Paediatric rheumatology

Serum calprotectin (S100A8/9), clinical and ultrasound assessment in patients with juvenile idiopathic arthritis

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Abstract

Objective
To explore the association between serum S100A8/9 (calprotectin), clinical and ultrasound (US) assessment in juvenile idiopathic arthritis (JIA) patients.

Methods
A total of 30 well-characterised consecutive patients (18 female) with non-systemic JIA and 20 age-matched healthy controls were included. Serum and plasma samples obtained the same day of the clinical and sonographical assessment were tested for calprotectin levels by ELISA. Clinical status was defined using Wallace criteria. Ultrasonographic B-mode and power Doppler (PD) assessment of 44 joints for each subject was performed.

Results
Clinically active disease was present in 14 patients, while 16 patients were active according to US evaluation. We found no differences in the serum/plasma calprotectin levels in clinically active disease group [29.6 (5.4–198.1) ng/ml; 12.6 (2.8–65.8) ng/ml] as compared with inactive disease group [24.8 (14.1–204.3); 12.7 (3.4–65.1)] ($p=0.73$; $p=0.29$). There was also no difference between US active disease [29.8 (5.4–204.3); 12.9 (2.8–65.8)] and US inactive disease [24.8 (12.1–197.1); 11.7 (3.4–44.2)] with regard to the serum/plasma calprotectin levels ($p=0.83$; $p=1.0$). Serum/plasma calprotectin levels correlated moderately with C-reactive protein (CRP) (Spearman $r=0.44$, $p=0.01$; Spearman $r=0.56$, $p=0.0021$).

Conclusion
To our knowledge, this is the first study to simultaneously examine the correlation between serum/plasma calprotectin levels, clinical and US assessment in JIA. Calprotectin was not associated with the disease status in JIA patients with low number of active joints and its levels were moderately correlated with CRP. Our preliminary study needs to be extended with a larger number of patients.

Key words
juvenile idiopathic arthritis, calprotectin, S100A8/9, ultrasonography

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Introduction

Juvenile idiopathic arthritis (JIA) is the most common inflammatory rheumatological condition affecting children and adolescents, with a prevalence of approximately 1 in 1,000 (1). There are seven categories in JIA but even within each category, patients have different clinical courses and outcomes (2). Published studies and collaborative efforts have identified some clinical predictors of poor prognosis (3, 4). Biomarker profiling is very important when it is combined with clinical features to predict disease phenotype, severity, course and can aid to select patients for early aggressive treatment (5, 6). Unfortunately, JIA still lacks reliable biomarkers for diagnosis or monitoring. Two well studied proteins S100A12, and the complex S100A8/A9 (also known as calprotectin or MRP8/14) have shown correlations with disease activity in JIA patients (7-13). However, all of those studies were conducted on relatively small cohorts (<100 patients for all). S100 proteins are secreted during neutrophil and monocyte activation. Calprotectin is an important proinflammatory factor of innate immunity. Inciarte-Mundo *et al.* showed that serum levels of calprotectin is more accurate biomarker than erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) to detect disease activity in patients with rheumatoid arthritis (RA) who are receiving anti-TNF treatment (14). In another study Frosch *et al.* determined serum calprotectin levels by ELISA in patients with systemic JIA, active and inactive oligoarticular JIA, in healthy controls and in patients with bacterial infections. The study revealed that serum calprotectin levels in patients with active systemic JIA were 120-fold higher than healthy controls and 12 times higher than active oligoarthritis group. Calprotectin was also significantly higher when compared to patients with various bacterial infections (15). A recent study showed no association between S100 proteins and articular disease activity in patients with JIA (16). This finding is also supported by another study, showing the lack of ability of S100 proteins in predicting flares in non-systemic polyarticular JIA (17). However, in systemic onset JIA (sJIA) serum level of S100 proteins were correlated with disease activity for systemic symptoms, and results were promising in monitoring disease course (7).

Previous reports about patients with active RA showed an association between S100 protein levels and ultrasonographic findings. Nordal *et al.* reported that the S100A12 protein was significantly associated with other inflammatory markers, clinical assessments as well as positive US evaluation. Their results supported that S100A12 is a potential marker of inflammation in RA patients (18). These proteins may also help clinicians to identify active synovitis in RA and PsA patients in clinical remission or with low disease activity treated with TNF inhibitors (14). Unfortunately, the standardisation of the S100 proteins assay is poor, with vast intra-assay and inter-assay variability (19).

There are several studies related to ultrasound (US) and flare prediction in RA, but the role of US to predict disease outcome is not yet well established for JIA. A few published reports have suggested that US may be more sensitive than clinical examination in detecting chronic joint inflammation for paediatric population (20-25), while US has not been shown to have a predictive value for relapse in patients with JIA (26). In our previous study, we found that US abnormalities were a strong predictor of relapse at individual patient but not at joint level (27). The role of US in clinical practice for JIA is therefore still debated (28).

The previously published studies showed discrepancy of calprotectin usage to define disease activity in different subtypes of JIA, which served as justification for studying its value to predict disease activity. Ultrasonography is increasingly being used as an extension to physical examination in paediatric rheumatology practice and its application goes beyond the detection of synovitis in small and large joints. The present study explores the correlations between serum levels of calprotectin and clinical as well as US assessment in a non-systemic JIA cohort.

Materials and methods

Patients

In this cross-sectional study, we included consecutively children with JIA, classified according to the International League of Associations for Rheumatology (ILAR) criteria (29). Patients were recruited from our outpatient rheumatology clinic between December 2015 and May 2016. We also included healthy subjects for the comparisons of biomarker levels. The study was approved by ethical committee of Milano Area 2 (690_2017), Italy and informed consent was obtained from each subject and/or their legal guardian.

At the date of clinical visit for study entry, each patient underwent detailed clinical assessment and the following data were recorded: sex, birth date, ILAR category, age at disease onset, number of clinically active joints, extra-articular involvement, medication list (*i.e.* NSAIDs, corticosteroids, DMARDs or biologic agents). Disease activity status was defined by an experienced paediatric rheumatologist according to Wallace criteria as clinical remission off medication-CR; clinical remission on medication-CRM; inactive disease-ID (30). For the purpose of the study, we defined as clinical inactive patients who were in clinical remission. Each subject underwent an US assessment on the same day after the clinical evaluation, performed blindly by a rheumatologist (ODL) with long experience in paediatric musculoskeletal US.

Biological samples

Morning blood samples were collected from patients after 12 hours of fasting on the day of the clinical and ultrasound examinations. Serum and plasma samples were immediately centrifuged and within two hours all sera were stored at -80°C until the analysis. Calprotectin was measured by QUANTA Lite Calprotectin ELISA (Research use only, Inova Diagnostics, San Diego, USA). Analysis of inflammatory markers included ESR and CRP, with upper normal levels of 20 mm/h for ESR and 1 mg/dl for CRP. ANA were detected by indirect immunofluorescence. A titre of 1:160 was chosen as a cut-off point for ANA positivity for at least two positive results at least 3 months apart.

Ultrasound

The US examination technique and the standard scans were based on published Outcome Measures in Rheumatology (OMERACT) guidelines and on the studies on paediatric US available at study entry (31-36). We adopted the recent OMERACT preliminary definition of synovitis in children (37). During the enrolment phase of the study there was no published article about the definition of synovitis in children. We reanalysed ultrasound images on the basis of recent definition of synovitis and there was no discrepancy detected.

In detail, in each joint, three elements were assessed by US: synovial hyperplasia, synovial effusion and power Doppler (PD) signal. Synovial hyperplasia was defined as an abnormal hypoechoic joint space, distinct from the intra-articular fat pad and non-displaceable with the transducer. Synovial effusion was detected as the presence of an abnormal hypoechoic or anechoic displaceable space within the joint. Synovial hyperplasia and synovial effusion were considered indifferently as a single abnormality in grey scale examination. PD signal was considered pathological in the presence of vessel dots only inside an area of synovial hyperplasia. In each joint, synovial hyperplasia, effusion and PD signal were graded semi-quantitatively on a 0–3 scale (31). A total of 44 joints (2 shoulders, 2 elbows, 2 wrists, 10 metacarpophalangeal, 10 proximal interphalangeal of hands, 2 hips, 2 knees, 2 subtalar, 2 tibiotalar and 10 metatarsophalangeal) were scanned in each subject for the presence of synovial hyperplasia/joint effusion and PD signal. To perform the examination, an Esaote MyLab 70 with a 6–18 MHz linear probe was used. The grey scale frequency was adjusted according to the depth of the joint (18 MHz for small superficial joints, 14–12 MHz for medium size and 10 or less for deeper joints); the highest frequencies were used for younger children. The PD frequency was set to 9–10 MHz for superficial joints and 8–6 MHz for medium and deep joints. The pulse repetition frequency was 750 Hz and the gain was set at a level just below the disappearance of the noise under the cortical bone (27).

Statistical analysis

Data were analysed using the Statistical Package for the Social Sciences (SPSS 15.0; SPSS Inc., Chicago, IL). Normal distribution was evaluated with Kolmogorov Smirnov test. Non-normally distributed variables were expressed as median (range) and normally distributed variables as mean ± Standard Deviation. Comparisons between groups of nominal variables were performed with the Chi-square test (or Fisher's exact test where appropriate) and for numeric variables with Mann-Whitney U-test. Pearson's correlation analysis was used to determine correlations between two variables. Both McNemar's test and Cohen's kappa coefficient were used to assess concordance between the Wallace Criteria and US findings. Concordance of κ coefficient was interpreted as follows: >0.81=high, 0.61–0.8=good; 0.41–0.6=moderate, 0.21–0.4=fair, 0.0–0.2=poor. *p*-values <0.05 were considered to be statistically significant.

Results

Clinical, ultrasound and laboratory assessment

In total, 30 patients with diagnosed non-systemic JIA (16 clinically active, 14 clinically inactive disease) were prospectively included in our study. Demographic, clinical and laboratory characteristics of our cohort are summarised in Table I. The median disease duration in the clinically active disease group was shorter than in the inactive group (*p*=0.001). Although the distribution of JIA subtypes was not equal between the two groups, there was no difference for the treatment regimens. No significant differences were found in WBC (*p*=0.31), ESR (*p*=0.05), CRP (*p*=0.29) and ferritin (*p*=0.61) levels between patients with clinically active and inactive disease. ANA positivity was also found to be equal between the groups.

Correlation between serum/plasma calprotectin levels

Although we observed a statistically

Table I. Demographic, clinical and laboratory findings.

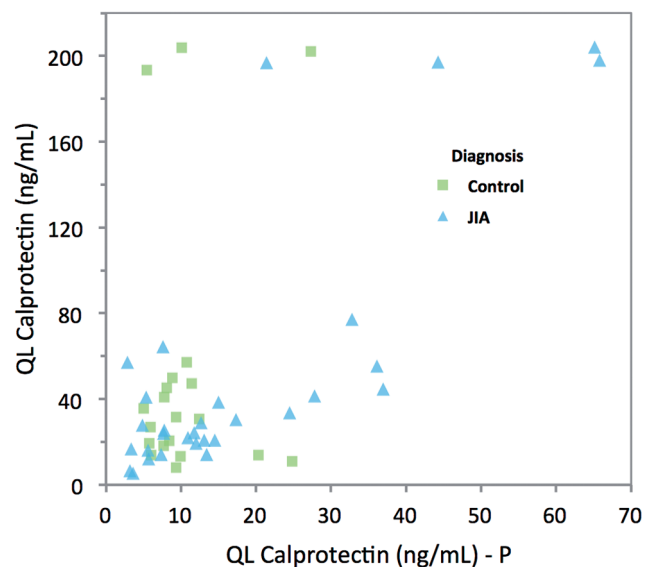|	Active disease (n=14)	Inactive disease (n=16)	Total (30)	p-value
Female	10 (71.4%)	8 (50.0%)	18 (60%)	0.23
Median age yrs (range)	10.7 (2.4-17.4)	10.5 (5.18-18.87)	10.6 (2-18)	0.80
Median age of disease onset, yrs (range)	7.9 (1.20-16.01)	3.09 (1.60-8.20)	4.2 (1.20-16.01)	0.14
Median disease duration, months (range)	19.3 (1.51-16.01)	73.49 (23.54-190.82)	58.4 (1.51-190.82)	0.001
Extra-articular involvement (uveitis)	2 (14.3%)	4 (25.0%)	6 (20%)	0.65
ANA positivity, n (%)	10 (71.4%)	12 (75.0%)	22 (73.3%)	1
WBC median, (range)	7.2 (4.0-10.6)	6.2 (3.3-10.70)	6.7 (4.0-10.7)	0.31
ESR mm/h, median, (range)	18.5 (3-67)	9.5 (2-20)	12 (2-67)	0.05
CRP mg/dl, median, (range)	0.3 (0.1-4.6)	0.2 (0.1-1.0)	0.2 (0.1-4.6)	0.29
Ferritin ng/ml, median, (range)	22 (5-270)	26.5 (14-119)	22 (5-270)	0.61
Alpha 2/globulin %, median (range)	11.5 (9.4-15.5)	10.4 (7.9-13.5)	11.28 (7.9-15.5)	0.11
ILAR category:				
Persistent oligoarticular, n (%)	3 (21.42%)	10 (62.5%)	13 (43.3%)	
Extended oligoarticular, n (%)	5 (35.7%)	3 (18.8%)	8 (26.6%)	
Polyarthritis (RF-), n (%)	4 (28.6%)	1 (6.3%)	5 (16.6%)	
Polyarthritis (RF+), n (%)	1 (7.14%)	0	1 (3.3%)	
ERA, n (%)	1 (7.1%)	1 (6.3%)	2 (6.6%)	
Psoriasic arthritis, n (%)	0	1 (6.3%)	1 (3.3%)	
Ongoing treatment:				
Corticosteroids	2 (14.3%)	0	2 (6.6%)	
DMARDs, n (%)	9 (64.3%)	10 (62.5)	19 (63.3%)	
Biological therapy, n (%)	1 (7.14%)	1 (6.3)	2 (6.6%)	

ANA: antinuclear antibodies; WBC: white blood cells; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; ERA: enthesitis-related arthritis; ILAR: International League Against Rheumatism; RF: rheumatoid factor; DMARD: disease-modifying anti-rheumatic drugs.

relevant correlation between serum and plasma levels (r=0.39, 95% Confidence interval 0.11–0.61), calprotectin levels were significantly higher in serum *versus* plasma (p<0.001). The difference was mostly driven by 7 samples that had very high calprotectin levels when measured in serum (around 500 mg/mL) (Fig. 1).

Plasma/serum calprotectin levels in the different patient subgroups

The median plasma calprotectin levels were 9.1 (5.0–7.3) ng/ml in healthy controls, 12.6 (2.8–65.8) ng/ml in clinically active disease and 12.7 (3.4–65.1) ng/ml in clinically inactive disease group. We found no differences for the plasma calprotectin levels in clinically active disease group as compared with inactive disease group (p=0.29). For both groups, plasma calprotectin levels were not statistically different from the healthy controls (p=0.92 for clinically active disease and p=0.06 for inactive disease). There was also no difference between plasma calprotectin levels in active [12.9 (2.8–65.8)] *vs.* inactive disease [11.7 (3.4–44.2)] defined based on US (p=1.0). For both US groups, plasma calprotectin levels were not statistically

Fig. 1. Correlation between serum/plasma calprotectin levels. Although a significant correlation was observed, significant difference was observed in seven samples. QL: QUANTA Lite; P: plasma; JIA: juvenile idiopathic arthritis.

different from healthy controls (p=0.21 for US active disease and p=0.4 for inactive disease). No difference in the plasma calprotectin levels were found between clinically and US active disease (p=0.91).

We performed the same comparisons with serum samples and we did not find statistically significant differences between patients and healthy controls, and between active and inactive patients according to clinical and

ultrasound evaluation. Figure 2 shows serum and plasma calprotectin levels in active and inactive groups, both clinically and sonographically in comparison with controls.

When we compared calprotectin levels between all active patients (clinically and sonographically, n=18) and inactive patients (n=12) the median serum/plasma calprotectin level were not significantly different neither in serum [29.8 (5.4–204.3) *vs.* 24.8 (14.1–197.1),
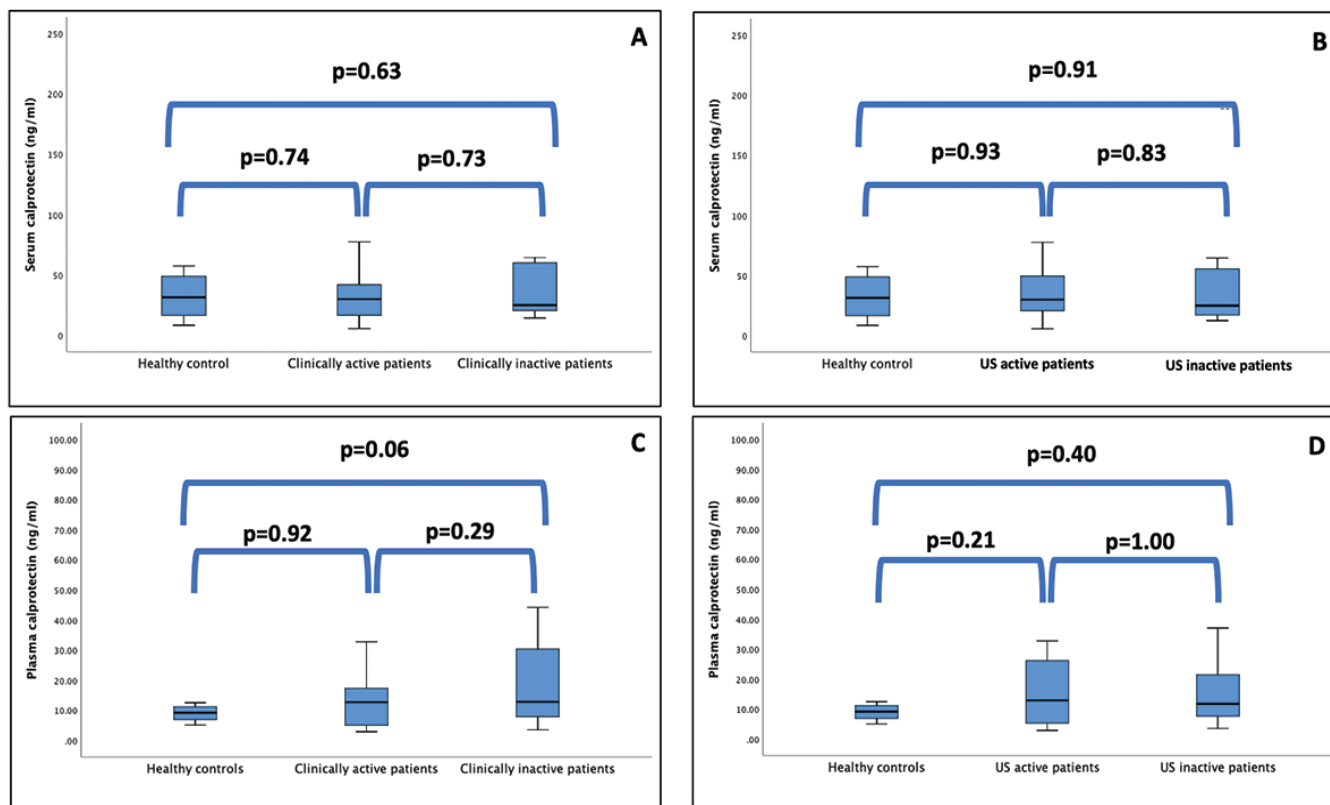
Fig. 2. Comparison of S100A8/9 (calprotectin) serum levels according to clinically and sonographically disease activity status.
A: serum calprotectin in healthy control, clinically active patients and clinically inactive patients.
B: serum calprotectin in healthy control, US active patients and US inactive patients.
C: plasma calprotectin in healthy control, clinically active patients and clinically inactive patients.
D: plasma calprotectin in healthy control, US active patients and US inactive patients.

$p=0.91$] nor plasma [12.3 (2.8–65.8) *vs.* 12.6 (3.4-44.2), ($p=0.58$)].

Correlation between calprotectin levels and other inflammatory markers
Calprotectin (serum/plasma) correlated moderately with CRP levels (Spearman r=0.44; $p=0.01$; r=0.56; $p=0.0021$) but not with ESR values (Spearman r=0.19; $p=0.325$; r=0.17; $p=0.39$) (Fig. 3).

Concordance of physical and ultrasound examination
We scanned 44 joints per patient by US for a total of 1320 joints in 30 JIA patients. The US examination was abnormal in 16/30 (53.3%) of patients, for at least one of grey scale elementary lesions (synovial hyperplasia/joint effusion), with or without abnormal PD signal. Among the 14 clinically active patients, one patient was active just because of the uveitis without any active synovitis. The median number of active joints in 13 active patients was 2 (1-26) according to our physical examination.

In these 13 patients the median number of active arthritis according to US was 1 (0–32) ($p=0.26$). US and physical examination agreed in 80% of cases. The concordance between US and physical examination to define synovitis in all joints was moderate (κ=0.60, 95% CI 0.32–0.89) (Fig. 4).

Comparisons between other biomarkers and disease activity
There were no significant differences in CRP levels between clinically active and inactive patients according to Wallace criteria ($p=0.29$). CRP levels were also not different between the sonographically active and inactive [0.21 (0.1–1.0)] patient groups ($p=0.85$). Similar results were obtained when we compared ESR levels and disease activity according to Wallace criteria and US evaluation ($p=0.05$ and $p=0.92$, respectively).

Discussion
In this cross-sectional study of patients with non-systemic JIA, serum/plasma

calprotectin levels have not been able to differentiate clinically or sonographically active from inactive disease. Interestingly, calprotectin levels were not able to differentiate patients with active disease from healthy controls. Moreover, our study did not find a correlation between calprotectin values and the presence of a clinical and/or ultrasound synovitis in patients with a prevalent oligoarticular pattern. The most represented ILAR category was, in fact, persistent oligoarthritis (13/30 patients) and, even in the presence of cases classified according to ILAR as extended oligoarthritis and polyarthritis, the joint count was very low in patients with active arthritis.

Previous studies on this subject have yielded conflicting results. A positive correlation between circulating calprotectin levels and disease activity at baseline and subsequent response to therapy with methotrexate or biologics has been previously shown. Alberdi-Saugstrup *et al.* investigated the asso-
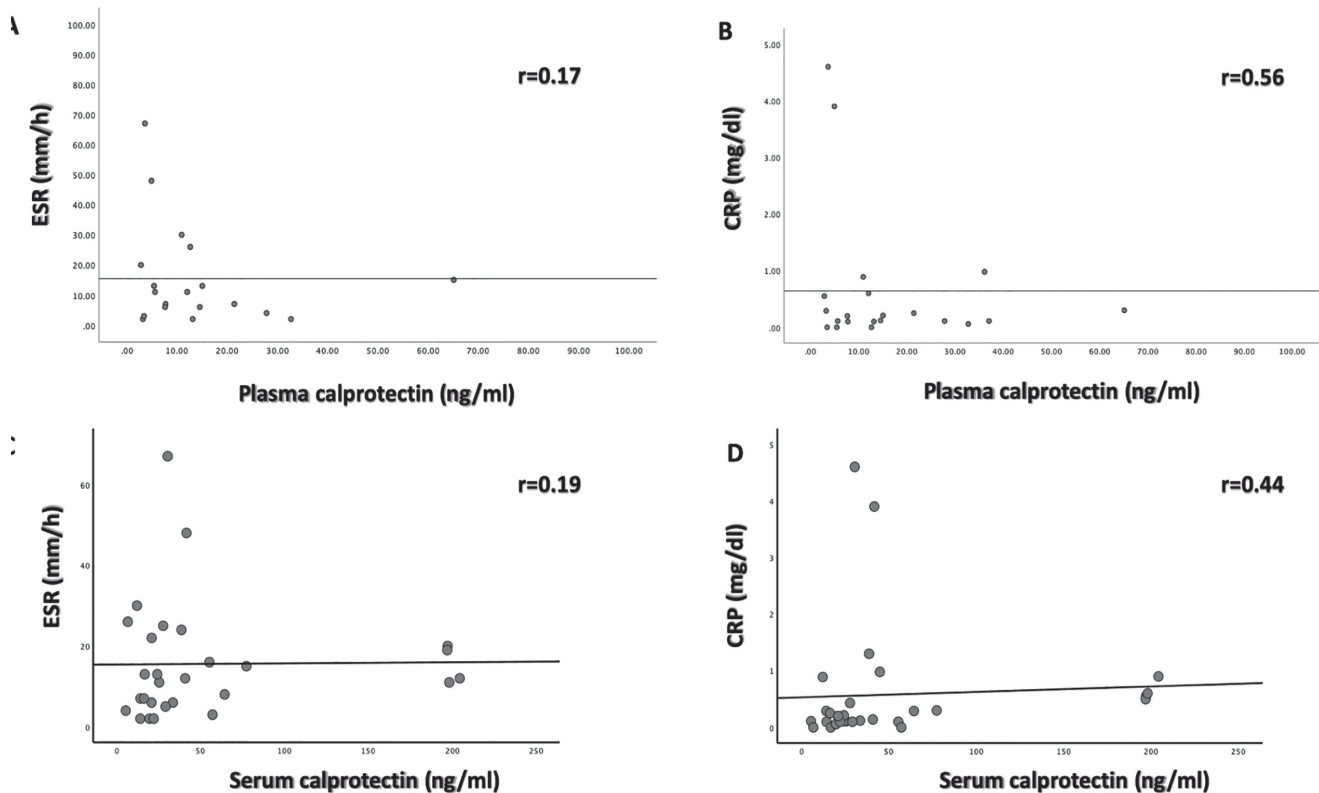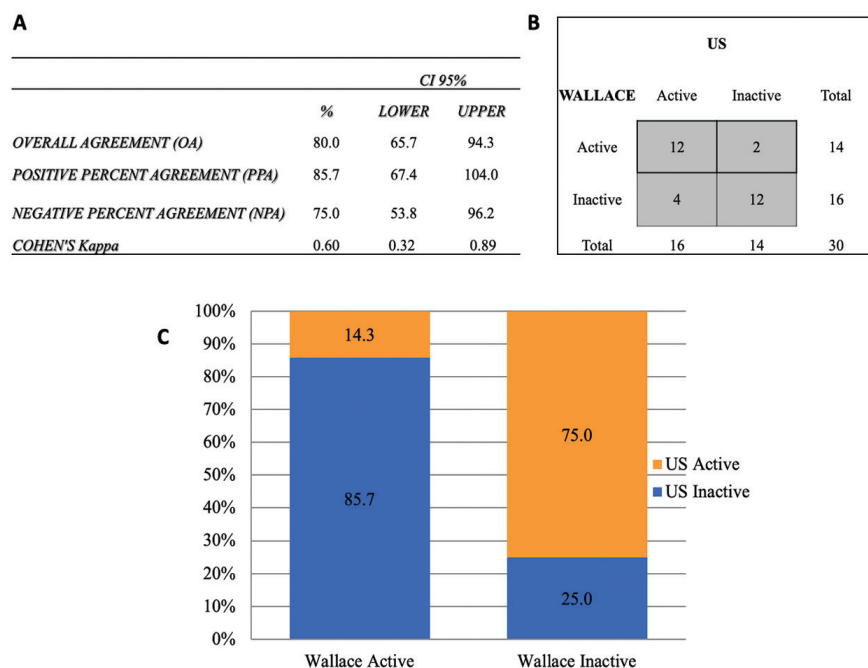
Fig. 3. Correlation between S100A8/9 (calprotectin) and other inflammatory biomarkers [C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR)]. **A**: correlation between plasma calprotectin and ESR. **B**: correlation between plasma calprotectin and CRP. **C**: correlation between serum calprotectin and ESR. **D**: correlation between serum calprotectin and CRP.

ciation between the pre-treatment level of calprotectin or hsCRP and found a significant positive association between the baseline concentration of calprotectin and CRP with achievement of an inactive disease after 1 year of treatment (38). Anink *et al.* performed a study in 88 non-systemic JIA and found that baseline calprotectin levels were higher in responders. They also compared patients who achieved inactive disease with those not achieving inactive disease and found higher serum calprotectin levels in patients with inactive disease (9). There are however also some published studies which are in agreement with our results. Recent published data on 72 patients with non-systemic JIA indicate that neither S100A8/9 (calprotectin) nor S100A12 were related to disease activity (16). In another prospective study, Hinze *et al.* aimed to determine the relationship between serum levels of S100A8/A9 (calprotectin) and S100A12 with clinically inactive disease in patients with polyarticular forms of JIA. Comparison of serum levels of S100A8/A9 (calpro-

tectin) and S100A12 between patients who maintained clinically inactive disease (n=106) and those who did not (n=24) revealed no significant differences between these patient groups (17). Our results revealed that there were no differences for the calprotectin levels in the clinically active *versus* inactive disease group. We conducted our study on a population with limited joint involvement. We found an absence of correlation between serum/plasma calprotectin levels and both clinical and US disease activity, and that the finding of median values of this inflammatory marker were not significantly different from that of a healthy control group.

To the best of our knowledge this is the first published study comparing serum/plasma calprotectin levels between active and inactive JIA patients as evaluated by US. US is a potentially valuable tool in paediatric clinical practice especially in the assessment of joints more difficult to examine clinically (*e.g.* shoulder, hip, ankle) (28). On the other hands, US usage in paediatric rheumatology is new and user depend-

ent, which can cause misdiagnosis. Adding objective biomarkers such as calprotectin could strongly supplement the ultrasound results. In our study, we were not able to show significant differences between the groups, but we acknowledge the small sample size. There are few studies in adult RA populations that demonstrated significant correlation between calprotectin and clinical and/or US scores (39, 40).

While ESR and CRP levels were not sensitive enough to assess clinically active and inactive disease in our cohort, there was a moderate correlation between calprotectin and CRP levels. Often, in the oligoarticular JIA category, the inflammatory markers are normal. It was demonstrated that S100A8 and S100A9 were strongly expressed in infiltrating neutrophils and monocytes within the inflamed joints, and could be found in significantly higher concentrations (about 20 times) in synovial fluid compared with serum in oligoarticular type of JIA (41). These findings support the hypothesis that calprotectin might be released at sites of inflammation and

A

	%	CI 95%	
		LOWER	UPPER
OVERALL AGREEMENT (OA)	80.0	65.7	94.3
POSITIVE PERCENT AGREEMENT (PPA)	85.7	67.4	104.0
NEGATIVE PERCENT AGREEMENT (NPA)	75.0	53.8	96.2
COHEN'S Kappa	0.60	0.32	0.89

B

	US		
WALLACE	Active	Inactive	Total
Active	12	2	14
Inactive	4	12	16
Total	16	14	30

C

Fig. 4. Concordance of physical examination and ultrasound.
A: agreement between US and clinical disease activity status. **B**: comparison between US and clinical disease. **C**: agreement of activity between Wallace and US.

thus may be reliable markers to monitor local activity of the phagocyte system during inflammatory processes (41). Of note, fecal calprotectin is known to be an important marker for inflammatory bowel diseases, and we have previously shown that fecal calprotectin levels were higher in JIA patients than in healthy controls, and that levels were significantly higher in patients with polyarticular than in those with oligoarticular disease (unpublished data).

S100A8 (MRP8) and S100A9 (MRP14) are calcium-binding proteins expressed in granulocytes, monocytes and macrophages during early differentiation stages. Since the activation of the innate immune system is a hallmark of the pathogenesis of sJIA, S100A8/A9 (calprotectin) serum/plasma concentrations seem to be a good candidate marker for monitoring disease activity, as has been already shown in other autoinflammatory diseases (42-44). High levels of S100 proteins were reported in serum from sJIA patients, and were significantly higher than those in patients with various bacterial infections, thus allowing to help in the clinical dilemma regarding the differential diagnosis between systemic JIA and infectious febrile diseases (15).

Calprotectin levels measured in blood matrices have been used to determine prediction of flares, response to treatment, maintenance of therapy, and differentiation of active from inactive disease in published articles (7-9, 17, 45, 46). We were not able to show any relation between calprotectin levels and disease activity. This is possibly related with sample size, heterogeneous group of patients, exclusion of systemic JIA, lack of follow-up data and lack of repeated measures of calprotectin levels. It is known that S100A8/9 levels vary in serum *versus* plasma, the former usually being higher than in the latter which is in keeping with our observations. Chelation of Ca2+ (EDTA) destructs heterodimers, resulting in an altered calprotectin structure (47), and it is hypothesised that such altered structure underlies the differences in calprotectin levels observed between serum and plasma (48). Indeed, in contrast to EDTA-plasma, serum contains freely available calcium which can cause generation of calprotectin dimers and oligomers. Therefore, in a sandwich ELISA, serum calprotectin levels can be artificially elevated (48, 49). It has also been reported that circulating calprotectin levels can be highly vari-

able throughout the day (50). Moreover, the kinetics of S100 protein may play a role. There has been no available information about the half-life of S100A12. It is presumably rather short (in the order of hours rather than days), similar to the half-life of the related S100A8/A9 proteins (51, 52). It is also known that processing time may influence calprotectin levels: we were able to process and store our samples within two hours from blood drawing. Our data provides additional evidence that serum and plasma matrix should not be used interchangeably for the measurement of circulating calprotectin. In addition, although not statistically significant, we observed a trend towards higher degree of correlation between circulating calprotectin levels and measures of disease activity when using plasma which is consistent to observations from RA (49).

The concordance between US and physical examination to define synovitis in all joints was moderate (=0.60, 95% CI 0.32–0.89). It has been suggested that US may be more sensitive in detecting joint inflammation in patients with JIA than the clinical assessment (21, 22, 25). However, the role of US-detected synovitis when clinical manifestations in JIA are absent is unknown. In our previous study, we found that US abnormalities are a strong predictor of relapse at individual patient but not at joint level (27). In our current study, concordance indicated a moderate agreement. Clinical and US assessment agreed in 80% of cases to define disease status. Among clinically active patients (n=14), 12 of them were also found active by US evaluation (85.7%). However, a quarter of the cases judged inactive by the paediatric rheumatologist showed the presence of subclinical synovitis (4/16, 25%) with US examination (Fig. 4). With no gold standard (*e.g.* magnetic resonance imaging) available, the jury is still out.

In conclusion of our small series of patients, serum levels of calprotectin were not associated with disease status as active or inactive both clinically or sonographically in children with non-systemic type of JIA, and its levels were moderately correlated with CRP. High

serum calprotectin levels could be related with a polyarticular disease either in clinical activity or in subclinical remission. Our study is limited by its cross-sectional nature, the relatively small sample size and the small number of patients in subgroups. This is a preliminary study which needs to be extended with further work including large number of patients and designed as prospectively, to outline the importance of calprotectin for use in clinical practice and for decision making protocols.

References

1. THIERRY S, FAUTREL B, LEMELLE I, GUILLEMIN F: Prevalence and incidence of juvenile idiopathic arthritis: A systematic review. *Joint Bone Spine* 2014; 81: 112-7.
2. GUZMAN J, HENREY A, LOUGHIN T *et al*.: Predicting which children with juvenile idiopathic arthritis will have a severe disease course: Results from the ReACCh-Out cohort. *J Rheumatol* 2017; 44: 230-40.
3. OEN K: Long-term outcomes and predictors of outcomes for patients with juvenile idiopathic arthritis. *Best Pract Res Clin Rheumatol* 2002; 16: 347-60.
4. OEN K, MALLESON PN, CABRAL DA *et al*.: Early predictors of longterm outcome in patients with juvenile rheumatoid arthritis: subset-specific correlations. *J Rheumatol* 2003; 30: 585-93.
5. DUURLAND CL, WEDDERBURN LR: Current developments in the use of biomarkers for juvenile idiopathic arthritis. *Curr Rheumatol Rep* 2014; 16: 406.
6. VAN DIJKHUIZEN EHP, AIDONOPOULOS O, TER HAAR NM *et al*.: Prediction of inactive disease in juvenile idiopathic arthritis: A multicentre observational cohort study. *Rheumatology* (Oxford) 2018; 57: 1752-60.
7. 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.
8. FOELL D, FROSCH M, SCHULZE ZUR WIESCH A, VOGL T, SORG C, ROTH J: Methotrexate treatment in juvenile idiopathic arthritis: When is the right time to stop? *Ann Rheum Dis* 2004; 63: 206-8.
9. ANINK J, VAN SUIJLEKOM-SMIT LW, OTTEN MH *et al*.: Mrp8/14 serum levels as a predictor of response to starting and stopping anti-TNF treatment in juvenile idiopathic arthritis. *Arthritis Res Ther* 2015; 17: 200.
10. FOELL D, WITTKOWSKI H, HAMMERSCHMIDT I *et al*.: Monitoring neutrophil activation in juvenile rheumatoid arthritis by s100a12 serum concentrations. *Arthritis Rheum* 2004; 50: 1286-95.
11. FOELL D, WULFFRAAT N, WEDDERBURN LR *et al*.: Methotrexate withdrawal at 6 vs 12 months in juvenile idiopathic arthritis in remission: A randomized clinical trial. *JAMA* 2010; 303: 1266-73.
12. KAHN R, BERTHOLD E, GULLSTRAND B *et al*.: Circulating complexes between tumour necrosis factor-alpha and etanercept predict long-term efficacy of etanercept in juvenile idiopathic arthritis. *Acta Paediatrica* 2016; 105: 427-32.
13. CATTALINI M, MADUSKUIE V, FAWCETT PT, BRESCIA AM, ROSE CD: Predicting duration of beneficial effect of joint injection among children with chronic arthritis by measuring biomarkers concentration in synovial fluid at the time of injection. *Clin Exp Rheumatol* 2008; 26: 1153-60.
14. INCIARTE-MUNDO J, RAMIREZ J, HERNANDEZ MV *et al*.: Calprotectin and tnf trough serum levels identify power doppler ultrasound synovitis in rheumatoid arthritis and psoriatic arthritis patients in remission or with low disease activity. *Arthritis Res Ther* 2016; 18: 160.
15. FROSCH M, VOGL T, SEELIGER S *et al*.: Expression of myeloid-related proteins 8 and 14 in systemic-onset juvenile rheumatoid arthritis. *Arthritis Rheum* 2003; 48: 2622-6.
16. 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 Online J* 2020; 18: 7.
17. 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: 451-9.
18. NORDAL HH, BRUN JG, HALSE AK, JONSSON R, FAGERHOL MK, HAMMER HB: The neutrophil protein s100a12 is associated with a comprehensive ultrasonographic synovitis score in a longitudinal study of patients with rheumatoid arthritis treated with adalimumab. *BMC Musculoskelet Disord* 2014; 15: 335.
19. STIGBRAND T, NYBERG L, ULLEN A, HAG-LID K, SANDSTRÖM E, BRUNDELL J: A new specific method for measuring s-100b in serum. *Int J Biol Markers* 2000; 15: 33-40.
20. PASCOLI L, WRIGHT S, McALLISTER C, ROONEY M: Prospective evaluation of clinical and ultrasound findings in ankle disease in juvenile idiopathic arthritis: Importance of ankle ultrasound. *J Rheumatol* 2010; 37: 2409-14.
21. MAGNI-MANZONI S, EPIS O, RAVELLI A *et al*.: Comparison of clinical versus ultrasound-determined synovitis in juvenile idiopathic arthritis. *Arthritis Rheum* 2009; 61: 1497-504.
22. FILIPPOU G, CANTARINI L, BERTOLDI I, PICERNO V, FREDIANI B, GALEAZZI M: Ultrasonography vs. Clinical examination in children with suspected arthritis. Does it make sense to use poliarticular ultrasonographic screening? *Clin Exp Rheumatol* 2011; 29: 345-50.
23. HASLAM KE, McCANN LJ, WYATT S, WAKEFIELD RJ: The detection of subclinical synovitis by ultrasound in oligoarticular juvenile idiopathic arthritis: A pilot study. *Rheumatology* (Oxford) 2010; 49: 123-7.
24. JANOW GL, PANGHAAL V, TRINH A, BADGER D, LEVIN TL, ILOWITE NT: Detection of active disease in juvenile idiopathic arthritis: Sensitivity and specificity of the physical examination vs ultrasound. *J Rheumatol* 2011; 38: 2671-4.
25. BRETON S, JOUSSE-JOULIN S, CANGEMI C *et al*.: Comparison of clinical and ultrasonographic evaluations for peripheral synovitis in juvenile idiopathic arthritis. *Semin Arthritis Rheum* 2011; 41: 272-8.
26. MAGNI-MANZONI S, SCIRE CA, RAVELLI A *et al*.: Ultrasound-detected synovial abnormalities are frequent in clinically inactive juvenile idiopathic arthritis, but do not predict a flare of synovitis. *Ann Rheum Dis* 2013; 72: 223-8.
27. DE LUCIA O, RAVAGNANI V, PREGNOLATO F *et al*.: Baseline ultrasound examination as possible predictor of relapse in patients affected by juvenile idiopathic arthritis (JIA). *Ann Rheum Dis* 2018; 77: 1426-31.
28. CIMAZ R, GIANI T, CAPORALI R: What is the real role of ultrasound in the management of juvenile idiopathic arthritis? *Ann Rheum Dis* 2020; 79: 437-9.
29. PETTY RE, SOUTHWOOD TR, MANNERS P *et al*.: International league of associations for rheumatology classification of juvenile idiopathic arthritis: Second revision, Edmonton, 2001. *J Rheumatol* 2004; 31: 390-2.
30. WALLACE CA, RUPERTO N, GIANNINI E: Preliminary criteria for clinical remission for select categories of juvenile idiopathic arthritis. *J Rheumatol* 2004; 31: 2290-4.
31. SZKUDLAREK M, COURT-PAYEN M, JACOBSEN S, KLARLUND M, THOMSEN HS, OSTERGAARD M: Interobserver agreement in ultrasonography of the finger and toe joints in rheumatoid arthritis. *Arthritis Rheum* 2003; 48: 955-62.
32. WAKEFIELD RJ, BALINT PV, SZKUDLAREK M *et al*.: Musculoskeletal ultrasound including definitions for ultrasonographic pathology. *J Rheumatol* 2005; 32: 2485-7.
33. COLLADO P, NAREDO E, CALVO C, CRESPO M: Assessment of the joint recesses and tendon sheaths in healthy children by high-resolution B-mode and power Doppler sonography. *Clin Exp Rheumatol* 2007; 25: 915-21.
34. KARMAZYN B, BOWYER SL, SCHMIDT KM *et al*.: Us findings of metacarpophalangeal joints in children with idiopathic juvenile arthritis. *Pediatr Radiol* 2007; 37: 475-82.
35. ROTH J, JOUSSE-JOULIN S, MAGNI-MANZONI S *et al*.: Definitions for the sonographic features of joints in healthy children. *Arthritis Care Res* (Hoboken) 2015; 67: 136-42.
36. COLLADO P, VOJINOVIC J, NIETO JC *et al*.: Toward standardized musculoskeletal ultrasound in pediatric rheumatology: Normal age-related ultrasound findings. *Arthritis Care Res* (Hoboken) 2016; 68: 348-56.
37. ROTH J, RAVAGNANI V, BACKHAUS M *et al*.: Preliminary definitions for the sonographic features of synovitis in children. *Arthritis Care Res* (Hoboken) 2017;69:1217-23.
38. ALBERDI-SAUGSTRUP M, NIELSEN S, MATHIESSEN P, NIELSEN CH, MÜLLER K: Low pretreatment levels of myeloid-related protein-8/14 and c-reactive protein predict poor adherence to treatment with tumor

necrosis factor inhibitors in juvenile idiopathic arthritis. *Clin Rheumatol* 2017; 36: 67-75.

39. HAMMER HB, FAGERHOL MK, WIEN TN, KVIEN TK: The soluble biomarker calprotectin (an s100 protein) is associated to ultrasonographic synovitis scores and is sensitive to change in patients with rheumatoid arthritis treated with adalimumab. *Arthritis Res Ther* 2011; 13: R178.

40. HURNAKOVA J, HULEJOVA H, ZAVADA J *et al.*: Serum calprotectin discriminates subclinical disease activity from ultrasound-defined remission in patients with rheumatoid arthritis in clinical remission. *PLoS One* 2016; 11: e0165498.

41. FROSCH M, STREY A, VOGL T *et al.*: Myeloid-related proteins 8 and 14 are specifically secreted during interaction of phagocytes and activated endothelium and are useful markers for monitoring disease activity in pauci-articular-onset juvenile rheumatoid arthritis. *Arthritis Rheum* 2000; 43: 628-37.

42. WITTKOWSKI H, FROSCH M, WULFFRAAT N, GOLDBACH-MANSKY R *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.

43. ASAN G, DERIN ME, DOĞAN HO, BAYRAM M, ŞAHIN M, ŞAHIN A: Can calprotectin show subclinical inflammation in familial mediterranean fever patients? *J Korean Med Sci* 2020; 35: e63.

44. WITTKOWSKI H, KUEMMERLE-DESCHNER JB, AUSTERMANN J *et al.*: Mrp8 and mrp14, phagocyte-specific danger signals, are sensitive biomarkers of disease activity in cryopyrin-associated periodic syndromes. *Ann Rheum Dis* 2011; 70: 2075-81.

45. GERSS J, ROTH J, HOLZINGER D *et al.*: Phagocyte-specific s100 proteins and high-sensitivity c reactive protein as biomarkers for a risk-adapted treatment to maintain remission in juvenile idiopathic arthritis: A comparative study. *Ann Rheum Dis* 2012; 71: 1991-7.

46. BOJKO J: Measurement of blood calprotectin (mrp-8/mrp-14) levels in patients with juvenile idiopathic arthritis. *Reumatologia* 2017; 55: 10-4.

47. KORNDÖRFER IP, BRUECKNER F, SKERRA A: The crystal structure of the human (s100a8/s100a9)2 heterotetramer, calprotectin, illustrates how conformational changes of interacting alpha-helices can determine specific association of two EF-hand proteins. *J Mol Biol* 2007; 370: 887-98.

48. NAESS-ANDRESEN CF, EGELANDSDAL B, FAGERHOL MK: Calcium binding and concomitant changes in the structure and heat stability of calprotectin (l1 protein). *Clin Mol Pathol* 1995; 48: M278-84.

49. NORDAL HH, FAGERHOL MK, HALSE AK, HAMMER HB: Calprotectin (s100a8/a9) should preferably be measured in edta-plasma; results from a longitudinal study of patients with rheumatoid arthritis. *Scand J Clin Lab Invest* 2018; 78: 102-8.

50. AL-BASSAM WW, AD'HIAH AH, MAYOUF KZ: Significance of calgranulins (s100a8, s100a9 and s100a12), ferritin and toll-like receptor 4 in juvenile idiopathic arthritis children. *The Egyptian Rheumatologist* 2020; 42: 147-52.

51. VAN ZOELEN MA, VOGL T, FOELL D *et al.*: Expression and role of myeloid-related protein-14 in clinical and experimental sepsis. *Am J Respir Crit Care Med* 2009; 180: 1098-106.

52. FOELL D, WITTKOWSKI H, KESSEL C *et al.*: Proinflammatory s100a12 can activate human monocytes via toll-like receptor 4. *Am J Respir Crit Care Med* 2013; 187: 1324-34.