TRANSLATIONAL SCIENCE

Molecular signature characterisation of different inflammatory phenotypes of systemic juvenile idiopathic arthritis

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Handling editor Josef S Smolen

► Additional material is published online only. To view please visit the journal online (http://dx.doi.org/10.1136/annrheumdis-2019-215051).

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Received 10 January 2019
Revised 14 March 2019
Accepted 2 April 2019
Published Online First 20 April 2019

ABSTRACT

Objectives The International League of Associations for Rheumatology classification criteria define systemic juvenile idiopathic arthritis (SJIA) by the presence of fever, rash and chronic arthritis. Recent initiatives to revise current criteria recognise that a lack of arthritis complicates making the diagnosis early, while later a subgroup of patients develops aggressive joint disease. The proposed biphasic model of SJIA also implies a 'window of opportunity' to abrogate the development of chronic arthritis. We aimed to identify novel SJIA biomarkers during different disease phases.

Methods Children with active SJIA were subgrouped clinically as systemic autoinflammatory disease with fever ($SJIA^{syst}$) or polyarticular disease ($SJIA^{poly}$). A discovery cohort of n=10 patients per SJIA group, plus n=10 with infection, was subjected to unbiased label-free liquid chromatography mass spectrometry (LC-MS/MS) and immunoassay screens. In a separate verification cohort ($SJIA^{syst}$, n=45; $SJIA^{poly}$, n=29; infection, n=32), candidate biomarkers were measured by multiple reaction monitoring MS (MRM-MS) and targeted immunoassays.

Results Signatures differentiating the two phenotypes of SJIA could be identified. LC-MS/MS in the discovery cohort differentiated $SJIA^{syst}$ from $SJIA^{poly}$ well, but less effectively from infection. Targeted MRM verified the discovery data and, combined with targeted immunoassays, correctly identified 91% ($SJIA^{syst}$ vs $SJIA^{poly}$) and 77% ($SJIA^{syst}$ vs infection) of all cases.

Conclusions Molecular signatures differentiating two phenotypes of SJIA were identified suggesting shifts in underlying immunological processes in this biphasic disease. Biomarker signatures separating SJIA in its initial autoinflammatory phase from the main differential diagnosis (ie, infection) could aid early-stage diagnostic decisions, while markers of a phenotype switch could inform treat-to-target strategies.

Key messages

What is already known about this subject?

- Insights into the biological basis of systemic juvenile idiopathic arthritis (SJIA) increasingly support the existence of an initial autoinflammatory phase of the disease offering a therapeutic 'window of opportunity'.
- Early initiation of effective treat-to-target strategies may change the course of SJIA (Still's disease) and prevent the development of a chronic polyarticular disease phenotype.

What does this study add?

- We identified molecular signatures discriminating SJIA phenotypes and separating SJIA from infection.
- Diagnostic biomarkers could aid early-stage therapeutic decisions in initial SJIA phases, while markers of a phenotype switch could inform treat-to-target strategies.

How might this impact on clinical practice or future developments?

- These findings may have future implications for improving SJIA classification criteria and for the development of precision medicine.

INTRODUCTION

Systemic juvenile idiopathic arthritis (SJIA, or Still's disease) is an autoinflammatory disorder of unknown pathogenesis that accounts for 10%–15% cases of juvenile idiopathic arthritis (JIA).[1] The International League of Associations for Rheumatology (ILAR) criteria for JIA classification define SJIA by the presence of quotidian fever at onset for a minimum of 2 weeks, a transient rash and chronic arthritis.[2] Early in disease, arthritis is minimal or absent, which complicates establishing the diagnosis when features also fitting differential diagnoses such as infections dominate. However, early diagnosis is key to initiate effective treat-to-target approaches.[3 4] In a subgroup of patients, stable remission status can be reached rapidly (monophasic disease),[5] though in most cases SJIA progresses to become recurrent or persistent. Recent findings on pathophysiology suggest a biphasic model of SJIA.[6 7] Innate immune dysregulation is present early with systemic features of an autoinflammatory disorder, while adaptive immunity is thought to dominate later phases with destructive joint disease. Therefore, initial immune dysregulation induced by unknown triggers may then drive autoimmune arthritis. A genetic association of SJIA with major histocompatibility complex class II specific allele HLA-DRB1*11 also suggests an autoimmune component.[8]

To cite: Gohar F, McArdle A, Jones M, *et al*. *Ann Rheum Dis* 2019;**78**:1107–1113.

Table 1 Clinical and laboratory characteristics of sampled patients

	Discovery cohort, n=30			Verification cohort, n=106		
	SJIA^{syst}	SJIA^{poly}	Infection	SJIA^{syst}	SJIA^{poly}	Infection
Patients, n	10	10	10	45	29	32
Gender (M:F)	4:6	3:7	4:6	30:15	11:18	11:21
Age at diagnosis, years (IQR)	n.a.	n.a.	6 (7)	5 (9)	4 (6)	6 (9)
Age at sample, years (IQR)	7 (5)	15 (6)	6 (7)	9 (8)	11 (5)	6 (9)
Phenotype at sampling, n						
Fever	10	0	10	40	0	27
Arthralgia	7	5	4	25	9	7
Arthritis	4	10	n.a.	14	29	n.a.
Active joints (mean)	1.3 (0–3)	3.6 (3–13)	n.a.	2 (0–5)	6 (5–23)	n.a.
Exanthema	6	0	3	18	0	6
Serositis	1	0	1	9	0	1
Hepatosplenomegaly	4	0	4	9	0	2
Phenotype after sampling						
Monophasic:chronic	7:3	0:10	n.a.	26:19	5:24	n.a.
Medication at sampling						
Antibiotics	1	0	5	0	2	16
NSAID	4	2	2	13	7	6
Methotrexate	2	5	0	6	16	0
Biologics	0	0	0	0	0	0
Steroid	3	2	0	15	12	2
Markers of inflammation at sampling						
ESR (mm/h)	95 (55)	5 (11)	60 (56)	80 (67)	11 (17)	68 (39)
Missing ESR, n	1	1	3	7	3	16
CRP (mg/dL)	11 (7.3)	0.2 (1.4)	13 (5.6)	12.8 (17.9)	0.4 (1.8)	9.7 (8.0)
Missing CRP, n	0	0	0	1	0	4
WCC (×10^9/L)	21 (12)	7 (5.3)	11.8 (7.0)	19 (10)	8 (4)	12 (14)
Missing WCC, n	0	0	0	1	0	4

Values are median (IQR) unless otherwise specified.
CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; NSAID, non-steroidal anti-inflammatory drug; SJIA^{poly}, systemic juvenile idiopathic arthritis with polyarthritis but systemic disease; SJIA^{syst}, SJIA with fever/systemic disease; WCC, white cell count; n.a., not available.

Early diagnosis and recognition of phenotypes could therefore be key to initiate effective treat-to-target–based management strategies during a window of opportunity and also to prevent the progression of SJIA into a more aggressive chronic arthritis phenotype. A revision of the ILAR criteria to facilitate the implementation of targeted and personalised management strategies is a current initiative.[3 9 10]

There is an unmet need for laboratory tests to diagnose and monitor SJIA disease activity.[11] Differential gene expression profiles in peripheral blood mononuclear cells and cytokine signatures in serum have been investigated in new-onset and active versus inactive SJIA.[12] Notably, several immune mediators including interleukin-1 (IL-1), IL-18 as well as the S100 proteins S100A12 and S100A8/A9 (MRP8/14, myeloid related protein 8/14) might be important players in the pathogenesis of SJIA.[13–15] S100A12 and MRP8/14 are highly elevated in SJIA compared with infection or other causes of fever of unknown origin[16–18] and are predictive for subclinical inflammatory activity and disease flares.[19–21]

We aimed to identify molecular markers of systemic-autoinflammatory and chronic-polyarticular SJIA phenotypes by applying unbiased label-free proteomics and multiplex immunoassays in a discovery cohort and targeted candidate biomarker analyses in a verification cohort.

PATIENTS AND METHODS

Patients

Serum samples were collected between 2009 and 2012 from 136 paediatric patients with SJIA or infections (clinically diagnosed and/or confirmed by serology/microbiology; see online supplementary table S1) attending various hospitals in Germany. In 2015 to 2016, treating clinicians retrospectively reported whether patients with SJIA developed a predominantly systemic or (poly)arthritic phenotype and a monophasic or chronic disease course. Patients were subgrouped as classical autoinflammatory (SJIA^{syst}) or chronic articular-dominant (SJIA^{poly}) based on the overall clinical disease course. Patients with SJIA^{poly} had a polyarthritic course (≥5 joints affected) and active arthritis at sampling, but no fever, rash, serositis, splenomegaly or generalised lymphadenopathy. In contrast, patients with SJIA^{syst} had a systemic course with fever, rash, serositis, splenomegaly, lymphadenopathy, elevated acute phase reactants or white cell count and <5 joints involved at time of sampling. No patient fulfilled the criteria for a diagnosis of macrophage activation syndrome (MAS).[22]

Ten patients each with either SJIA^{syst}, SJIA^{poly} or infection (total n=30) formed the 'discovery cohort'. Another 106 patients (45 SJIA^{syst}, 29 SJIA^{poly} and 32 infections; table 1) formed the 'verification cohort'. Patients were not included in both cohorts. At sampling, all patients had clinically active disease (eg, active joints or fever) and laboratory signs of inflammation (eg, elevated acute phase reactants). In the verification cohort, disease duration was significantly shorter in patients with SJIA^{syst} (median 0.1 years; range, 0.1–8.3) compared with SJIA^{poly} (median 4.7 years; range, 0.1–10; p=0.001). Patients using biological treatments at time of sampling were excluded. The study overview is shown in online supplementary figure S2.

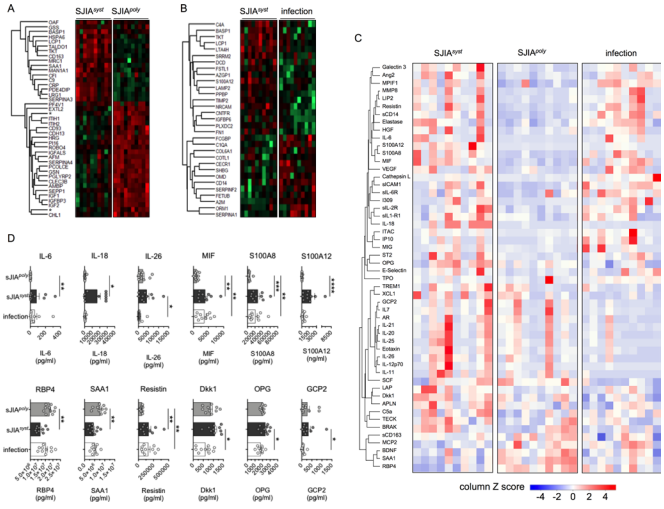

Figure 1 Proteomic and immune assay analyses in the discovery cohort. Serum was analysed by LC-MS/MS in a discovery cohort with the differences between the phenotypes shown as follows: (A) SJIA with systemic disease (SJIA^{syst}, n=10) vs SJIA with polyarticular disease (SJIA^{poly}, n=10) and (B) SJIA with systemic disease (SJIA^{syst}, n=10) vs infections (n=10). Heatmaps are shown with red squares representing overexpressed proteins and green representing lower expression (*indicates peptides without protein assignment). (C) Sera of identical patients as in (A) and (B) were analysed by 150-plex bead array Luminex assay. Analytes differentiating between groups with a p value <0.08 (see online supplementary table S4) are shown as heatmap based on Spearman rank correlation and average linkage clustering. S100A12 levels in respective sera were determined by ELISA and data are included in the heatmap. (D) Top performing serum markers based on significant separation of SJIA^{syst} from SJIA^{poly} or infections are shown as box-and-whisker plots (10th–90th percentile). Black full circles indicate outliers. Data were analysed by Kruskal-Wallis test and corrected for multiple comparisons, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

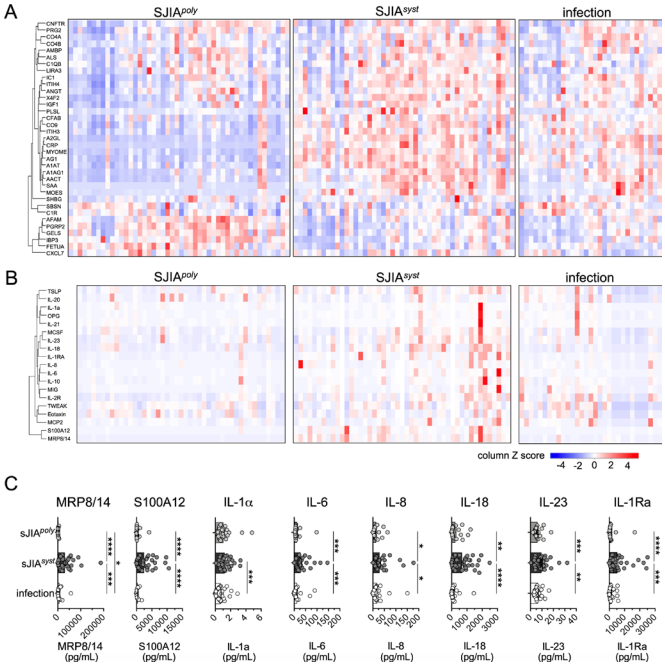

Figure 2 Proteomic and immune assay analyses in the verification cohort. (A) Heatmap of MRM peak area data for peptides derived from the indicated proteins as detected in sera of patients with systemic juvenile idiopathic arthritis with systemic (SJIA^{syst}, n=46) or polyarticular disease (SJIA^{poly}, n=47) or infections (n=32) based on Spearman rank correlation and average linkage clustering. (B) Heatmap of serum marker concentrations by bead array assay in cohorts as described in (A) based on Spearman rank correlation and average linkage clustering. (C) Box-and-whisker plots (10th–90th percentile) of top performing serum markers. Black full circles indicate outliers. Data were analysed by Kruskal-Wallis test and corrected for multiple comparisons, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Serum collection

Samples were collected in routine clinical settings. A 20 min centrifugation of the blood sample (collected in a serum gel tube) was performed within 2 hours of collection and the serum directly separated from the cell pellet. Serum was aliquoted into 1.5 mL or 2 mL Eppendorf tubes and posted at room temperature, to be stored at −80°C on arrival after the measurement of serum S100 proteins. Discovery cohort samples were subjected to unbiased label-free proteomics using liquid chromatography mass spectrometry (LC-MS/MS) and a broad multiplexed immunoassay screen (Luminex). Multiple reaction monitoring MS (MRM-MS) and targeted Luminex assays were performed on verification cohort samples. All samples were analysed for S100A12 and MRP8/14 by ELISAs (figures 1 and 2).

Proteomic analysis

Serum for LC-MS/MS analysis was prepared by depletion of the 14 most highly abundant proteins using a MARS Hu-14 affinity depletion column (Agilent Technologies, P/N 5188-6557) and visually confirmed using SDS chromatography as previously described.[23] Depleted samples were further concentrated by centrifugation and then subjected to in-solution tryptic digestion followed by desalting using C18 zip tips (online supplementary methods S3). Technical reference samples were included. Samples were stored at −80°C before analyses using a Q-Exactive mass spectrometer.[24] LC-MS/MS data were analysed by MaxQuant (V.1.4.1.3) with protein identifications generated using Andromeda.[25] Qualitative analysis was performed using PEAKS Studio (BSI) and Spectrum Mill (Agilent). Statistical analyses of the LC-MS/MS data were performed using Perseus (V.1.4.1.3).

MRM analysis of the verification cohort

To evaluate the candidate biomarkers identified during discovery, a unique MRM assay was generated, incorporating as many candidate proteins as possible (online supplementary methods S3).

Bead array assays and ELISA

In the discovery cohort, a total of 150 immune-related proteins (online supplementary table S4) were screened by a bead-based (Luminex) multiplex panel.[26] Proteins with little or no variation between samples (less than 75% unique values) or proteins with low expression levels were excluded. In the verification cohort, a customised panel of 17 cytokines was designed using commercially available Luminex analytes (eBioscience): interleukin (IL) 1 alpha (IL1α), IL1 receptor antagonist (IL1RA), IL2 receptor (IL-2R), IL6, IL8, IL10, IL18, IL20, IL21, IL23, macrophage colony stimulating factor, osteoprotegerin, thymic stromal lymphopoietin, monocyte chemotactic protein 2, eotaxin, TNF-like weak inducer of apoptosis (TWEAK) and monokine induced by gamma interferon.[24 27 28] S100A12 and MRP8/14 concentrations were measured in all serum samples

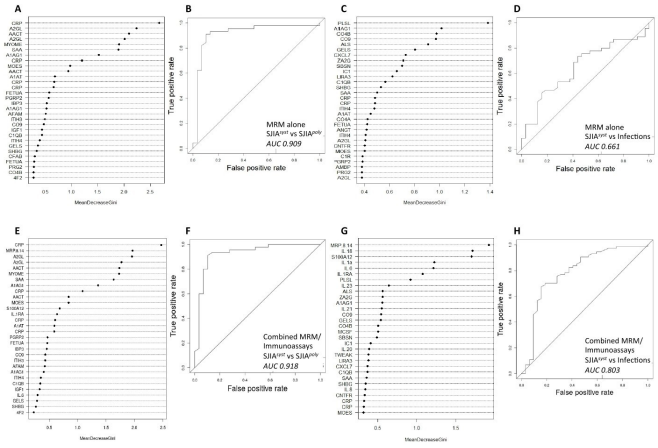

Figure 3 Predictive power of biomarker signatures. The accuracy of either the proteomic MRM panel alone (upper panels A–D) or a combination of MRM and immunoassays Luminex/ELISA (lower panels E–H) was analysed using a Random Forest model with 'leave-one-out cross-validation' statistical method. In the top rank list of the Random Forest models, the same protein could be identified by multiple peptides. Based on the ranked markers, receiver operating characteristic (ROC) curves were plotted for different comparisons. The analyses were performed for comparison groups SJIA^{syst} vs SJIA^{poly} (A, B or E, F, respectively) and for the differentiation of SJIA^{syst} vs infections (C, D or G, H, respectively). The ROC curve and area under the curve (AUC) are shown.

using standardised in-house ELISAs as previously reported.[29 30] Both assays included reference internal control sera with established cut-off values and were performed blinded to the patient characteristics.

Data analysis

Random Forest models were used to discriminate between the clinical groups (Random Forest package in R V.4.3.2). Reported performance measures were cross-validated in all models using leave-one-out cross-validation. Measurements of accuracy included the classification rate (percentage of total cases correctly classified by the model) and the area under the receiver operating characteristic (ROC) curve (AUC), calculated using the pROC package in R V.3.4.4. The Random Forest model provides a measure of importance of each variable contributing towards the overall performance, calculated using the Gini decrease in impurity (higher decrease in impurity means the variable is more predictive). In each model, the overall importance of variables was taken as the average decrease in the Gini decrease in impurity over each of the *n* random forest models run (n=number of samples in a given cohort). Kruskal-Wallis test, corrected for multiple comparisons, was applied to compare biomarkers between the groups. Data are shown as median (IQR), unless otherwise specified. Network analyses were performed using the GeneMANIA platform by applying Gene Ontology network weighting based on biological processes.[31]

RESULTS

Clinical and laboratory characteristics of the cohorts

Clinical characteristics and laboratory markers of participants are detailed in table 1. Patients with infection had similarly high levels of C-reactive protein (CRP), WCC and erythrocyte sedimentation rate as patients with SJIA^{syst}. Frequencies of fever and exanthema were highest in SJIA^{syst} followed by infection and lowest in SJIA^{poly}. Patients with infection and SJIA^{poly} had similar frequencies of joint pain and hepatosplenomegaly, which were lower than in SJIA^{syst}.

LC-MS/MS proteomic analysis in the discovery cohort

Univariate analysis revealed 41 differentially expressed proteins between SJIA^{syst} and SJIA^{poly} and 31 non-overlapping proteins differing between SJIA^{syst} and infections (total 72 candidate markers). Heat maps showing upregulated and downregulated proteins indicated good separation of the groups (figure 1). Some markers that discriminated groups in the immunoassay panel (eg, SAA1, S100A12 and sCD163) were also discovered by unbiased proteomics and thus acted as independent confirmation.

Proteomic analysis in the verification cohort

Of the 72 differentially expressed proteins from the LC-MS/MS discovery phase, 48 could be included in the MRM assay. Univariate and multivariate analysis of the data confirmed the presence of protein signatures capable of differentiating SJIA^{syst} from SJIA^{poly} and separately SJIA^{syst} from infection (figure 2A). The top 30 performing peptides and their recognised proteins for each analysis are shown in the variable importance plot in figure 3A–D. Interestingly, a CRP peptide was the top-ranked peptide for SJIA^{syst} versus SJIA^{poly} and also featured in the top 30 for SJIA^{syst} versus infection. CRP featured more than once in the list due to the inclusion of several different peptides in the assay panel. Analyses using the Random Forest model distinguished the SJIA^{syst} phenotype from SJIA^{poly} with 91% accuracy (sensitivity 86%, specificity 93%; table 2). Patients with SJIA^{syst} were less well distinguished from those with infection (accuracy, 62%; sensitivity, 38%; specificity, 80%). ROC curves showed a good differentiation of the two SJIA phenotypes with an AUC of 0.91 (95% CI 0.83 to 0.99), which outperformed SJIA^{syst} versus infection (AUC 0.66, 95% CI 0.54 to 0.79).

Inflammatory markers analysed by immune assays

In the discovery cohort, Luminex and ELISA analyses showed differences between the groups (figure 1). However, sample size limited statistical analysis. Therefore, a verification panel including commercially available analytes that were discriminative in the discovery cohort or in published literature, allowing a semitargeted reproducible approach, was designed. Univariate analyses of the best-performing individual markers in differentiating SJIA^{syst} and SJIA^{poly} measured by ELISA and Luminex are shown in figure 2B and levels of most important markers per group are plotted in figure 2C. Variable importance plots ranking each of the markers tested within the combined analysis revealed that MRP8/14, IL18 and S100A12 were the most important variables for differentiating SJIA^{syst} from infection, whereas MRP8/14, S100A12 and IL-1Ra were the top variables differentiating SJIA^{syst} from SJIA^{poly} (online supplementary figure 5). The ROC curve showed a good differentiation of the two SJIA phenotypes (AUC 0.90, 95% CI 0.81 to 0.98), similar to the discrimination of SJIA^{syst} and infection (AUC 0.84, 95% CI 0.75 to 0.93).

Lastly, to compare the performance of known biomarkers, ROC analyses of the single markers MRP8/14, S100A12, IL18 and ferritin were performed in the verification cohort (summarised in online supplementary figure S6 and online supplementary table S7). For the distinction between SJIA^{syst} versus infection, the single markers S100A12 (AUC 0.81) and MRP8/14 (AUC 0.82) performed almost as well as the immune assay panel (AUC 0.84). The best discriminator between SJIA^{syst}

Table 2 Accuracy of the proteomic marker panels

	SJIA^{syst} vs SJIA^{poly}			SJIA^{syst} vs infection		
	MRM	Immune assays	Combined	MRM	Immune assays	Combined
Sensitivity	0.86	0.76	0.86	0.80	0.69	0.69
Specificity	0.93	0.91	0.93	0.38	0.82	0.82
Accuracy	0.91	0.85	0.91	0.65	0.77	0.77
PPV	0.89	0.85	0.89	0.80	0.73	0.73
NPV	0.91	0.85	0.91	0.57	0.79	0.79
LR+	14.33	8.44	14.33	1.28	4.06	4.06
LR–	0.15	0.26	0.15	0.53	0.39	0.39
AUC	0.909 (0.828–0.990)	0.895	0.918	0.661	0.840	0.803

AUC, area under the curve; LR–, negative likelihood ratio; LR+, positive likelihood ratio;MRM, multiple reaction monitoring; NPV, negative predictive value; PPV, positive predictive value; SJIA^{poly}, systemic juvenile idiopathic arthritis with polyarthritis but systemic disease;SJIA^{syst}, SJIA with fever/systemic disease.

and SJIA^{poly} was MRP8/14 (AUC 0.93), which was also ranked high in the variable importance plots of the multiplex analyses.

Accuracy of multimodular analysis combining proteomic and immune assays

The top 30 variables in the combined multimarker panels comprising MRM, ELISA and Luminex were ranked (figure 3E–H). The top five discriminating biomarkers for SJIA^{syst} versus SJIA^{poly} were CRP, leucine-rich alpha-2-glycoprotein (A2GL), MRP8/14, alpha-1-antichymotrypsin (AACT) and myomegalin (myome); for SJIA^{syst} versus infection, MRP8/14, IL18, S100A12, IL1α and IL6. MRP8/14 was the only marker featuring in the top five of both panels. Eleven biomarkers were common to both panels: CRP, MRP8/14, AACT, serum amyloid A (SAA), alpha-1-acid glycoprotein 1 (A1AG1), moesin (MOES), S100A12, IL1RA, gelsolin (GELS), IL6 and sex hormone–binding globulin. In the combined model, distinction between SJIA^{syst} versus SJIA^{poly} was possible with an overall accuracy of 90.5% with 67/74 cases correctly identified (AUC 0.93). Accuracy of the SJIA^{syst} versus infection model was 76.6% and 59/77 cases were correctly classified (table 2). The combined multimodal panel (MRM and immunoassay) improved the distinction between SJIA^{syst} and SJIA^{poly}, but not SJIA^{syst} from infections, with immunoassays outperforming MRM.

To better understand potential relationships between the diverse identified markers, network analyses based on Gene Ontology biological process annotations using the GeneMANIA platform (figure 4 and online supplementary figures S8, S9) were performed. Serum marker panels identified by the combined Random Forest models to discriminate SJIA^{syst} from SJIA^{poly} (figure 3E) or SJIA^{syst} from infections (figure 3G) revealed multiple associations between each other as well as additional proteins that were not in the analysed panels (figure 4A,B). The top performing markers (mean Gini decrease >0.5) of both Random Forest model panels associated best with members of the IL1 signalling pathway, namely IL1R2 (figure 4). The association with IL1 signalling appeared more pronounced with markers in the panel differentiating SJIA^{syst} from SJIA^{poly}, with IL1R2 further linking to IL1β and IL1R1 (figure 4B), which was not pronounced for the separation of SJIA^{syst} from infections.

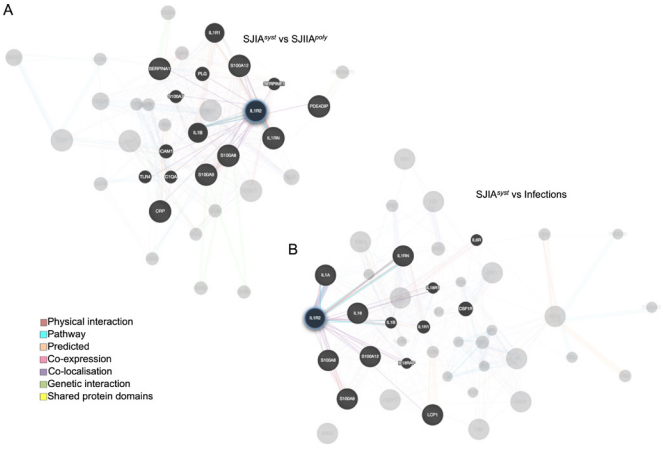

Figure 4 Association of identified discriminating serum markers. Plots show GeneMANIA-generated networks seeded with the proteins identified by Random Forest analysis discriminating SJIA^{syst} from SJIA^{poly} (A) and SJIA^{syst} from infections (B) above a mean Gini decrease cut-off of 0.5. Seeded markers are depicted as hatched circles of uniform size, while those that were added as relevant based on gene ontology biological process annotations are depicted as solid circles. Circle size is proportional to the number of interactions. The most relevant identified associations are highlighted.

DISCUSSION

Using proteomic analyses and immunoassays, signatures of serum proteins that distinguish two clinical phenotypes of SJIA and help differentiate autoinflammatory SJIA from infections were discovered. Proteomics has been relatively underused in paediatric rheumatology with most studies focusing on synovial fluid protein expression in JIA.[32 33] Proteomic analyses have, however, identified serum protein profiles of SJIA and revealed biomarkers for monitoring response to therapy in SJIA.[28 34] Clinical heterogeneity is a well-recognised feature of JIA and assumed to have a biological basis,[35 36] with differential PBMC gene expression profiles found in patients with SJIA versus non-systemic JIA.[12] Serum cytokine profiles have so far predominantly focused on discriminating active and inactive SJIA, or predicting response to treatment. With that regard, specifically RNA expression studies have been performed.[37–39] However, recent analyses failed to show distinct transcriptional profiles that could be attributed to diverse subphenotypes of SJIA.[39 40]

This study is the first aiming to systematically discriminate SJIA phenotypes with proteomic biomarkers. Correct discrimination of SJIA^{syst} from SJIA^{poly} was achieved in over 90% of cases using any of the three identified biomarker panels (MRM alone, ELISA/Luminex alone and combined). Our study included a number of markers with reported potential value for the diagnosis of SJIA.[41–43] Of these, IL18 is not regularly measured due to technical limitations in performing bioassays, while the routine use of IL6 is still limited for various reasons.[44 45] In the MRM assay, peptides of S100A12, MRP8/14 and the bead assay–measured

cytokines were below the limit of detection for MRM and were therefore excluded from panels. Lack of available analytes and/or low sensitivity are limitations of proteomic analyses and may explain the variation in results from the different approaches. The use of the combined approach could overcome this to some extent. However, our analyses of MRP8/14 and S100A12 by immunoassays show that these single analytes, partially already in routine care, perform very well as surrogate markers.

Interestingly, a number of markers in our combined panel that discriminated SJIA^{syst} from SJIA^{poly} were also identified in a published panel of markers that differentiated flare from quiescent SJIA.[28] The common markers were alpha-2-macroglobulin (A2M or A2GL), alpha-1-acid glycoprotein 1 (A1AG1 or AGP1), serpin a3/alpha-1-antichymotrypsin (AACT), GELS, SAA, MRP8/14 and CRP, which as part of the full panel published by Ling *et al* also differentiated SJIA from acute febrile illnesses. Of these biomarkers, A1AG1, GELS, SAA, MRP8/14 and CRP featured in both the SJIA^{syst} versus SJIA^{poly} and the SJIA^{syst} versus infection comparisons. Kininogenin (KLKB1), a high molecular weight protein which plays a role in the pathogenesis of inflammatory reactions, was previously identified by MS and also featured among the top gene ontology associated markers in the SJIA^{syst} from SJIA^{poly} network analysis performed here.[28]

A differentiation of phenotypes is not currently included in SJIA classification criteria. As knowledge of underlying immunological processes increases, leading to new treatment strategies,[46] it is important that treat-to-target approaches are supported by reliable biomarkers. The primary target is disease remission, and the available data support the hypothesis of a therapeutic window of opportunity in the autoinflammatory phase of the disease.[4] Our patients with clinically discriminated SJIA phenotypes had significantly different disease durations, which itself supported the biphasic model of SJIA. It is therefore important to start therapy early, which requires timely diagnosis before chronic arthritis develops. Recent data show that about half of the patients treated as SJIA do not have arthritis and therefore do not fulfil the ILAR classification criteria,[47] resulting in SJIA often being a diagnosis of exclusion. Here, the tested biomarker panel can help the earlier differentiation of SJIA versus infections. Another important aspect of treat-to-target protocols is the monitoring of the therapeutic response to check for necessary treatment adaptation. Phenotype switches occurring during the clinical course may require a corresponding therapy adjustment. The identification of underlying immunological imbalances could be facilitated by biomarker panels as described here.

Our study has a number of limitations. The sample size was relatively small. The quality of some samples may have been suboptimal for unbiased proteomic profiling, although clinically useful diagnostic biomarkers should be robust and stable.[48] Multiple preanalytical factors are thought to affect results, including the handling, shipping and storage of samples.[49] However, internal evaluation of the impact of freeze–thawing samples for MRM analysis and tryptic digestion of proteins before proteomic analysis showed that any preanalytical proteolysis had no effect on the final measurements (data not shown).

CONCLUSION

In summary, differing biomarker profiles between two phenotypes of SJIA were identified, strengthening the biological basis for subphenotypes in SJIA. Moreover, separate panels discriminating patients with SJIA^{syst} from those with infections were established. Biomarker panels were measurable using MRM, ELISA and Luminex assays or a combination of these, which improved the accuracy of the discrimination of SJIA^{syst} from infection, but not the discrimination of SJIA^{syst} from SJIA^{poly}, which already performed very well with single-platform panels.

The identified protein signature of SJIA versus infections can help to establish an early diagnosis. The discrimination of SJIA subphenotypes may improve the understanding of the pathophysiology underlying different disease phases and courses, which may inform future treat-to-target strategies. Future work could include biomarker measurements at specific time points including at diagnosis and flare as well as in established phenotype switches in a larger cohort.

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Acknowledgements The authors would like to thank the patients and colleagues who contributed their time and samples to the bio-bank collections. We are also thankful to Melanie Saers, Susanne Schleifenbaum, Sabrina Fuehner (Muenster) and Kieran Wynne (Dublin) for their excellent technical assistance in performing the experiments and assays.

Collaborators Antje Hellige; Andreas Urban; Aleš Janda; Annette Jansson; Bernd-Ulrich Keck; Boris Hügle; Eggert Lilienthal; Elisabeth Weißbarth-Riedel; Frank Weller-Heinemann; Gerd Ganser; Gerd Horneff; Gert Reutter-Simon; Gertrud Wannenmacher; Gregor Dückers; Gundula Böschow; Hans-Iko Huppertz; Hartwig Lehmann; Jeannine Rettschlag; Jens Berrang; Johannes-Peter Haas; Jürgen Brunner; Markus Knuf; Kirsten Mönkemöller; Klaus Tenbrock; Markus Hufnagel; Prasad Thomas Oommen; Rainer Berendes; Sandra Hansmann; Thomas Berger; Toni Hospach.

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Funding FG was supported by the EU FP7 EUTRAIN (European Translational Training for Autoimmunity and Immune manipulation Network) project grant (ref. 289903) until December 2016. FG, AM, NC, ML, SdR, SRP and DF received funding from the EU FP7 MIAMI (Monitoring innate Immunity in Arthritis and Mucosal Inflammation) project grant (ref. 305266). BH is funded by the HRB (Health Research Board) ref. LP-HSR-2017-021. The AID-Net was funded by the German Ministry of Research and Education (BMBF 01GM1512A/D). The UCD Conway Institute and Proteome Research Centre is funded by the Programme for Research in Third Level Institutions, as administered by Higher Education Authority of Ireland.

Competing interests OF declares the receipt of research grants and personal fees from Pfizer, Abbott and personal fees from Roche, Amgen, UCB, Celgene and Lilly. DF declares the receipt of research grant support and honoraria from Pfizer, Novartis, Sobi and Chugai-Roche. SRP declares honoraria from Celgene and is founder of Atturos, a UCD spin-out company developing advanced diagnostic tests. The other authors declare no conflicts of interest.

Patient consent for publication Not required.

Ethics approval University of Münster (reference 2009-031-f-S and 2014-637-f-S).

Provenance and peer review Not commissioned; externally peer reviewed.

Data sharing statement All data relevant to the study are included in the article or uploaded as supplementary information.

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