

Potential role of calprotectin as a monitoring biomarker for clinical and sonographic activity and treatment outcome in recent-onset rheumatoid arthritis

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Background

Calprotectin is a protein released during the activation and turnover of leukocytes. It can be used as a biomarker of inflammatory diseases such as rheumatoid arthritis (RA).

Aim

The current study aimed to measure the serum level of calprotectin in RA patients, recently diagnosed and after initiation of treatment, to determine its association with clinical disease, synovial inflammation determined by Ultrasound (US), and its relation to therapy when compared with other inflammatory markers.

Patients and methods

A total of 32 patients with recent RA and 20 healthy individuals were assessed for serum calprotectin level (enzyme-linked immunosorbent assay). C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) were also measured in patients who were reassessed 4 months after initiation of therapy. Disease activity was evaluated by the disease activity score of 28 joints (DAS28), and US assessment was performed.

Results

The mean level of serum calprotectin was significantly higher ($P < 0.001$) than that of controls. At baseline, there were significant ($P < 0.001$) correlations of calprotectin serum level with DAS28, ESR, CRP, grayscale, and power Doppler (PD) synovitis scores. After therapy, all except DAS28 and ESR significantly correlated with calprotectin serum level. Calprotectin was shown to be better ($P = 0.001$) than CRP ($P = 0.922$) and ESR ($P = 0.104$, $r^2 = 0.495$) in predicting power Doppler synovitis score. Calprotectin results showed higher sensitivity in predicting disease activity at the stage of active inflammation.

Conclusion

Serum calprotectin level is strongly associated with clinical, laboratory, and US parameters of inflammation in recent-onset RA. Calprotectin is a confident biomarker for monitoring the treatment outcome in RA patients.

Keywords:

calprotectin, disease activity score 28, rheumatoid arthritis, ultrasound

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Introduction

Rheumatoid arthritis (RA) is a chronic systemic inflammatory autoimmune disease that is more common among women. It is characterized by progressive joint destruction, presence of autoantibodies, and an increased risk for cardiovascular complications [1].

Treatment of patients with RA requires proper evaluation of the inflammatory activity of the disease, which depends on the acute-phase protein C-reactive protein (CRP) and the erythrocyte sedimentation rate (ESR) [2].

Calprotectin is a member of the S100 protein family. It is a heterodimeric complex of S100A8/A9 [3], myeloid-related protein (MRP8/MRP14) [4], L1

protein [5], and cystic fibrosis antigen [6], released during activation and turnover of leukocytes [7]. It could be used as an imperative biomarker of numerous inflammatory diseases, including RA [8,9].

Calprotectin is associated with joint damage and is an indicator of radiographic progression in patients with RA [10,11]. Circulating calprotectin levels elevate with disease activity and decrease after successful treatment; it could be the most sensitive biomarker of disease

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activity in RA compared with acute-phase proteins mainly of hepatic origin [12,13].

Musculoskeletal ultrasonography (US) is widely used as an imaging tool for evaluating disease activity in RA [14]. Several studies have reported that US examination is a highly sensitive method for assessment of subclinical synovitis [15]. Hammer *et al.* [16] suggested in their study that serum level of calprotectin is significantly associated with US examination of disease activity in RA patients. Our objective was to measure the serum level of calprotectin in recently diagnosed RA patients at baseline and after 4 months of treatment initiation to determine its association with clinical and US parameters of synovial inflammation and its relation to treatment outcomes compared with other markers of inflammation.

Patients and methods

This study was carried out in two phases: first, a case-controlled study, and second a longitudinal clinical study in which patients were prospectively followed up for 4 months after the initiation of appropriate treatment.

This study was carried out at the Rheumatology and Rehabilitation Outpatient Clinic and Inpatient Department of Benha University Hospitals from May 2014 until May 2015. It was approved by the Ethical Committee of the Rheumatology and Rehabilitation Department at Benha University. Informed consent was taken from the participants before enrollment in the study.

This study was conducted on 52 individuals who were divided into 2 groups.

- (1) Thirty-two patients with a recent-onset RA (<6 months) that was defined according to the American College of Rheumatology/European League Against Rheumatism (EULAR) 2010 classification [17].
- (2) The control group consisting of 20 healthy individuals age and sex matched to the patient group.

Exclusion criteria.

- (1) Patients not fulfilling the American College of Rheumatology criteria for the diagnosis of RA or had another rheumatic disease.

- (2) Patients younger than 18 years.
- (3) Patients with symptom duration more than 6 months.
- (4) Patients receiving disease-modifying anti-rheumatic drugs or glucocorticoids.

All patients were subjected to the following:

- (1) Full history taking and thorough clinical examination with stress on the locomotor system.
- (2) Disease activity assessment using the disease activity score for 28 joints (DAS28) [18].
- (3) Evaluation of clinical response to treatment as defined by the EULAR response criteria [19]: good responders had a $DAS28 \leq 3.2$ and >1.2 decrease in DAS28, whereas moderate responders were defined as having (a) $DAS28 \leq 3.2 + >0.6 \leq 1.2$ decrease in DAS28, (b) $DAS28 \leq 5.1 + >3.2 + >0.6$ decrease in DAS28, or (c) $DAS28 > 5.1 + >1.2$ decrease in DAS28. Nonresponders were defined as having <0.6 decrease in DAS28 or a $DAS28 > 5.1 + \leq 1.2$ decrease in DAS28.

Laboratory investigations

Blood samples of 5 ml were collected from all participants. For RA patients, samples were collected before therapy (baseline) and 4 months after the start of therapy. Blood was used for measurement of the following:

- (1) Complete blood picture using the automated hematology system (Sysmex XE 5000) (Sysmex, Canada) [20].
- (2) ESR by means of the Westergren method [21].
- (3) CRP using latex agglutination (CRP-Latex Cromatest) (Biotrax Testing, USA).
- (4) Assessment of serum anti-cyclic-citrullinated peptide antibodies determined by standard enzyme-linked immunosorbent assay (ELISA) kits (MyBioSource ACCPA, BioSource, Germany). ELISA was carried out according to the manufacturer's instructions.
- (5) Calprotectin serum levels using an ELISA test kit (Quantikine ELISA, R&D Systems parent company, USA), Human S100A8/S100A9 Heterodimer Immunoassay (R&D Systems parent company, USA), catalog number DS8900) according to the manufacturer's instructions. Our cutoff level for abnormal results was 2290 ng/ml (cutoff value = mean of negative control + 2SD).

Ultrasound imaging

A commercially available Logiq e real time scanner (General Electric Medical Systems, Milwaukee,

Wisconsin, USA) was used for the ultrasound (US) examination with a high-frequency (8–13 MHz) linear transducer. US assessment of the examined joints was performed based on the EULAR guidelines for musculoskeletal ultrasound in rheumatology on the same day as the clinical examination [22]. Grayscale (GS) and power Doppler (PD) examinations were performed using a 12-joint US assessment (bilateral elbow, wrist, second metacarpophalangeal, third metacarpophalangeal, knee, ankle). Synovitis in GS was graded semiquantitatively as follows: grade 0, absent; grade 1 (mild), small hypoechoic/anechoic line under the joint capsule; grade 2 (moderate), the joint capsule is elevated parallel to the joint area; and grade 3 (severe), strong distension of the joint capsule.

PD was also graded semiquantitatively from 0 to 3: 0=none, 1=minor, 2=moderate, and 3=major presence. The total US score was calculated as the sum of the synovitis and PD scores of each joint [23]. US examination was performed by a single observer who was blinded to the condition of the patients.

Statistical analyses

The data were summarized in the form of mean \pm SD, median, and range for quantitative data and as frequency and proportion for qualitative data. The *t*-test used to compare patients and controls for normal variables (age and sex) and the Mann–Whitney U-test was used as a nonparametric alternative.

The Kruskal–Wallis test (χ^2) was used for comparison between the different study groups for non-normally distributed quantitative data to detect significant differences.

The Wilcoxon signed-rank test was used to examine changes from baseline in the US, clinical, and laboratory results during follow-up for non-normally distributed data.

Correlations were analyzed using Spearman's rank correlations to detect correlations between all non-normally distributed data.

Linear regression models were used to assess the association between US scores as the dependent variable and different markers.

The discriminatory capacity of calprotectin was assessed with receiving operating characteristic curves with activity yes/no (yes DAS28>3.2) as the gold standard; the best cutoff in terms of sensitivity and specificity was identified. The area under the curve was calculated.

After the calculation of each of the test statistics, the corresponding distribution tables were consulted to get the '*P*' value (probability value). Statistical significance was accepted at *P* value less than or equal to 0.05 (significant), whereas a *P* value greater than 0.05 was considered nonsignificant.

All statistical analyses were carried out with SPSS, version 20.0 for Windows (SPSS Inc., Chicago, Illinois, USA).

Results

Thirty-two RA patients (age range 19–51 years) with a mean age of 33.44 \pm 7.8 years and 20 age-matched and sex-matched apparently healthy control (age range 19–63 years) with a mean age of 31.23 \pm 6.98 years were included in the study. Patients' clinical and laboratory features are shown in Table 1. Regarding

Table 1 Comparison between RA patients and controls as regard demographic, clinical and laboratory characteristics at baseline evaluation

Variable		Patients (No.=32)		Controls (No.=20)		<i>P</i>
		No.	%	No.	%	
Sex	Female	20	62.5	12	60	<i>P</i> >0.05
	Male	12	37.5	8	40	
Age (years)	Mean \pm SD	33.44 \pm 7.8		31.23 \pm 6.98		<i>P</i> >0.05
Swollen joint count	Mean \pm SD	4.99 \pm 2.98		—————		—
Tender joint count	Mean \pm SD	5.72 \pm 4.03		—————		—
DAS 28	Mean \pm SD	4.37 \pm 0.97		—————		—
Calprotectin (ng/L)	Mean \pm SD	3057 \pm 1527		1268 \pm 511		<i>P</i> <0.001
ESR (mm/1 st hr)	Mean \pm SD	39 \pm 17.28		—/—		—
CRP (mg/dl)	Mean \pm SD	34.78 \pm 24.97		—————/		—
RF U/ml	Mean \pm SD; (range)	/ 36.41 \pm 11.25		—————		—
Anti CCP U/ml	Mean \pm SD; (range)	96.85 \pm 55.47		—————		—

extra-articular manifestation in RA patients, six patients (18.8%) had subcutaneous nodules and four patients (12.5%) had interstitial pulmonary fibrosis.

All patients were receiving nonsteroidal anti-inflammatory drugs, whereas 20 patients (62.5%) were receiving methotrexate (12.5–20 mg/week) in combination with hydroxychloroquine, seven patients (21.88%) were on leflunomide (20 mg/day) in combination with hydroxychloroquine, and five patients (15.62%) were receiving a combination of methotrexate and leflunomide. Twenty patients (62.5%) were on corticosteroids, with a dose ranging from 5 to 20 mg/day.

The mean baseline serum calprotectin level in the RA patients (3057±1527 ng/l) showed a highly statistically significant ($P<0.001$) increase as compared with the mean serum level in the controls (1268±511 ng/l).

After 4 months of medical treatment, all clinical parameters, inflammatory markers, and ultrasound parameters improved.

There was a statistically significant decrease in the median DAS ($P<0.001$), calprotectin level ($P<0.001$), ESR ($P<0.001$), CRP ($P<0.001$), GS score ($P<0.001$), PD signal ($P<0.001$), and total ultrasound score ($P<0.001$) after medical treatment compared with their median values at baseline (Table 2).

Ten patients (31.25%) had good improvement, 12 patients (37.5%) had moderate improvement, and 10 patients (31.25%) reached remission according to the EULAR response criteria.

Regarding the relation between different disease activity grades and different inflammatory markers, there was a statistically significant difference in the

disease activity grades in relation to mean calprotectin ($P<0.05$), ESR ($P<0.05$), and CRP ($P<0.05$) at baseline evaluation (Fig. 1).

Serum calprotectin levels at baseline showed a statistically significant positive correlation with ESR ($r=0.646$, $P<0.001$), CRP level ($r=0.5$, $P<0.001$), DAS ($r=0.611$, $P<0.001$), GS score ($r=0.732$, $P<0.001$),

Figure 1

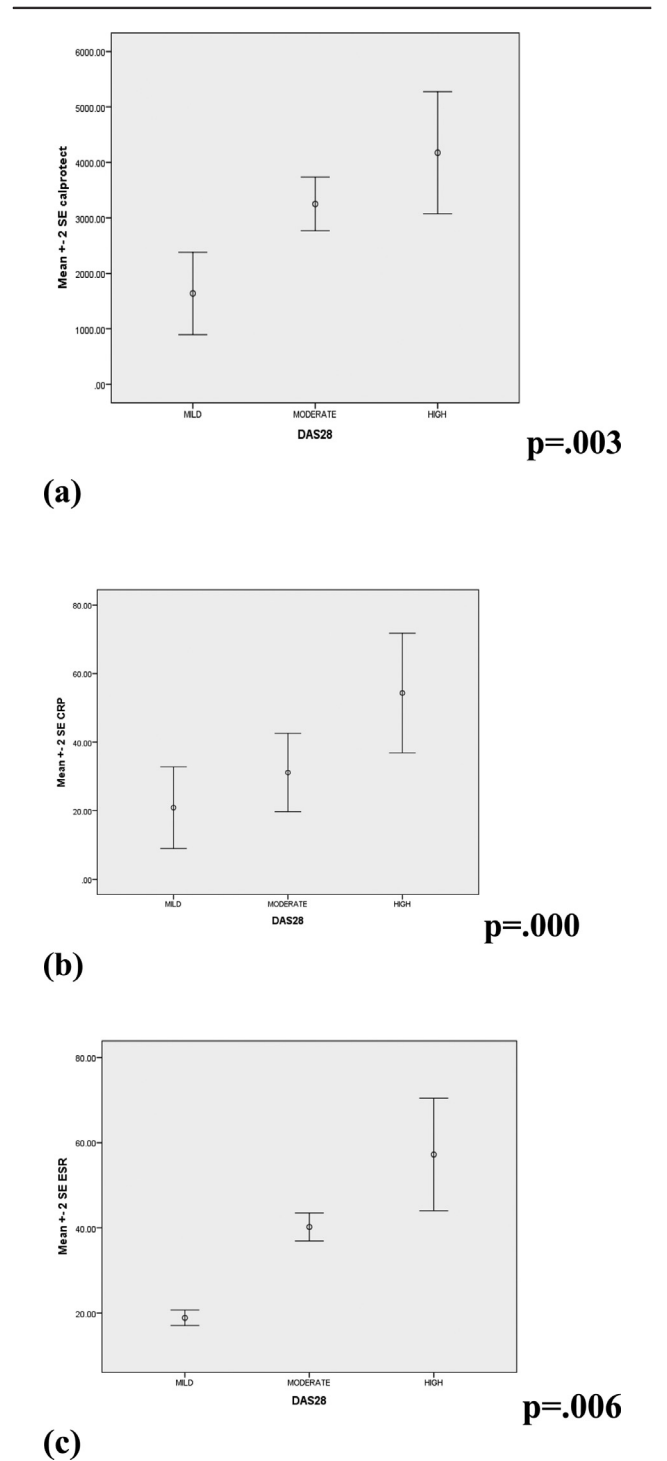


Table 2 Comparison of US, clinical and laboratory findings in RA patients at baseline evaluation and after 4 months.

Variable	At base line Median (range)	After 4 months Median (range)	Test *	P value
DAS28	4.45 (2.93–6.22)	3.19 (2.6–4.6)	-4.918	.000**
ESR (mm/1 st hr)	39 (15–94)	24 (7–53)	-4.863	.000**
CRP (mg/dl)	24 (0–96)	12 (0–48)	-4.717	.000**
GS	12.5 (3–24)	2 (0–8)	-4.786	.000**
PD	4.5 (0–14)	1 (0–4)	-4.655	.000**
Total US score	17(3–38)	3(0–12)	-4.785	.000**
Calprotectin (ng/L)	2974 (514–7217)	933 (512–2832)	-4.723	.000**

*The Wilcoxon signed rank test is used. **Highly statistical significant. GS, Gray scale; PD, power Doppler; RF, rheumatoid factor; US, ultrasound.

a, b, c Error plots of the different markers regarding different disease activity grades.

PD signal ($r=0.725$, $P<0.001$), and total US scores ($r=0.688$, $P<0.001$). Also, serum calprotectin levels at follow-up showed a statistically significant correlation with the CRP ($r=0.375$, $P<0.05$), GS score ($r=0.748$, $P<0.001$), PD signal ($r=0.58$, $P<0.001$), and total US scores ($r=0.687$, $P<0.001$) (Table 3).

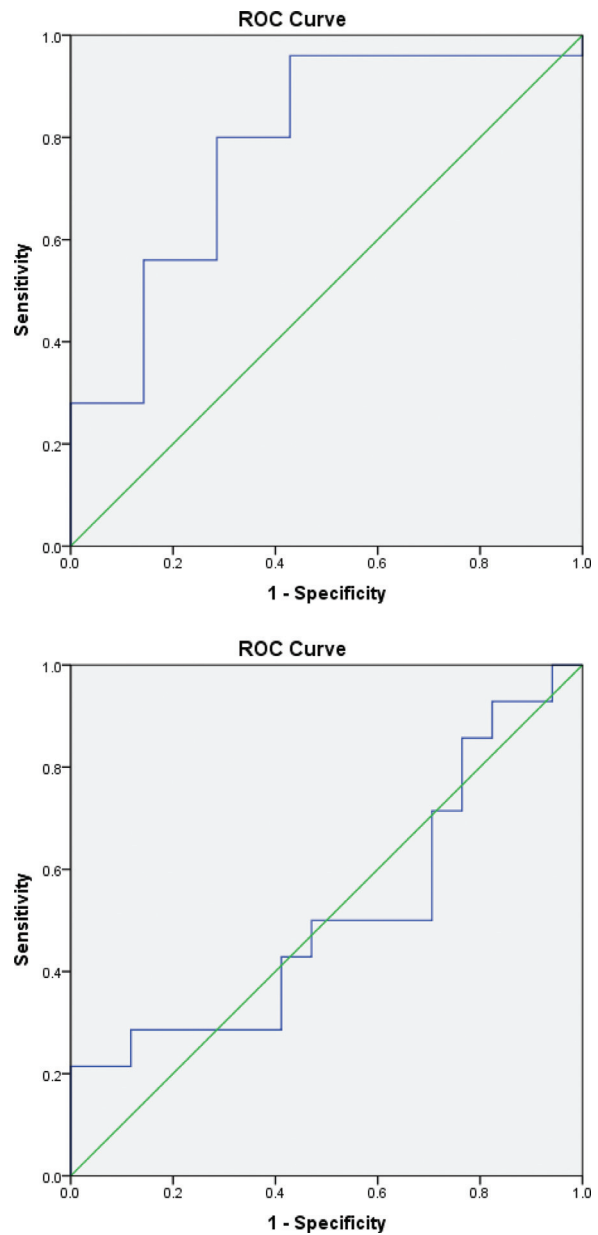
There was a statistically significant positive linear relationship between sonographic scores of the disease and serum levels of different inflammatory markers (Table 4). At baseline the relationship was highly significant (F -ratio=16.157 and $P<0.0001$) and accounted for 75% of the total variance in the dependent variable. Baseline calprotectin was shown to be better ($P=0.001$) than CRP ($P=0.922$) and ESR ($P=0.104$) at predicting the PD synovitis score.

The association after 4 months' evaluation was highly significant (F -ratio=13.516 and $P<0.0001$) and accounted for 73% of the total variance in the dependent variable. Also, follow-up calprotectin was shown to be better ($P<0.001$) than CRP ($P=0.477$) and ESR ($P=0.568$) at predicting the PD synovitis score.

The receiving operating characteristic curve of the relation between DAS28 and calprotectin level at baseline (Fig. 2a) revealed an area under the curve of

0.783 at 95% CI, which means that calprotectin level was a fair predictor with statistically significantly higher

Figure 2



(2-a) ROC curve of the relation between DAS and Calprotectin marker at base line assessment. (2-b) ROC curve of the relation between DAS and different markers after 4 months.

Table 3 Correlation between Calprotectin serum levels at baseline and after four months evaluation in RA patients with various disease parameters

Variable	Calprotectin at base line	Calprotectin after 4 months
GS	0.732**	0.748**
PD	0.725**	0.475**
Total US score	0.688**	0.687**
ESR (mm/1 st hr)	0.646**	0.154
CRP (mg/dl)	0.500**	0.375*
RF	0.15	0.17
Anti CCP	0.18	0.14
DAS28	0.611**	0.124

* Statistical significant, **Highly statistical significant. GS, Gray scale; PD, power Doppler; RF, rheumatoid factor; US, ultrasound.

Table 4 linear regression between total US score of the disease and different markers levels at base line and after four months assessment.

Model		Unstandardized Coefficients		Standardized Coefficients	T	Sig.	F	R square
		B	Std. Error	Beta				
At base line	CRP	.004	.043	.012	.099	.922	16.157	.757
	ESR	.144	.085	.292	1.68	.104		
	Calprotectin	.004	.001	.671	3.74	.001		
After 4 months	CRP	-.022	.031	-.098	-.722	.477	13.516	.738
	ESR	.016	.028	.074	.579	.568		
	Calprotectin	.005	.001	.910	7.272	.000		

results ($P=0.024$). The optimal cutoff point for calprotectin level to predict the DAS28 was 1310 with a sensitivity of 96% and specificity of 43%, whereas at follow-up the area under the curve was 0.513 at 95% CI [calprotectin level after 4 months was not a fair predictor of DASs, with nonstatistically significant results ($P=0.905$)]. The optimal cutoff point of calprotectin level in predicting the DAS was 782 with a sensitivity of 71% and specificity of 70% (Fig. 2b).

Discussion

Calprotectin is expressed by synovial tissue macrophages localized in the lining layer adjacent to the cartilage–pannus junction [24]. It is claimed to be produced by the inflamed synovium and may reflect the amount of activated synovial macrophages [7].

In the present study, we found a highly significant increase in the serum level of calprotectin in recent-onset RA patients compared with controls. This is in agreement with the results of Andrés Cerezo *et al.* [13] on patients with recent RA and with other reports on a previously established disease [16]. As calprotectin is a small molecule that can easily diffuse outside the inflamed synovium to be detected in the bloodstream [5], serum levels of calprotectin strongly correlate with its level in the synovial fluid and significantly associate with clinical disease activity and acute-phase reactants [25,26]. These data were obvious in our study on RA patients, in whom statistically significant correlations were detected between the level of serum calprotectin, acute-phase reactants, disease activity, ultrasound GS, and PD synovitis scores.

Some RA patients do not exhibit high levels of CRP while the disease is active. In addition, there is rising evidence that calprotectin may be elevated even with normal acute-phase reactants [27].

The levels of calprotectin after 4 months of treatment have been found to reflect the level of synovial inflammation, as both calprotectin level and cases of GS/PD US-detected synovitis reduced significantly. The decrease in calprotectin serum levels, but not CRP, was considered a significant predictor of improvement in RA-related swollen joints [12]. In our study, we used ultrasound assessment, GS, and PD to ensure an accurate assessment of joint inflammation and disease activity in RA, as followed by Hurnakova *et al.* [28]. This is a modality that reflects the clinical activity and might be more sensitive than a clinical examination [29].

There was a significant positive linear relationship between our sonographic scores and the serum levels of different inflammatory markers, in which baseline and follow-up calprotectin results were shown to be better than CRP and ESR for predicting and following PD synovitis, supporting the hypothesis that calprotectin is a predictive marker of inflammation by US than CRP or ESR. That is why some studies considered it as a promising biomarker of RA disease activity [10], psoriatic arthritis [30], and juvenile idiopathic arthritis [31].

Subclinical synovitis is important in the assessment of RA patients, and both GS and PD activity have been described to be associated in patients with RA in clinical remission [32]. PD activity was found to be associated with erosions [33]. Our finding of a correlation between calprotectin level and US scores suggests that in recent-onset RA patients a normal level of calprotectin may be considered a potential marker of improvement and response to medication, which can indirectly reflect radiographic remission. These results support the study of Hammer *et al.* [15] in which there was a significant correlation between serum calprotectin level and US examination in 20 established RA patients. Our results have shown that calprotectin had a sensitivity of 96% and specificity of 43% with a statistical significance to predict the disease activity. After treatment, no statistically significant results were found that could be related to the effect of treatment on the inflammatory status.

Although several studies have found a relationship between calprotectin serum level with rheumatoid factor and anti-cyclic-citrullinated peptide antibodies in patients with RA [10,34,35], this was not supported in our results, as we found no statistically significant correlations for these antibodies.

Calprotectin has been documented to predict a 10-year radiographic progression in recognized RA patients [11]. This study was not adequately long to consider the outcome of changes in calprotectin levels on the progression of radiographic changes. We could suppose that a decrease in calprotectin serum levels over time, coupled with a decrease in disease activity, would lead to inhibition of more structural damage to the joints.

Conclusion

Serum calprotectin is strongly associated with clinical, laboratory, and US parameters of active inflammation in recent-onset RA. However, its level declines with

improvement after therapy. This demonstrates that calprotectin can monitor the treatment outcome in RA patients. Calprotectin is a reliable biomarker for prediction of synovial inflammation in US assessment; however, larger longitudinal studies with longer follow-up durations are needed to establish this finding.

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Conflicts of interest

There are no conflicts of interest.

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