitis	30	34.5 [21-43]	1 (3.3)	26.7	European caucasian: 36.7 Eastern caucasian or Northern African: 40Sephardi Jewish: 3.3Mizrahi Jewish: 0African: 10Asian: 0Mixed: 10	77.5 [36-174]	19 [8.5-21]	None: 9 (30)Colchicin: 11 (36.7) Corticosteroids: 8 (26.7) median dose: 35 mg/dayanti-TNF: 4 (13.3)Methotrexate: 4 (13.3) Apremilast: 3 (10)NSAID: 2 (6.7) Leflunomide: 1 (3.3)Azathioprine: 1 (3.3)Cyclophosphamide: 1(3.3) Mycophenolate mofetil: 1 (3.3)IVIg: 1 (3.3)
	Recurrent pericarditis (RP)	18	19.5 [15-34]	7 (38.9)	50	European caucasian: 72.2 Eastern caucasian or Northern African: 11.1Sephardi Jewish: 0Mizrahi Jewish: 0African: 11.1 Asian: 0Mixed: 5.6	29.5 [12-75]	21 [16.75-21]	None: 1 (5.6)Colchicin : 13 (72.2) NSAID: 9 (50)Corticosteroids: 9 (50) median dose: 10 mg/dayIL-1 inhibition: 3 (16.7)Aspirin: 2 (11.1) Azathioprine: 1 (5.6)
	Takayasu's vasculitis	14	34 [45.5-54.5]	0 (0)	85.7	European caucasian: 64.3 Eastern caucasian or Northern African: 14.3Sephardi Jewish: 0Mizrahi Jewish: 0African: 21.4Asian: 0Mixed: 0	52 [12-114]	19 [16-20.25]	None: 4 (28.6)Corticosteroids: 6 (42.9) median dose: 55 mg/dayAzathioprine: 5 (35.7) Methotrexate: 2 (14.3)Anti-TNF: 2 (14.3)Aspirin: 2 (14.3)Mycophenolate mofetil: 1 (7.1)

Continued

Table 1 Continued

Diseases		Number of patients	Age(median, [IQR])	Number of individuals ≤ 16 yo (%)	% of females	Ethnicity (%)	Disease duration (months, median, [IQR])	Disease activity at inclusion(median, [IQR])	Ongoing treatment at inclusion(patient number (%))
	Schnitzler's syndrome	11	68 [61-76]	0 (0)	63.6	European caucasian: 90.9 Eastern caucasian or Northern African: 9.1Sephardi Jewish: 0Mizrahi Jewish: 0African: 0Asian: 0Mixed: 0	48 [34-160.5]	20.5 [16.5-21.5]	None: 7 (63.6)IL-1 inhibition : 3 (27.3)NSAID: 1 (9.1)Corticosteroids: 1 (9.1) Dose: 10 mg/day Aspirin: 1 (9.1)
	Neutrophilic dermatosis	5	52 [31-67]	1 (20)	60	European caucasian: 40 Eastern caucasian or Northern African: 0Sephardi Jewish: 20Mizrahi Jewish: 0African: 40 Asian: 0Mixed: 0	12 [0.3-168]	16 [11.5-21.5]	None: 2 (40)Colchicin: 2 (40) Corticosteroids : 1 (20) Dose: 5 mg/ dayDapsone: 1 (20)
	Kawasaki disease	1	4	1 (100)	0	European caucasian: 0 Eastern caucasian or Northern African: 0Sephardi Jewish: 0Mizrahi Jewish: 0African: 0Asian: 0Mixed: 100	0.2	14	None : 1 (100)
Non inflammatory controls		49	31 [27-42]	2 (4)	53.1	European caucasian: 75.5 Eastern caucasian or Northern African: 8.2Sephardi Jewish: 0Mizrahi Jewish: 0African: 4.1Asian: 4.1Mixed: 8.2	NA		

Corticosteroids dose is given as prednisone equivalent.
*Disease activity is assessed through a composite score which is the sum of fatigue (from 0: Absent - to 10: Extreme), patient global assessment (from 0: Inactive disease – to 10: Extremely active disease) and physician global assessment (from 0: Inactive disease – to 10: Extremely active disease).
IL, Interleukin; IQR, Interqurtile range; IVIg, Intravenous Immunoglobulins; JAK, Janus Kinase; NSAID, Non-Steroidal Anti Inflammatory Drugs; SAIDs, Systemic AutoInflammatory Diseases; TNF, Tumor Necrosis Factor; yo, year old.

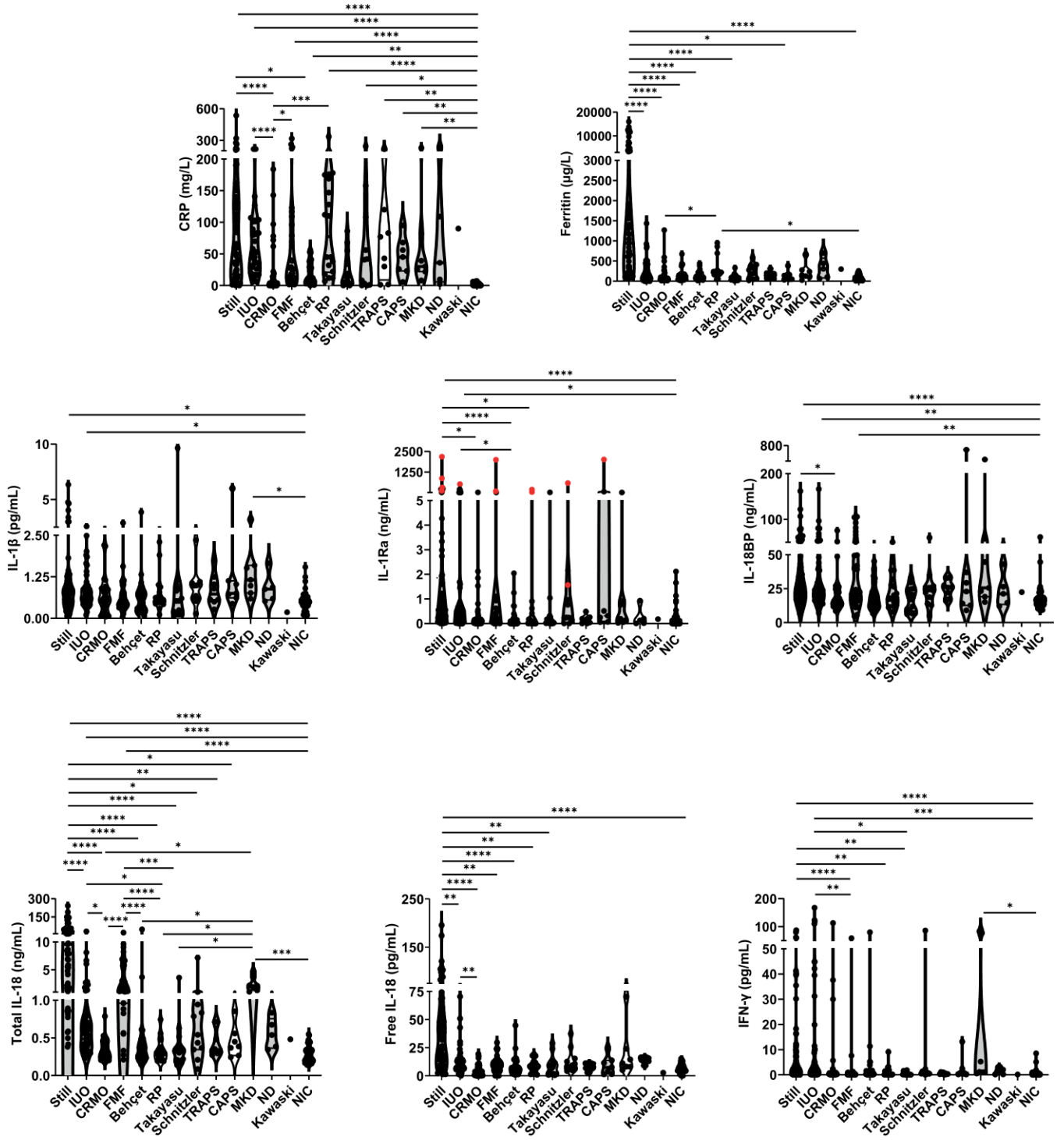


Figure 1 Levels of biomarkers of inflammation, cytokines and their inhibitors at first visit in the whole cohort. Individual measurements are represented as dots. For each disease group and non-inflammatory controls (NICs), results are shown as median (thick bars) and first and third quartiles (thin bars). On the graph showing serum levels of IL-1Ra, red dots represent patients treated with anakinra at the time of blood sampling. Comparisons between groups are performed using the Kruskal-Wallis test. Only significant differences are shown. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. CAPS, cryopyrin-associated periodic syndrome; CRMO, Chronic Recurrent Multifocal Osteomyelitis; FMF, familial Mediterranean fever; IUO, inflammation of unknown origin; MKD, Mevalonate Kinase Disease; ND, neutrophilic dermatosis; RP, recurrent pericarditis; TRAPS, TNF receptor-associated periodic syndrome.

Median IL-1β levels in the whole SAID cohort were 0.64 pg/mL (0.48–0.89). They were significantly higher in patients with Still's disease, IUO and MKD (respectively, 0.76 (0.46–1.13), 0.72 (0.52–0.91) and 1.16 (0.87–1.55) pg/mL) than in controls (0.49 (0.38–0.62) pg/mL). IL-1Ra serum levels were significantly higher in patients

with Still's disease and IUO (respectively, 0.55 (0.20–1.78) and 0.37 (0.11–0.65) ng/mL) than in patients with Behçet's disease and controls (respectively, 0.03 (0–0.22) and 0.10 (0–0.26) ng/mL). IL-1Ra levels were also significantly higher in Still's disease than in CRMO (0.14 (0.03–0.28) ng/mL). Of note, some patients with the highest serum levels of IL-1Ra were treated with anakinra (recombinant human IL-1Ra) at the time of blood collection, thus further statistical analyses were performed after exclusion of these cases. IL-1Ra serum levels remained higher in Still's disease and IUO than in Behçet's disease ($p<0.0001$ and $p=0.0102$, respectively) and controls ($p<0.0001$ and $p=0.0308$). IL-1Ra serum levels in Still's disease were also higher than in RP ($p=0.0165$).

Serum levels of total IL-18 were significantly more elevated in patients with Still's disease, IUO, FMF and MKD than in controls, with the highest levels in Still's disease (9.61 ng/mL (1.84–53.73)). Total IL-18 concentrations were also significantly higher in Still's disease than in most diseases, but not in comparison to FMF and MKD. Of note, serum levels of total IL-18 above 46 ng/mL were only measured in patients with Still's disease and present in 31% of these patients. Free IL-18 levels were the highest in patients with Still's disease (35.98 pg/mL (14.60–60.91)). As opposed to total IL-18, free IL-18 concentrations were significantly higher in patients with Still's disease than in FMF ($p=0.0016$). Extremely high levels of free IL-18 (≥ 70 pg/mL) were specifically present in Still's disease, but only in a minority of patients (11/61, 18%). IL-18BP serum levels were significantly higher in Still's disease, IUO and FMF than in controls. They did not differ significantly between Still's disease and FMF ($p>0.9999$). A profile of total IL-18, free IL-18 and IL18BP serum levels in patients with Still's disease or FMF is shown in online supplemental figure 1. Serum levels of IFN-γ were significantly higher in patients with Still's disease, IUO and MKD than in controls. They were also significantly higher in Still's disease and IUO than in FMF and Takayasu, and higher in Still's disease than in RP.

IL-18BP stabilises IL-18 in the circulation

Serum levels of total IL-18 were considerably higher than those of free IL-18 in all measured samples. In addition, we observed a discrepancy between total and free IL-18 levels in FMF patients that was not explained by higher IL-18BP concentrations. We, therefore, decided to examine whether IL-18BP, in addition to its inhibitory role, could act as a stabiliser of IL-18 in the serum via the formation of IL-18/IL-18BP complexes. For these experiments, we used knockout mice deficient in IL-18BP (*Il18bp*$^{-/-}$) and wild-type (WT) mice that were either naïve or submitted to repeated injections of CpG, a toll-like receptor 9 agonist, that is, known to induce manifestations comparable to macrophage activation syndrome.[23] Total IL-18 levels were significantly lower in *Il18bp*$^{-/-}$ than in WT mice, both in naïve and CpG-stimulated conditions. Treatment of *Il18bp*$^{-/-}$ mice with recombinant IL-18BP resulted in an increase in total IL-18 concentrations comparable to those present in WT mice (online supplemental figure 2).

Correlations between cytokine levels and disease activity

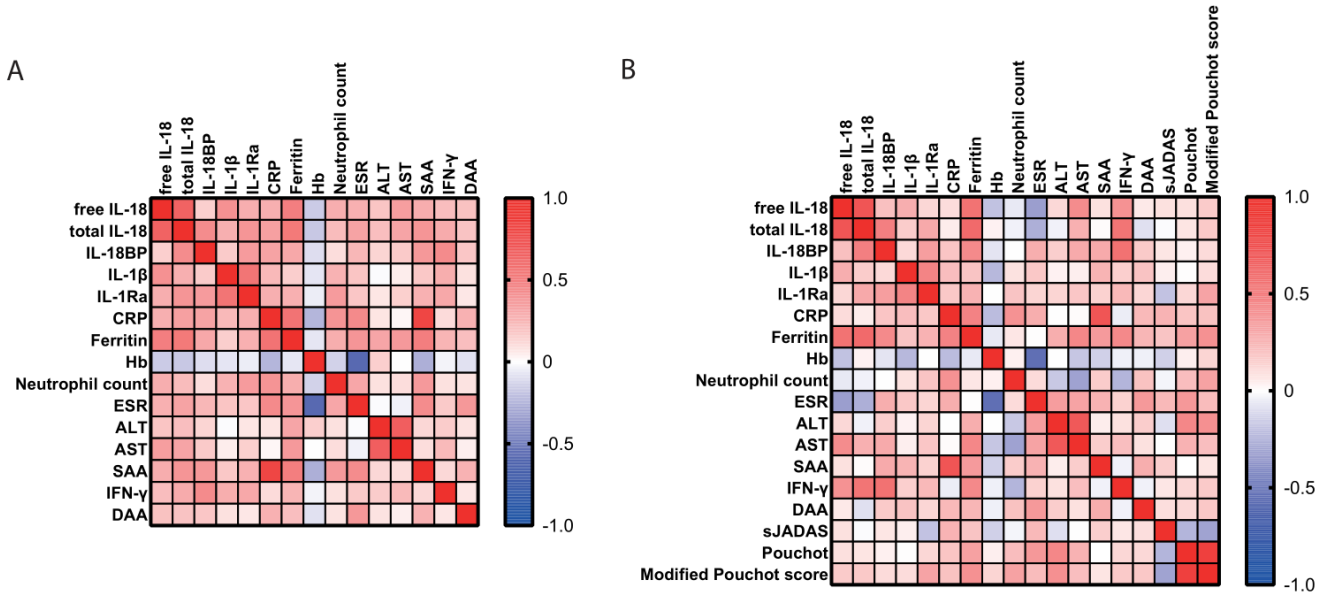
As depicted in figure 2A, we established correlations between the levels of each cytokine and their inhibitors and other potential biomarkers of disease activity (CRP, ferritin, ESR, neutrophil count, haemoglobin level, transaminase levels and SAA) or disease activity scores (Disease activity assessment for the whole cohort, sJADAS, Pouchot and modified Pouchot's score for Still's disease).

In the whole SAID cohort, the strongest correlation was observed between ferritinaemia and total and free IL-18 levels (r=0.527 and 0.489, respectively) (figure 2A). AST also correlated with total and free IL-18 (r=0.321 and 0.346, respectively). The levels of total and free IL-18 correlated significantly (r=0.645). IL-18BP correlated with total IL-18 (r=0.432) and IFN-γ (r=0.442), but only weakly with free IL-18 (r=0.149). IL-1β correlated with IL-1Ra (r=0.552), free IL-18 (r=0.411) and total IL-18 (r=0.274, $p<0.0001$). We found a robust correlation between CRP and SAA (r=0.804). Ferritinaemia correlated with CRP (r=0.553) and SAA (r=0.500). The disease activity assessment score correlated with markers of inflammation (r=0.373, 0.266, 0.262 for ESR, CRP and SAA, respectively), and only weakly with free and total IL-18 and IL-18BP (r=0.201, $p=0.002$; r=0.198, $p=0.002$; r=0.183, $p=0.005$, respectively) and not with IL-1β and IL-1Ra.

Given the specificities of Still's disease in terms of cytokine profile and ferritin levels, and as it represented the largest sample size (61 cases), we decided to analyse this group of patients separately (figure 2B). There was a positive correlation between serum levels of ferritin and IL-18 (r=0.600 for total IL-18, r=0.558 for free IL-18) and IL-18BP (r=0.444). Free IL-18 correlated with AST (r=0.432). Levels of IFN-γ correlated with total and free IL-18 (r=0.550 and r=0.404), as well as with IL-18BP levels (r=0.565) and ferritin (r=0.448). Total IL-18, but not free IL-18, correlated with IL-18BP (r=0.491). IL-1β correlated with IL-1Ra (r=0.470). None of the clinical scores (Disease activity assessment score as well as dedicated sJADAS, Pouchot's or modified Pouchot's scores) correlated with the levels of cytokines or their inhibitors. We also compared the levels of total and free IL-18 in patients with Still's disease according to the clinical presentation at inclusion (ie, presence or absence of cutaneous rash, type of musculoskeletal involvement—arthralgia vs arthritis, myalgias) but found no significant differences between subgroups (data not shown).

Identification of diagnostic biomarkers for Still's disease

Figure 3 shows that total IL-18 was the most sensitive and specific biomarker for Still's disease with an area under the curve (AUC) of 0.9103 ($p<0.0001$). Both free IL-18 and ferritin were a bit less powerful than total IL-18 (AUC=0.8295, $p<0.0001$ and 0.8549, $p<0.0001$).



Biomarkers	Correlated biomarkers	r	p
Free IL-18	Total IL-18	0.645	<0.0001
	IL-1β	0.411	<0.0001
	Ferritin	0.496	<0.0001
Total IL-18	IL-18BP	0.432	<0.0001
	Ferritin	0.527	<0.0001
IL-18BP	IFN-γ	0.442	<0.0001
IL-1β	IL-1Ra	0.552	<0.0001
CRP	Ferritin	0.553	<0.0001
	ESR	0.444	<0.0001
	SAA	0.804	<0.0001
Ferritin	SAA	0.500	<0.0001
Hemoglobin	ESR	-0.574	<0.0001
ESR	SAA	0.438	<0.0001
ALT	AST	0.666	<0.0001

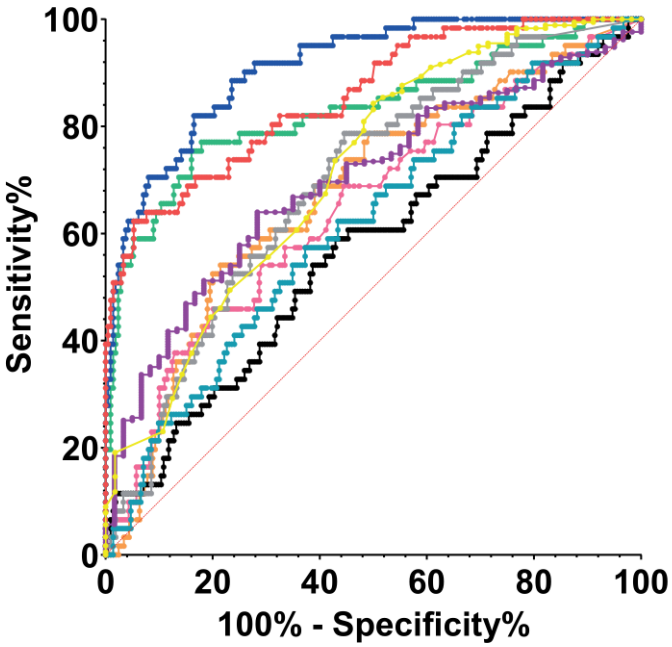
Biomarkers/scores	Correlated biomarkers/scores	r	p
Free IL-18	Total IL-18	0.719	<0.0001
	IFN-γ	0.404	0.0012
	Ferritin	0.558	<0.0001
	AST	0.432	0.0009
Total IL-18	IL-18BP	0.491	<0.0001
	IFN-γ	0.550	<0.0001
	Ferritin	0.600	<0.0001
IL-18BP	IFN-γ	0.565	<0.0001
	Ferritin	0.444	0.0003
IL-1β	IL-1Ra	0.470	0.0001
CRP	Ferritin	0.475	0.0001
	Neutrophil count	0.407	0.001
	SAA	0.719	<0.0001
Ferritin	AST	0.455	0.0004
	IFN-γ	0.448	0.0003
Hemoglobin	ESR	-0.548	0.006
ALT	AST	0.691	<0.0001
	Pouchot score	0.458	0.0005
	Modified Pouchot score	0.414	0.002
Pouchot score	Modified Pouchot score	0.847	<0.0001

Figure 2 Correlations between cytokines and other markers of disease activity. Spearman correlation analyses were performed in the whole disease cohort (A) and in patients with Still's disease only (B) between measured cytokines and their inhibitors and biological and clinical markers of disease activity collected at first visit (V1). Results are shown as heatmaps, with r coefficient represented as gradation of red (for positive correlation) and blue (for negative correlation). Strongest correlations, namely r values >0.4 or <–0.4 are shown in tables below, with corresponding p values. ALT, alanine transaminase; AST, aspartate transaminase; CRP, C reactive protein; DAA, disease activity assessment; ESR, erythrocyte sedimentation rate; Hb, haemoglobin; SAA, serum amyloid A.

Other biomarkers, including IL-1Ra, IFN-γ and CRP, were less useful to differentiate Still's disease from other autoinflammatory diseases (figure 3). Combining two biomarkers (including total IL-18) did not allow reaching a better AUC than total IL-18 alone (data not shown). A total IL-18 cut-off value of 1.372 ng/mL was associated with the diagnosis of Still's disease with a sensitivity and specificity of 81.97% and 83.49%, respectively.

Changes in cytokine levels and their inhibitors in patients with Still's disease

28 patients with guSAID who were not treated at the time of V1 had a blood collection during visit 2 (3±1 months after V1). We analysed the variations of serum levels of cytokines and inhibitors between V1 and V2 in Still's disease, which was the largest represented group at V2 (10 cases). Eight patients were still under treatment at V2. One received prednisone, which was stopped 1 month before V2. The last patient was treated with non-steroidal anti-inflammatory drugs only. Figure 4A shows IL-1β, IL-1Ra, total IL-18, free IL-18, IL-18BP and IFN-γ levels at V1 and V2. Free IL-18 levels decreased significantly between V1 and V2. Extremely high levels of IL-1Ra were observed at V2 in one patient treated with anakinra. When removing patients treated with anakinra (n=4), the serum levels of IL-1Ra between V1 and V2 were also not significantly different. Figure 4B shows that the levels



Biomarkers	AUC	p
Total IL-18	0.9103	<0.0001
Ferritin	0.8549	<0.0001
Free IL-18	0.8296	<0.0001
AST	0.7146	<0.0001
SAA	0.6967	<0.0001
IL-1Ra	0.6961	<0.0001
IFN-γ	0.6758	<0.0001
CRP	0.6493	0.0004
IL-18BP	0.6157	0.0059
IL-1β	0.5716	0.0882

Figure 3 ROC curves of total and free IL-18, IFN-γ, ferritin and CRP as diagnostic biomarkers of Still's disease. The sensitivity and 100%-specificity of each biomarker to differentiate Still's disease from other autoinflammatory diseases are shown. Areas under the curve (AUC) and p values are reported in the table for each biomarker. Cytokines, ferritin, CRP, SAA and AST levels at first visit (V1) were used for analyses. AST, aspartate aminotransferase; CRP, C reactive protein; ROC, receiver operating characteristic; SAA, serum amyloid A.

of free IL-18 decreased significantly in patients achieving complete remission at V2 (p=0.0079) but not in patients in partial remission.

DISCUSSION

We measured serum levels of IL-1β, IL-18 and their inhibitors, as well as IFN-γ, in a unique prospective cohort of 273 patients with SAID and showed that Still's disease differs from other SAID with a specific cytokine profile characterised by elevated total and free IL-18 as well as increased concentrations of IFN-γ. Also, we established a strong correlation between serum levels of IL-18 and ferritin, but not with other biological or clinical markers of disease activity. Moreover, we could not identify a particular clinical presentation of Still's disease associated with higher IL-18 levels. Drawing ROC curves, we demonstrated that total IL-18 allowed the diagnosis of Still's disease, among other SAID, with good sensitivity and specificity, ahead of ferritin and free IL-18, and was a much more accurate diagnostic marker than any other measured cytokines and inhibitors. Finally, longitudinal analyses in Still's disease patients untreated at inclusion showed that, among cytokines, free IL-18 was the only measured parameter that significantly decreased after treatment, particularly in patients who achieved complete remission as assessed by treating physicians.

Several publications have indicated that serum levels of IL-18 are elevated in Still's disease patients, in both children, the so-called sJIA, as well as in AOSD, now consensually considered as a unique disease.[5 7] IL-18 is a potent pro-inflammatory cytokine, the biological activity of which is tightly regulated by various mechanisms, including its naturally occurring inhibitor, IL-18BP, that binds to IL-18 as a decoy receptor and prevents its interaction with IL-18 receptors (IL-18R).[14] Importantly, the binding affinity of IL-18BP to its ligand is largely superior to that of IL-18R, thus considerably limiting the biologic activity of IL-18.[15] IL-18BP is present in large excess in the blood, so that most of circulating IL-18 is biologically inactive.[16] The available commercial immunoassays for IL-18 detection do not distinguish IL-18 present in IL-18BP/IL-18 complexes and free bioactive IL-18. We previously developed and validated an immunoassay that allows specific measurement of free IL-18 and demonstrated that free IL-18 was uniquely detectable in Still's disease, in comparison to other rheumatic diseases like rheumatoid arthritis, psoriatic arthritis, ankylosing and systemic lupus erythematosus spondylarthritis,[9] or some mSAID, including CAPS or FMF.[17 18]

Noteworthy, Priori *et al* had previously published that serum levels of IL-18 were significantly higher in Still's disease than in sepsis, a condition that can mimic a flare of SAID.[19] Additionally, in a collaborative study, we showed that IL-18 levels were not elevated in critically ill COVID-19 patients as opposed to patients with Still's disease and macrophage activation syndrome.[20]

We show that free IL-18 is specifically elevated in adult and paediatric patients with Still's disease, in a large and diverse cohort of monogenic and guSAID. Of note, a few patients diagnosed as having IUO, who, by definition, did not fulfil the diagnostic criteria for other SAID, displayed high levels of free IL-18, raising the hypothesis that some

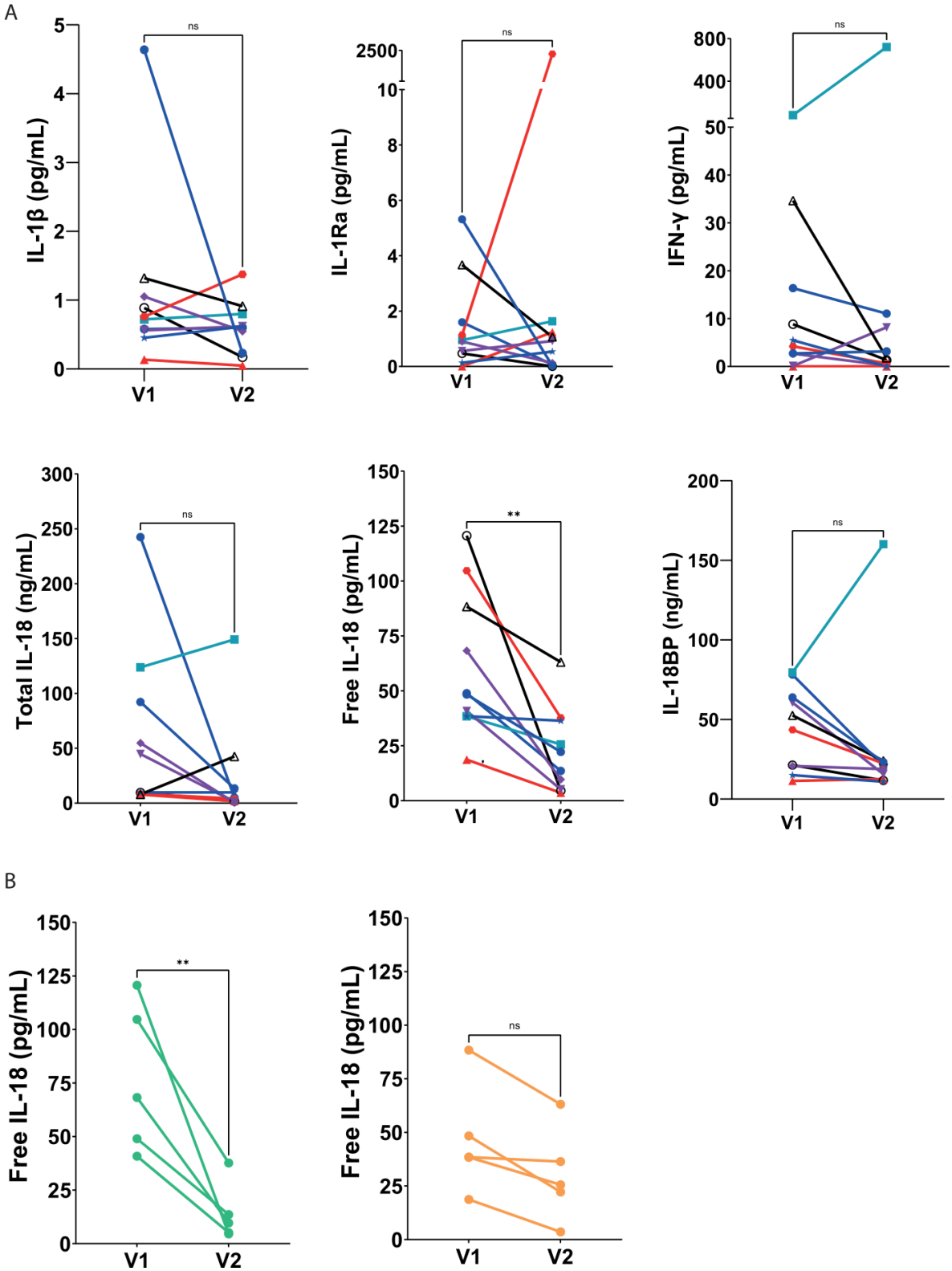


Figure 4 Levels of cytokines and their inhibitors at first and second visits in Still's disease. Serum levels of IL-1β, IL-1Ra, IFN-γ, total IL-18, free IL-18 and IL-18BP of 10 patients with Still's disease are individually reported at V1 and V2 and compared between both visits using the Mann-Whitney test (ns=p>0.05; **p<0.01 but >0.001) (A). Each symbol represents a single patient. Empty symbols and black lines represent patients without any treatment at V2. Blue symbols and lines represent patients treated with prednisone alone at V2. Red symbols and lines represent patients treated with anakinra alone at V2. Violet symbols and lines represent patients treated with prednisone and anakinra at V2. Teal blue squares and lines represent one patient receiving prednisone and tocilizumab at V2. Serum levels of free IL-18 are additionally reported according to disease activity at V2, with patients achieving complete remission in green, and patients in partial remission **at** V2 in orange (B). Again, the Mann-Whitney U test was used to compare free IL-18 levels between V1 and V2 (ns=p>0.05; **p<0.01 but >0.001).

of these patients may have been misclassified as IUO instead of Still's disease, because of incomplete clinical presentation.

Total IL-18 levels were not statistically different in FMF and Still's disease. In contrast, free IL-18 was not detected in FMF sera. This result is not related to higher levels of IL-18BP—which could have buffered free IL-18—in FMF. Rather, although the difference is not statistically significant, it can be explained by total IL-18 levels in FMF patients that do not reach the extremely high levels present in some patients with Still's disease. These extremely high levels of total IL-18 specifically found in patients with Still's disease in our cohort (>46 ng/mL) may overwhelm the binding capacity of IL-18BP, resulting in the detection of circulating free IL-18. The presence of total IL-18 in the absence of free IL-18 can be explained by the stability of IL-18BP/IL-18 complexes in the circulation. Consistent with this finding, we previously observed that on treatment with recombinant IL-18BP, serum levels of free IL-18 became rapidly undetectable, while total IL-18 levels remained unchanged for several days.[21] Levels of total IL-18 are also significantly lower in IL-18BP deficient mice, where they can be restored by the administration of recombinant IL-18BP. Although these experiments have not been performed in human samples, we hypothesise that a similar stabilising effect of IL-18BP takes place in human circulation due to comparable IL-18BP structure and biology.[14] We, therefore, hypothesise that, in FMF patients, IL-18 released from pyroptotic cells, coincidently with IL-1β upon pyrin inflammasome activation, is rapidly complexed with IL-18BP and detected in the ELISA for total IL-18. One of the potential differences between FMF and Still's disease is the kinetics of inflammatory flares, which are substantially longer in Still's disease than in FMF, with higher and longer levels of IL-18 production. We and others have shown that blocking IL-18 is effective in some patients with Still's disease.[8 22 24]

Of note, not all patients with Still's disease had elevated free IL-18. The order of magnitude of free IL-18 levels was relatively low even in patients with Still's disease, as previously reported.[9 17] Free IL-18 levels up to 15 pg/mL must be considered as normal since healthy controls had values in this range. Importantly, we have observed that low levels of free IL-18 cannot be used to rule out a response to Tadekinig alfa in adults with Still's disease.[8] On the other hand, the highest levels (70 pg/mL and above) of free IL-18 appear to be specific to Still's disease. This relative lack of sensitivity explains that the determination of total IL-18 exhibits a better diagnostic accuracy than free IL-18 as demonstrated by AUC values.

As previously reported, ferritin levels correlated nicely with total and free IL-18 in Still's disease[9] but also in the whole cohort, though slightly less strongly. A robust correlation between IL-18 and IFN-γ was present in Still's disease, reflecting the role of IL-18 as an IFN-γ-inducing factor. Similarly, we observed a correlation between IFN-γ and IL-18BP that is consistent with the stimulatory effect of IFN-γ.[25] Of note, there was no correlation between IL-18 and clinical scores of disease activity as well as between IL-18 and the recently published Still's activity score[26] (data not shown). It is plausible that this finding is due to the inclusion of patients with active disease according to predefined recruitment criteria.

IL-1β and IL-1Ra measurements were far less informative to distinguish a specific subset of patients with SAID. Although we used a highly sensitive technique to measure IL-1β, its serum levels remained very low, even in diseases that respond to IL-1 blockers, like CAPS, FMF or Schnitzler's syndrome.[6]

Despite providing original and convincing insights for diagnostic strategies in SAID, and especially Still's disease, we recognise that our work has some noticeable limitations. Indeed, although this is to our knowledge the largest cohort of SAID ever built, some diseases such as mSAID were underrepresented while Still's disease was the largest group. This may have impaired our ability to find significant differences in the measured biomarkers as well as to identify potential associations with specific clinical presentations in mSAID and other rare guSAID. Some other mSAID, such as interferonopathies, were not included in the study. Nonetheless, we previously published that free IL-18 serum levels were not detectable in patients with CANDLE and SAVI.[17] The inclusion of children in our study was also lower than that of adults, particularly in the case of Still's disease and healthy controls, thus impairing age-matched comparison. The number of patients with longitudinal follow-up was relatively scarce, thus limiting the power of our analysis regarding the clinical relevance of cytokines as biomarkers of response to therapy.

In conclusion, the result of this unique SAID cohort demonstrated that serum levels of IL-18 were higher in patients with Still's disease and that total IL-18 was the best biomarker to distinguish these patients from other SAID.

Author affiliations
[1]Department of Medicine, University Hospitals, Geneva, Switzerland
[2]Department of Pathology and Immunology, University of Geneva, Geneva, Switzerland
[3]Immunology, Erasmus MC, Rotterdam, The Netherlands

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Collaborators Serge Amselem (Childhood genetic diseases, Hôpital Trousseau, Sorbonne Université, Paris, France); Jordi Anton (Pediatric rheumatology. Hospital Sant Joan de Déu. Institut de Recerca Sant Joan de Déu. Universitat de Barcelona, Spain); Tadej Avcin (Department of Allergology, Rheumatology and Clinical Immunology, University Children's Hospital, University Medical Centre Ljubljana, Ljubljana, Slovenia); Alexandre Belot (Centre International de Recherche en Infectiologie (CIRI), Univ Lyon, Lyon, France); Violeta Bittermann (Commissariat À L'énergie Atomique Et Aux Énergies Alternatives (CEA), Centre National de Recherche en Génomique Humaine (CNRGH), Université Paris-Saclay, Evry, France); (Anne Boland) Department of Rheumatology. University Hospital Ramón y Cajal. Madrid, Spain; Alina Boteanu (APHP, Saint Louis Hospital, Paris Cité University, Paris, France); Jean-David Bouaziz (Novo Nordisk Foundation

Center for Protein Research, University of Copenhagen, Copenhagen, Denmark); Søren Brunak (Department of biomedical and clinical sciences, University of Milano, Italy); Sonia Caccia (Rheumatology Unit Department of Medical Sciences, Surgery and Neurosciences, University of Siena, Siena, Italy); Luca Cantarini (OWKIN France, Paris, France); Sabrina Carpentier (Department of Immunology, Université de Paris, Paris, France); Arturo Hernandez Cervantes (Department Recherche Clinique et Innovation, Hôpital de La Pitiè Salpetrière, Paris, France); Emna Chabaane (Institut Curie, Paris, France; PSL Research University, Paris, France; Mines Paris Tech, Fontainebleau, France); Fanny Coffin (Laboratory of Rheumatology, GIGA-Research, CHU Liège, University of Liège, Liège, Belgium); Dominique de Seny (AMBIOTIS SAS, Toulouse, France); Henri de Soyres (Pediatric rheumatology department and CEREMAIA, Bicêtre hospital APHP, University of Paris Saclay, France); Jean-François Deleuze (Internal Medicine Ospedale Fatebene fratelli, Milano, Italy); Marc Dubourdeau (Department of Immunology, Erasmus University Medical Center, Rotterdam, the Netherlands); Perrine Dusser (Laboratory of Immunobiology, Center for Clinical, Experimental Surgery and Translational Research, Biomedical Research Foundation of the Academy of Athens, Athens, Greece); Emanuele Bizzi (Department of Rheumatology, Pitie Salpetriere University Hospital, Sorbonne Université, UPMC, Paris, France); Stefan J Erkeland (Department of Pediatric Rheumatology and Immunology, University of Muenster, Muenster, Germany); Evangelos Andreakos (Unit of Medical Technology and Intelligent Information Systems, Department of Materials Science and Engineering, University of Ioannina, Greece); Bruno Fautrel (Department of Internal Medicine, University of Geneva, Geneva, Switzerland); Dirk Foell (Internal medicine department, Tenon hospital, Sorbonne University, Paris, France); Dimitrios I. Fotiadis (Department of Microbiology, Immunology and Transplantation, Rega Institute for Medical Research, KU Leuven, Leuven, Belgium); Cem Gabay (Département de Médecine Interne et Immunologie Clinique, University of Lille, Lille, France); Apolline Gallois (Department of Internal Medicine, Division of Rheumatology, Marmara University, School of Medicine, Istanbul, Turkey); Sara Garcia (Rheumatology Department. Groupe Hospitalier Paris Saint-Joseph, Paris, France); Sophie Georgin-Lavialle (Pediatric immuno-rheumatology, Dept Pediatrics, CHUV, University Hospital of Lausanne, Lausanne, Switzerland); Charlotte Girard (Laboratory of Adaptive Immunology, Department of Microbiology, Immunology and Transplantation, KU Leuven, Leuven, Belgium); Irina Giurgea (Division of Rheumatology, Bambino Gesù Children's Hospital, IRCCS, Roma, Italy); Mieke Gouwy (Department of Internal Medicine, Hospices Civils de Lyon, University of Lyon, Lyon, France); Eric Hachulla (Institute of Allergology, Charité - Universitätsmedizin Berlin, Berlin, Germany); Haner Direskeneli (National Amyloidosis Centre, Royal Free London NHS Foundation Trust and University College London, London, UK); Gilles Hayem (Rheumatology Unit, 1st Department of Propaedeutic Internal Medicine, University of Athens, Medical School, Athens, Greece); Thomas Henry (Department of Pathology, University of Cambridge, Cambridge, United Kingdom); Michael Hofer (Clinical Immunology department, Strasbourg University Hospital, Strasbourg France); Stephanie Humblet-Baron (Leeds Institute of Rheumatic and Musculoskeletal Medicine, St James's University Hospital, University of Leeds, Leeds, UK); Philippe Hupé (Internal Medicine Department, Nantes Université, Nantes, France); Antonella Insalaco (Department of Biological Applications and Technology, University of Ioannina, Greece); Yvan Jamilloux (Rheumatology Department, Bordeaux University Hospital and Bordeaux University, Bordeaux, France); Isabella Friis Jørgensen (Paris-Cité University and Pediatric Hematology-Immunology and Rheumatology Department, APHP, Necker University Hospital, Paris, France); Sonia Karabina (Department of Internal Medicine and Clinical Immunology, Pitie Salpetriere University Hospital, Sorbonne Université, UPMC, Paris, France); Peter D Katsikis (European Clinical Research Infrastructure Network (ECRIN), Paris, France); Andres Jimenez Kaufmann (VIB Center for Inflammation Research (IRC), Ghent, Belgium); Christoph Kessel (Department of Pediatrics, Division of Rheumatology, Hacettepe University Faculty of Medicine, Ankara, Turkey); Isabelle Koné-Paut (Rheumatology Unit, Department of Medicine, University of Padova, Italy); Keshia Koutekissa (Department of Pathology & Clinical Bioinformatics, Erasmus MC, Rotterdam, The Netherlands); Karoline Krause (Division of Rheumatology, Department of Internal Medicine, Hacettepe University Faculty of Medicine, Ankara, Turkey); Helen Lachmann (Department of Internal Medicine, Erasmus MC, Rotterdam, The Netherlands); Katerina Laskari (Department of General Internal Medicine, UZ Leuven & Department of Microbiology, Immunology, and Transplantation, KU Leuven, Leuven, Belgium); Adrian Liston (Department of Pediatric Immunology and Infectious Diseases, Erasmus MC, Rotterdam, The Netherlands); Thibault Mahevas (Department of Pediatrics, University Hospitals Leuven, Leuven, Belgium).Thierry Martin, Nasima Matsa, Patrick Matthys, Michael F. McDermott, Stephane Mitrovic, Aura Moreno Vega, Yvonne M Mueller, Marco Francesco Natale, Antoine Néel, Maria Papadaki, Costas Papaloukas, Christophe Poulet, Nicolas Poursac, Sebastian Proost, Paul Proost, Pierre Quartier, Jeroen Raes, Julien Roméjon, David Saadoun, Sarhan Yaiche, Léa Savey, Sinisa Savic, Savvas Savvides, Seza Ozen, Paolo Sfriso, Vassili Soumelis, Sigrid Swagemakers, Marie-Elise Truchetet, Umut Kalyoncu, Paul Van Daele, Harmen J. G. van de Werken, Peter van der Spek, Rogier van Wijck, Steven Vanderschueren, Clementien Vermont, Matheus Vieira, Carine Wouters

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ORCID iD

Cem Gabay http://orcid.org/0000-0001-6853-3063

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