

## RESEARCH ARTICLE

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# The soluble biomarker calprotectin (a S100 protein) is associated to ultrasonographic synovitis scores and is sensitive to change in patients with rheumatoid arthritis treated with adalimumab

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## Abstract

**Introduction:** Calprotectin (MRP8/MRP14, S100A8/A9) is associated with disease activity in patients with rheumatoid arthritis (RA). Ultrasonography (US) is a reliable method for evaluation of synovitis (B-mode (BM) and power Doppler (PD)). The present objectives were to explore in RA patients the associations between calprotectin and a comprehensive US examination, as well as the responsiveness of calprotectin compared to other inflammatory markers during anti-TNF treatment.

**Methods:** A total of 20 RA patients starting treatment with adalimumab were examined longitudinally by US (BM and PD (semi-quantitative scores 0 to 3) of 78 joints, 36 tendons/tendon groups and 2 bursae) and clinically at baseline and after 1, 3, 6 and 12 months. Associations between the US sum scores and the inflammatory markers calprotectin, serum amyloid A (SAA), CRP and ESR were explored by correlation and linear regression analyses, and the response to treatment was assessed by Standardized Response Mean (SRM).

**Results:** The inflammatory markers, clinical examinations and US sum scores improved during treatment ( $P < 0.001$ ). Of the inflammatory markers, calprotectin had the highest correlation coefficients with the total BM and PD sum scores (median (range) 0.59 (0.37 to 0.76) for BM and 0.56 (0.38 to 0.72) for PD). Even higher correlations were found between calprotectin and sum US scores of reduced number of joint counts. Calprotectin made a considerable contribution to total US sum scores in the linear regression analyses ( $P = 0.001$  to 0.031) and among the inflammatory markers, calprotectin had the highest SRM (0.84 at one month).

**Conclusions:** Calprotectin was associated with the sum scores from a comprehensive US assessment and was responsive to change during anti-TNF treatment. Thus, examination of this leukocyte protein could be of additional value in the assessment of RA patients on biologic treatment.

**Keywords:** Calprotectin, acute phase proteins, rheumatoid arthritis, ultrasonography, anti-TNF treatment

## Introduction

Assessment of inflammatory activity is of pivotal importance for the optimal treatment of patients with rheumatoid arthritis (RA). Up till now the erythrocyte sedimentation rate (ESR) and the acute phase protein C-reactive protein (CRP) are the most widely used laboratory markers for evaluation of inflammatory activity in

patients with RA. Serum amyloid A (SAA) (an apo-lipoprotein associated with high-density lipoprotein in plasma) is produced in the liver after stimulation by the pro-inflammatory cytokines IL-1, IL-6 and TNF-alpha, and 1, 000-fold increases may be found during severe inflammation [1]. Thus, it acts as an acute phase protein like CRP, but may be more sensitive to inflammation [2].

Calprotectin is a major leukocyte protein, constituting 40 to 60% of the cytosolic protein in neutrophil granulocytes as well as being a major monocyte/macrophage protein [3-5]. Calprotectin (also named MRP-8/MRP-14

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[6] and S100A8/A9 [7]) is a calcium-binding S100 protein [8] and it is one of the damage-associated molecular pattern molecules (DAMPs) highly up-regulated in various autoimmune disorders [9]. The protein is released during activation and turnover of leukocytes [10] and calprotectin levels are strongly associated with disease activity in RA patients [11,12]. Concentrations of calprotectin are high in synovial fluid in RA patients in contrast to low levels in osteoarthritis patients [13] and calprotectin is associated to radiographic damage and is a predictor of radiographic progression in RA patients [12,14].

Ultrasonography (US) is a valid and reliable tool for detecting synovitis in patients with RA [15-17], and joints, tendons and bursae may be examined. The extent of synovitis is usually scored semi-quantitatively (scale 0 to 3) both for grey scale (or B-mode (BM)) synovitis (including combined evaluation of synovial hypertrophy and effusion) and power Doppler (PD) vascularization, and the US scores have been shown to be sensitive for improvement during biological treatment in RA patients [17-20]. A recently published study from our group showed a high degree of reliability when we used a similar scoring system as presently applied [21]. There is no consensus on the optimal number of joints and tendons to be assessed for US evaluation of inflammatory activity in RA patients, but similar sensitivity to change has been found for US examinations of different combinations of joints and tendons during biological treatment [22].

The objectives of the present study were to examine the associations between the levels of calprotectin and a comprehensive as well as reduced US joint scores and to explore the responsiveness of calprotectin compared to other inflammatory markers during biologic treatment in patients with RA.

## Materials and methods

### Patients

The present study is part of a work described in detail previously [23]. In short, a comprehensive US assessment was performed in 20 patients with RA [24] (median (range) age 53 (21 to 78) years, disease duration 7.5 (1 to 26) years, 15 women, 70% IgM rheumatoid factor positive) the same day as they started treatment with adalimumab and after 1, 3, 6 and 12 months. Adalimumab (as their first biological medication) was dosed 40 mg every other week, all patients were on methotrexate as co-medication, 14 patients used additional prednisolone (median (range) dose 7.5 (3.75 to 15) mg) and two patients were on daily non-steroidal anti-inflammatory drug.

The patients gave written consent according to the Declaration of Helsinki, and the study was approved by the local ethics committee (the regional committee for medical and health research ethics (REK), South-East).

### Laboratory examinations

The traditional inflammatory markers included ESR and CRP (both analyzed by use of in-house standard methodology, with upper normal levels of 20 mm/h for ESR and 4 mg/l for CRP). Serum and EDTA plasma from all the five examinations were frozen at  $-70^{\circ}\text{C}$  for assessment of SAA and calprotectin after completion of the clinical study. SAA was measured in serum by immunonephelometry using an N-Latex SAA reagent (Dade Behring Inc., Marburg, Germany) with normal levels  $\leq 6.4$  mg/l [25]. All plasma samples were analyzed for calprotectin at the same time by use of ELISA as described previously (using kits from Calpro AS, Norway) [13,26], with normal levels  $\leq 0.91$  mg/l. Assessments of calprotectin in plasma have shown coefficients of variation of 5% within and 13% between assays [27].

### Clinical evaluations

One of two study nurses, with more than five years experience with joint counts in clinical studies, assessed 40 joints for tenderness and swelling (proximal interphalangeal 1 to 5, metacarpophalangeal 1 to 5, wrist, elbow, shoulder, knee, ankle and metatarsophalangeal 1 to 5) and they scored the global disease activity caused by RA on a VAS scale (assessor's global VAS). They were blinded for the results of the US examinations. Patient's evaluation of disease activity was assessed (VAS), and DAS28 [28] was calculated.

### Ultrasonography

All patients were assessed by extensive US examinations (performed by one experienced sonographer, HBH) with use of a 5 to 13 MHz probe (Siemens Antares, Sonoline, Siemens Medical Solutions, 1230 Shorebird Way Mountain, CA, USA) with fixed settings optimal for power Doppler signals in more superficial joints. The joints assessed bilaterally by use of standard projections [29] included proximal interphalangeal 1 to 5, metacarpophalangeal 1 to 5, carpometacarpal 1 to 5, wrist (radiocarpal, intercarpal and radioulnar joints), elbow, shoulder (glenohumeral and acromioclavicular joints) hip, knee, ankle (talocrural joint), 4 major foot joints (talonavicular, subtalar, calcaneocuboidal and cuneonavicular joints), tarsometatarsal 1 to 5, metatarsophalangeal 1 to 5 and the interphalangeal joint of the first toe (a total of 78 joints), in addition to tendons bilaterally in the wrists (at the level of the radiocarpal joint: all the 6 compartments dorsally and the flexor digitorum superficialis and profundus, flexor pollicis longus and flexor carpi radialis tendons at the palmar side), in the ankles (at the level of the malleol and distal tibia; medial, anterior and lateral tendon groups) as well as the long biceps tendon (a total of 36 tendons or tendon groups) and subdeltoid bursa bilaterally. The arthritis, tenosynovitis and bursitis were all scored for BM presence of synovial

**Table 1 Median (range) of the laboratory, clinical and US scores**

|                                    | Baseline             | 1 month                | 3 months              | 6 months               | 12 months             |
|------------------------------------|----------------------|------------------------|-----------------------|------------------------|-----------------------|
| Calprotectin, mg/l (normal ≤ 0.9l) | 2.02 (0.56 to 20.44) | 0.92 (0.32 to 18.86)** | 0.93 (0.28 to 6.30)** | 0.94 (0.24 to 22.02)** | 1.08 (0.36 to 17.60)  |
| SAA mg/l (normal ≤ 6.4)            | 10.60 (2.17 to 300)  | 6.94 (1.37 to 270)*    | 4.19 (0.70 to 38.5)** | 4.39 (1.61 to 176)*    | 8.37 (1.12 to 136)    |
| CRP, mg/l (normal ≤ 4)             | 4.5 (1 to 136)       | 2.0 (1 to 163)         | 2.5 (1 to 26)**       | 2.5 (1 to 78)          | 4.5 (1 to 86)         |
| ESR, mm/h (normal ≤ 20)            | 20 (3 to 67)         | 13 (3 to 108)**        | 14.5 (2 to 65)**      | 14.5 (5 to 99)         | 17.5 (3 to 78)        |
| DAS28                              | 5.32 (3.40 to 7.70)  | 4.20 (1.56 to 8.90)**  | 4.26 (0.83 to 7.02)** | 4.36 (1.59 to 7.58)**  | 4.33 (1.40 to 6.60)** |
| Assessors VAS                      | 26 (6 to 55)         | 15 (4 to 52)*          | 14 (3 to 40)**        | 11 (2 to 57)           | 10 (3 to 44)**        |
| No of swollen joints (of 40)       | 8 (1 to 19)          | 5 (0 to 22)            | 6 (0 to 16)**         | 4 (0 to 16)*           | 4 (0 to 14)**         |
| No of tender joints (of 40)        | 12 (0 to 23)         | 9 (0 to 35)            | 7 (0 to 16)**         | 6 (0 to 16)**          | 7 (0 to 21)**         |
| BM score                           | 53 (6 to 151)        | 39 (3 to 121)**        | 35 (6 to 94)**        | 24 (0 to 86)**         | 27 (1 to 96)**        |
| PD score                           | 33 (0 to 119)        | 21 (0 to 83)**         | 16 (2 to 53)**        | 14 (0 to 76)**         | 13 (0 to 72)**        |

\* $P < 0.05$ , \*\* $P < 0.01$  (Wilcoxon signed rank test for changes from baseline to the different follow-up examinations).

BM, B-mode; CRP, C-reactive protein; DAS28, Disease Activity Score 28 joints; ESR, erythrocyte sedimentation rate; PD, power Doppler; SAA, serum amyloid A; Assessors VAS, assessors global disease activity score on a visual analogue scale (0 to 100).

hypertrophy and fluid (combined) and presence of vascularization (PD) on a 4-point scale: 0 = none, 1 = minor, 2 = moderate or 3 = major presence. The US examiner was blinded for US results from the previous examinations as well as for the results from the clinical joint assessments and laboratory tests performed the same day.

### Statistics

Wilcoxon signed rank test was used to examine changes from baseline of the US, clinical and laboratory results during follow-up. Correlations were analyzed by use of Spearman's rank correlations. Linear regression analysis was performed for all examinations with total sum score BM or sum score PD as the dependent variable with age, sex, disease duration and DAS28 as well as each of the biomarkers (one at a time) as the independent variables. A  $P$ -value  $< 0.05$  was considered statistically significant. The responsiveness to change was explored by calculation of the Standardized Response Mean (SRM) as the mean change divided by the SD of the change (definitions of SRM results:  $< 0.50$  = small,  $0.50$  to  $0.80$  = moderate and  $> 0.80$  = large responses).

### Results

The laboratory, clinical and US results improved significantly during the study (Table 1). Significant cross-

sectional correlations were found between the inflammatory biomarkers and both BM and PD total sum scores at most of the examinations, with calprotectin having the highest correlation coefficients (Table 2). Figure 1 illustrates the associations between calprotectin and total sum US results at baseline and after three months. Of the correlation analyses between calprotectin levels and US sum scores of different reduced numbers of joints and tendons (the 7-, 12-, 28- and 44-joint scores [22]), the 12-joint score was found to have the highest correlation coefficients at different examinations (median (range)  $0.59$  ( $0.53$  to  $0.83$ ) ( $P = 0.02$  to  $< 0.001$ ) for BM and  $0.58$  ( $0.42$  to  $0.72$ ) ( $P = 0.06$  to  $< 0.001$ ) for PD).

To assess the validity of the US examination, the 28 joints included in the DAS28 were explored for similarity in detecting arthritis by correlating the number of joints identified by clinical evaluation of swelling (yes vs. no) and BM score ( $\geq 1$  vs. 0). The median (range) correlations were  $0.58$  ( $0.43$  to  $0.85$ ) ( $P = 0.06$  to  $< 0.001$ ). When the sum BM scores of the 28 joints were correlated with the number of joints with clinical swelling, even higher coefficients were found ( $0.67$  ( $0.48$  to  $0.87$ ) ( $P = 0.03$  to  $< 0.001$ )).

The median (range) correlation coefficients between the inflammatory biomarkers and assessors global VAS (at the five examinations) were  $0.55$  ( $0.37$  to  $0.74$ ) for

**Table 2 Spearman's rank correlations between the laboratory variables and sum B-mode/power Doppler scores**

|              | Baseline   | 1 month    | 3 months      | 6 months     | 12 months     |
|--------------|------------|------------|---------------|--------------|---------------|
| Calprotectin | 0.45*/0.38 | 0.37/0.53* | 0.76**/0.72** | 0.60**/0.56* | 0.59**/0.60** |
| SAA          | 0.42/0.37  | 0.30/0.30  | 0.58**/0.37   | 0.61**/0.51* | 0.48*/0.45*   |
| CRP          | 0.33/0.34  | 0.04/0.11  | 0.49*/0.35    | 0.59**/0.43  | 0.57**/0.55*  |
| ESR          | 0.37/0.48* | 0.36/0.35  | 0.45*/0.42    | 0.32/0.33    | 0.35/0.40     |

\* $P < 0.05$ , \*\* $P < 0.01$

CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; SAA, serum amyloid-A

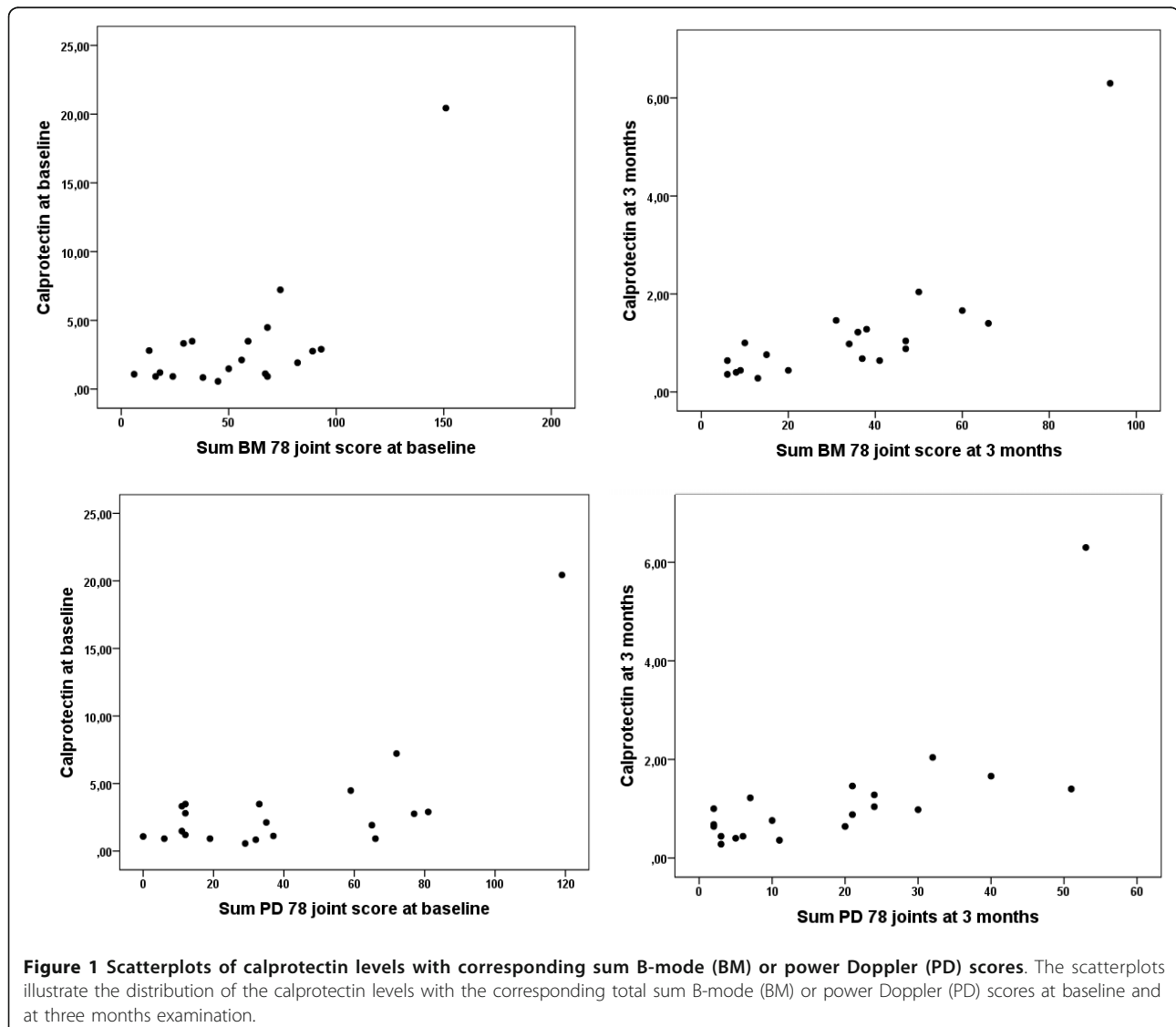
calprotectin, 0.57 (0.41 to 0.73) for SAA, 0.59 (0.39 to 0.80) for CRP and 0.45 (0.33 to 0.65) for ESR (correlation coefficients  $\geq 0.43 = P < 0.05$ ).

Both calprotectin and CRP contributed significantly to explain total sum score BM as the dependent variable in linear regression analyses performed for all examinations during the study ( $P = 0.001$  to  $0.017$  for calprotectin and  $P = 0.012$  to  $0.038$  for CRP), SAA contributed significantly at baseline and six months ( $P = 0.045$  and  $0.032$ ) and ESR at one and three months ( $P = 0.030$  and  $0.048$ ). With total sum score PD as the dependent variable, calprotectin contributed significantly at all examinations ( $P = 0.003$  to  $0.031$ ), CRP contributed significantly at baseline, 6 and 12 months ( $P = 0.001$  to  $0.026$ ), SAA at 6 months ( $P = 0.002$ ) and ESR at 3 and 6 months ( $P = 0.007$  and  $0.044$ ).

Calprotectin was found to have higher responsiveness to change than CRP, SAA and ESR (Table 3), with large SRM at 1 month, moderate at 3 and 6 months and small at 12 months examination.

## Discussion

To our knowledge, this is the first study to examine the associations between levels of calprotectin and BM/PD scores from a comprehensive US examination. All the inflammatory biomarkers, clinical assessments and US sum scores improved during treatment with adalimumab. Significant correlations were found between the levels of calprotectin and the US scores and the validity of calprotectin as an inflammatory marker was supported by associations with the clinical evaluation of disease activity. In addition, calprotectin was found to have substantial responsiveness to biological treatment.



**Table 3 Standardized response mean (SRM) of the changes from baseline of the four laboratory markers**

|              | SRM<br>1 month | SRM<br>3 months | SRM<br>6 months | SRM<br>12 months |
|--------------|----------------|-----------------|-----------------|------------------|
| Calprotectin | -0.84          | -0.63           | -0.63           | -0.32            |
| SAA          | -0.44          | -0.55           | -0.38           | -0.30            |
| CRP          | -0.10          | -0.43           | -0.15           | -0.12            |
| ESR          | -0.33          | -0.71           | -0.03           | -0.13            |

CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; SAA, serum amyloid-A.

Calprotectin is released from leukocytes during inflammation. Thus the present objective was to perform an extensive US examination of joints, tendons and bursae to capture the total amount of inflammation. However, the scoring system does not differ between small and large joints. Hence, adjusting for the joint size could have improved the correlations between calprotectin levels and sum US scores. When reduced numbers of joints were analyzed, higher coefficients were found. The strongest correlations were found with the 12-joint score [17], which includes bilateral examination of the elbow, wrist, MCP 2 and 3, knee and ankle in addition to the medial and lateral tendon compartments in the ankle. Thus, in this score most of the joints are relatively large, which may explain the stronger correlations with calprotectin.

The correlations between the laboratory markers and the total sum US scores increased during the study, with highest coefficients between calprotectin and US scores (both comprehensive and reduced number of joints) at the three-month examination (Figure 1). Calprotectin levels have been found to reflect the ongoing inflammation, and at the three-month examination both calprotectin and the US detected synovitis were significantly reduced. Since this is a small study, the different levels of association between calprotectin and US pathology during biologic medication should be explored in larger studies.

Calprotectin is expressed in synovial tissue macrophages localized in the lining layer adjacent to the cartilage-pannus junction [30] and high levels of calprotectin have been found in synovial fluid from RA patients [13]. Calprotectin is suggested to play an active role in the inflammation by amplifying pro-inflammatory cytokine responses via activation of NF- $\kappa$ B and p38 mitogen-activated protein kinase in RA [31]. Calprotectin has been shown to be a reliable marker of inflammation in several rheumatic joint diseases like RA [11,12], juvenile idiopathic arthritis [32,33] and psoriatic arthritis [34]. The present study indicates that calprotectin levels are associated with the overall inflammation in RA patients, including both the amount of synovial hypertrophy explored by BM and the extent of vascularization evaluated by PD.

The regression analyses showed calprotectin to be independently associated with both total sum BM and PD scores, even when DAS28 was included in the equation. Thus, calprotectin was able to give information beyond a clinical composite score. Subclinical inflammation is of major importance in the evaluation of RA patients, and both BM and PD activity have been described in RA patients being in clinical remission [35]. A recent study of patients with juvenile idiopathic arthritis in remission showed that the level of calprotectin was associated with risk of relapse after discontinuing methotrexate [36]. A definition of clinical remission in RA was recently presented by ACR and EULAR [37]. The present finding of associations between calprotectin and the US sum scores suggest that in RA patients a normal calprotectin level could support a clinical evaluation of remission. This important issue should be further explored.

PD activity has been found to be related to the development of joint erosions [38] and we have previously found calprotectin to be associated with radiographic damage [12,14]. Calprotectin plays a major role in the local inflammatory process during RA [9,31], and the possible association between PD activity and calprotectin levels should, therefore, be investigated in a larger study.

The evaluation of US synovitis scores is dependent on the operator. Much work has been done to improve the reliability of US scoring [15-17]. For the joint scores included in the present study there were high intra-reader intra-class correlations for evaluation of stored images (described previously [23]). In addition, the present BM and PD joint scoring was similar to the scoring system used in a US atlas recently applied in a reliability study where we found high intra- and inter- reader reliability [21].

Strengths of the present longitudinal study were that only one experienced sonographer performed the comprehensive US assessments and that all the RA patients used a biologic medication supposed to cause improvement. However, an obvious weakness is the limited number of patients included.

## Conclusions

Calprotectin was found to have high association to US assessments of RA patients, indicating that calprotectin reflects ongoing inflammation. In addition, calprotectin levels decreased during treatment with adalimumab, and had higher sensitivity to change than the traditional inflammatory markers. In contrast to the acute phase proteins being produced in the liver, calprotectin is released from activated leukocytes during inflammation. Our results support that calprotectin may be used as a marker of inflammatory activity in RA patients on biologic treatment.

## Abbreviations

BM: B-mode or grey scale; CRP: C-reactive protein; DAMPs: damage-associated molecular pattern molecules; DAS28: disease activity score (including 28 joints); ESR: erythrocyte sedimentation rate; IL: interleukin; PD: power Doppler activity; RA: rheumatoid arthritis; SAA: serum amyloid A; SRM: standardized response mean; TNF: tumor necrosis factor; US: ultrasonography; VAS: visual analogue scale.

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## Authors' contributions

HBH was the principal investigator for the study, responsible for the study design and performance of all the ultrasonographic examinations as well as writing the manuscript. MKF was responsible for the quantification of calprotectin and giving constructive comments on the manuscript. TNW was responsible for the quantification of serum amyloid A and giving constructive comments on the manuscript. TKK facilitated the performance of the study, giving input to the study design and giving constructive comments on the manuscript. All the authors have read and approved the manuscript for publication.

## Competing interests

The authors declare that they have no competing interests.

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