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ORIGINAL ARTICLE

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Calprotectin (S100A8/A9) should preferably be measured in EDTA-plasma; results from a longitudinal study of patients with rheumatoid arthritis

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ABSTRACT

Calprotectin (S100A8/A9), a protein expressed in neutrophils and monocytes/macrophages in circulation and inflamed tissue, is associated with measures of disease activity in rheumatoid arthritis (RA) patients both when measured in ethylenediaminetetraacetic acid (EDTA)-plasma and in serum. We wanted to explore if EDTA-plasma or serum should be preferred for calprotectin as a marker of disease activity. Calprotectin was analysed in EDTA-plasma and serum by enzyme-linked immunosorbent assay (ELISA) at baseline in 141 RA patients, starting biologic disease-modifying anti-rheumatic drugs (bDMARDs), and after three months. Differences between plasma and serum levels of calprotectin were assessed by Wilcoxon signed rank test. Variability was assessed by quartile coefficient of dispersion. Spearman's test explored correlations between calprotectin in plasma and serum and between calprotectin (plasma or serum) and clinical/ultrasound (US) measures of disease activity. Bland Altman plots were used for method comparisons. Conventional inflammatory markers were evaluated for comparison. Calprotectin had similar variability when measured in plasma and serum, but there was a significant difference in concentrations between plasma and serum ($p < .001$). The correlation coefficients at baseline between calprotectin measured in plasma/serum and measures of disease activity were $r_s = 0.62/0.46$ for sum power Doppler score (PD), $r_s = 0.60/0.48$ for assessor's global visual analogue scale (VAS), $r_s = 0.59/0.43$ for sum grey scale (GS) score and $r_s = 0.47/0.37$ for swollen joint count of 32, all $p < .001$. Similar differences were found after three months. Calprotectin measured in plasma showed the strongest associations with assessments of disease activity, and EDTA-plasma should preferably be used when evaluating disease activity in RA patients.

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Introduction

Macrophages are often the dominating inflammatory cells in the synovial sublining area, in the intimal lining layer and pannus–cartilage junction in rheumatoid arthritis (RA) patients [1, 2]. Neutrophils are the most abundant cells in the synovial fluid and can be found at the pannus–cartilage junction [2,3]. The calcium-binding protein calprotectin, a hetero-complex of S100A8 and S100A9, also called myeloid-related protein (MRP) 8/14, is mainly expressed in leukocytes; in circulating neutrophils and monocytes and in neutrophils and macrophages of inflamed tissue [4–6]. It is a major protein in neutrophils, comprising up to 60% of the total protein content in cytosol [7]. Compared with the acute-phase reactant C-reactive protein (CRP), which indirectly reflects inflammatory reactions by its interleukin (IL)-6 induced production in the liver, calprotectin levels reflect the leukocyte activation and turnover during inflammation, and strong associations have been shown between the level of calprotectin and disease activity in several inflammatory diseases [8–11].

Ultrasound (US) is a sensitive method for the evaluation of synovitis in RA [12]. Grey scale (GS) US detects the hypertrophied synovia and the increased synovial fluid of inflamed RA joints. Power Doppler (PD) assessments reflect the vascular flow in the synovia and correlate with the macrophage infiltrate in the sublining area of the synovia [13] as well as with the number of leukocytes in the synovial fluid of RA patients [14].

Calprotectin is a promising marker of inflammation in RA patients, and plasma or serum measurements have shown calprotectin to be associated with clinical disease activity [9,15–24] as well as being more strongly associated with disease activity than CRP and erythrocyte sedimentation rate (ESR) [9,22,23,25–28]. Calprotectin was also associated with US assessments in RA patients [25–29]. In addition, calprotectin has been found to predict response to methotrexate and biologic disease-modifying anti-rheumatic drugs (bDMARD) [28,30,31], was related to radiological damage in a cross-sectional study [9] and could predict radiological progression in two longitudinal studies [29,32].

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In the clinical studies [9,15–32] calprotectin was measured by enzyme-linked immunosorbent assay (ELISA), either in plasma or in serum. Already in 1980 and later in 1990, ethylenediaminetetraacetic acid (EDTA)-plasma was suggested as the preferred choice for measuring calprotectin in blood [33,34]. Calprotectin levels were stable in EDTA-plasma, while they increased in serum samples during storage at 4 °C. There may therefore be differences when calprotectin is measured in plasma versus in serum. There are to our knowledge no previous comparative studies on the two methods regarding the associations with disease activity in RA patients. The present objective was to explore whether calprotectin levels measured in EDTA-plasma or in serum have the highest association with disease activity in a longitudinal study of RA-patients starting with bDMARD [28].

Methods

Patients and blood sampling

From a previously described cohort of 141 patients with RA [28], according to the 1987 revised American Rheumatism Association classification criteria [35], EDTA-plasma and serum were collected before starting with bDMARD and after three months of treatment. The three months follow-up examination was chosen as a time point when we expected to see an effect of the bDMARD treatment and to be able to explore plasma and serum differences cross-sectionally and longitudinally. Blood was collected on EDTA tubes for immediate centrifugation within 30 minutes for 15 minutes at 1400 relative centrifugal force (RCF)/3000 revolutions per minute (rpm), and serum tubes were allowed 30 min for clotting before centrifugation for 15 minutes at 1400 RCF/3000 rpm. The upper third of the plasma samples (avoiding buffy coat) was used for calprotectin assessments. Samples were frozen at once after centrifugation, at −70 °C, until thawing for analysis.

Ethics and trial registration

The study was approved by the Norwegian Regional Committee for Medical and Health Research Ethics South East (reference number 2009/1254). The patients gave written consent according to the Declaration of Helsinki. Trial registration: ANZCTR, Australian New Zealand Clinical Trials Registry, ACTRN12610000284066. http://www.anzctr.org.au

Clinical disease activity

Clinical data were obtained as previously described [28]. Patient's evaluation of joint pain on a visual analogue scale (VAS) 0–100 mm and patient's and assessor's global VAS 0–100 mm (that is, how the patient and a trained study nurse evaluated the general disease activity) were registered. The study nurse examined the number of tender and swollen joints of 32 (bilateral shoulders, elbows, wrists, metacarpo-phalangeal (MCP) joints 1–5, proximal interphalangeal (PIP) joints 1-5, knees, ankles and metatarsophalangeal (MTP) joints (MTP 1–5 scored as one joint)). A validated disease activity score of 28 joints (DAS28) [36] was calculated (including the joints mentioned above, excluding ankles and MTP joints). This is the clinical disease activity score most often used for RA patients and includes the patient's global VAS, the number of tender and swollen joints of 28 and ESR. In addition, GS synovitis (combined score of hypertrophied synovia and effusion in a joint) and PD (reflecting the vascularisation of synovitis) US findings of 36 joints and 4 tendon sheaths (bilateral elbows, radiocarpal (RC) joints, midcarpal (MC) joints, radioulnar (RU) joints, MCP 1–5, PIP 2–3, knees, ankles (tibiotalar joints), MTP 1–5, extensor carpi ulnaris (ECU) and tibialis posterior (TP) sheaths) were scored by an experienced sonographer (HBH) on a 4-point semi-quantitative scale (0 = normal, 1 = minor, 2 = moderate, 3 = major presence) [37]. Sum scores of GS and PD were used in the calculations.

Laboratory methods

Calprotectin was measured with an ELISA from CALPRO AS (Lysaker, Norway) according to the instructions of the manufacturer. EDTA-plasma and serum from the same patient were primarily analysed on the same plate. Mikrotek Emax plate reader and Softmax Pro software v.5.4 (Molecular Devices, Sunnyvale, CA) were used to read the plates. According to the manufacturer, intra-assay coefficient of variation (CV) was median (range) 5.2 (1.9–9.7) %, testing six EDTA-plasma samples with mean concentrations of 294–4391 ng/mL. Intra-assay CV was median (range) 7.3 (1.5–11.9)%, testing six serum samples with mean concentrations of 393–3983 ng/mL. Inter-assay CV was median (range) 6.2 (2.6–13.0)%, testing six EDTA-plasma samples with mean concentrations of 373–6053 ng/mL. Inter-assay CV was median 7.1 (5.1–8.6)%, testing six serum samples with mean concentrations of 443–5081 ng/mL. Intra- and inter-assay precisions were found using DS2 automated ELISA system from Dynex technologies, Chantilly, VA, USA. The Westergren method was used for ESR, and CRP was measured by turbidimetry.

Statistics

IBM SPSS version 23, R version 3.4.0 for Windows, www.R-project.org and GraphPad Prism 5 were used for the statistical calculations and figures. Wilcoxon signed rank test was used to compare values of calprotectin measured in plasma and serum at baseline and after three months. Quartile coefficient of dispersion was used to compare variabilities of calprotectin when measured in plasma and in serum. Spearman's rank correlation coefficients explored the associations between calprotectin measured in plasma/serum and clinical/US evaluations of disease activity. Bland Altman plots were used for method comparisons. p-values $<.05$ were regarded as significant.

Missing data

For three patients, all data were missing at the three months examination, and for one patient, joint pain was registered neither at baseline nor after three months.

Results

Lower calprotectin values were found in plasma than in serum (Figure 1). Calprotectin measured in plasma versus serum had median (iqr) values 1149 (698–1949) versus 1837 (1263–3058) ng/mL at baseline and 676 (444–1122) versus 1268 (802–1756) ng/mL after three months. The differences between values measured in plasma and serum were significant at baseline and after three months ($p < .001$). Quartile coefficients of dispersion in plasma and serum were similar both at baseline/after three months, 0.47/0.43 for plasma and 0.42/0.37 for serum. Both in plasma and serum calprotectin decreased significantly ($p < .001$) after three months.

Spearman's correlation coefficient (r_s) between calprotectin measured in plasma and serum was 0.79 ($p < .001$) at baseline and 0.73 ($p < .001$) after three months. Figure 2 shows the Bland Altman plots of agreement between calprotectin levels measured in individual patients in serum and plasma (a) at baseline and (b) after three months treatment with bDMARD. The mean difference between serum and plasma (bias) is highly positive, both at baseline (663 ng/mL) and after three months (519 ng/mL), because of generally higher serum levels. We see a tendency of lower degree of agreement (wider scatter) between serum and plasma levels with increasing average levels.

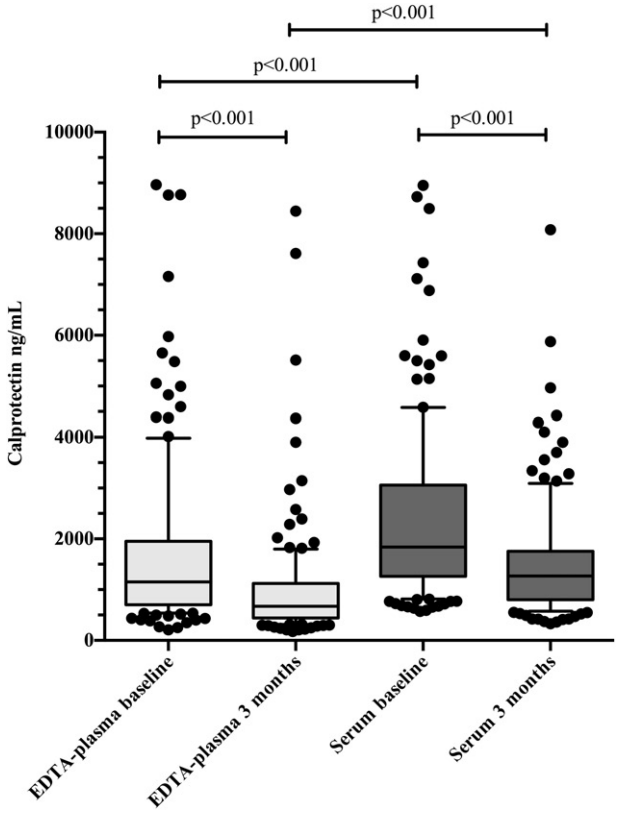

Figure 1. Median levels (boxes interquartile range, whiskers 10–90 percentile) of calprotectin levels measured in EDTA–plasma and serum at baseline and after three months treatment with biologic disease-modifying drugs.

Calprotectin measured in plasma was more strongly associated with measures of disease activity (US and clinical) than in serum at baseline (Table 1) and after three months (Table 2). Calprotectin had the highest correlation coefficients with the more objective assessments of disease activity, that is, the US scores, assessor's global VAS and number of swollen joints.

Compared with CRP, calprotectin measured in plasma correlated consistently more strongly with US and clinical measures of disease activity at both time points [28]. When measured in serum, calprotectin had more often similar correlation coefficients as CRP with US and clinical measures of disease activity (Tables 1 and 2). Compared with ESR, calprotectin measured both in plasma [28] and in serum correlated consistently more strongly with sum US scores, assessor's VAS and number of swollen joints (Tables 1 and 2).

To explore the stability of calprotectin, we reanalysed 136 of the baseline plasma samples that were thawed for the first analysis and then stored in the freezer (−70 °C) for two years. Median (range) levels of calprotectin were 1177 (206–2961) ng/mL first time analysed and 1092 (147–2916) ng/mL second time analysed, meaning there were no significant changes of calprotectin levels. The correlation coefficient r_s was 0.98.

Discussion

In this study, we found calprotectin levels to be lower but with similar variability when measured in EDTA-plasma compared with serum. Of highest importance was the finding that calprotectin in plasma had stronger associations with all the measures of disease activity compared with calprotectin in serum. Calprotectin assessed in plasma had also stronger correlations with measures of disease activity than CRP, while serum calprotectin had similar correlations as CRP. This suggests a greater potential for calprotectin as a new biomarker in RA when measured in plasma.

The lower levels of calprotectin in EDTA-plasma compared with serum are consistent with the findings of Fagerhol et al and Dale [33,34]. In these studies, EDTA was found to have a stabilizing effect over several days on the calprotectin levels, while the calprotectin levels increased dramatically in serum after three days of storage. Our samples were frozen immediately after centrifugation. Accordingly, the difference between plasma and serum might not necessarily be explained by time. On the other hand, to obtain serum, blood has to coagulate for 30–60 minutes before centrifugation, and calprotectin may be released from leukocytes during this time, probably because of cell activation and cell death, while plasma can be separated immediately.

The stabilizing effect of EDTA on calprotectin levels (compared with other anticoagulants and in serum), could according to Dale be due to an inhibition of a calcium-dependent transport across the cell membrane [34]. This is an interesting hypothesis, because calprotectin is found to be

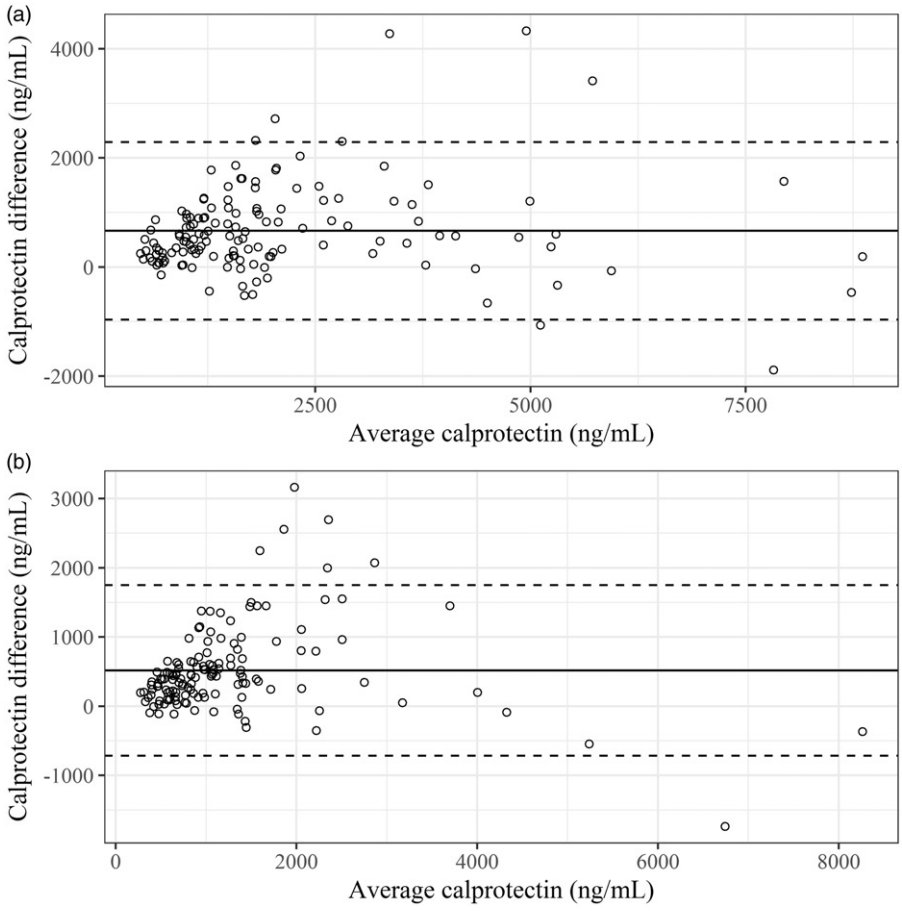

Figure 2. Differences between calprotectin measured in serum and EDTA-plasma (serum-plasma) plotted against average (serum + plasma)/2 (a) at baseline and (b) after three months. The dashed lines show 95% limits of agreement and the solid lines show the mean difference between serum and plasma concentrations of calprotectin.

Table 1. Spearman rank correlation coefficients (r_s) between P-calprotectin, S-calprotectin, CRP and ESR and US and clinical measures of disease activity at baseline ($n = 141$).

	P-calprotectin[a]	S-calprotectin	CRP[a]	ESR[a]
Sum GS score	0.59***	0.43***	0.41***	0.19*
Sum PD score	0.62***	0.46***	0.47***	0.30***
DAS28 with ESR	0.49***	0.40***	0.47***	0.67***
Swollen joints of 32	0.47***	0.37***	0.30***	0.22**
Assessor's global VAS	0.60***	0.48***	0.46***	0.46***
Patient's global VAS	0.27***	0.24**	0.18*	0.28**
Joint pain VAS[#]	0.32***	0.30***	0.24**	0.33***
Tender joints of 32	0.17*	0.16	0.15	0.27**

CRP: C-reactive protein; DAS28: disease activity score; ESR: erythrocyte sedimentation rate; GS: grey scale; P: ethylenediaminetetraacetic acid plasma; PD: power Doppler; S: serum, US; ultrasound, VAS: visual analogue scale,
[#]$n = 140$,
*$= p \leq .05$,
**$= p \leq .01$,
***$= p \leq .001$,
[a]Data are published earlier, ref. [28], under the Creative Commons Attribution License 4.0.

Table 2. Spearman rank correlation coefficients (r_s) between P-calprotectin, S-calprotectin, CRP and ESR and US and clinical measures of disease activity after three months ($n = 138$).

	P-calprotectin[a]	S-calprotectin	CRP[a]	ESR[a]
Sum GS score	0.38***	0.28***	0.20*	0.01
Sum PD score	0.46***	0.32***	0.25**	0.13
DAS28 with ESR	0.44***	0.38***	0.43***	0.59***
Swollen joints of 32	0.41***	0.31***	0.27**	0.06
Assessor's global VAS	0.48***	0.42***	0.33***	0.21*
Patient's global VAS	0.28***	0.24**	0.20*	0.23**
Joint pain VAS[#]	0.27**	0.21*	0.21*	0.20*
Tender joints of 32	0.19*	0.21*	0.05	0.09

CRP: C-reactive protein; DAS28: disease activity score; ESR: erythrocyte sedimentation rate; GS: grey scale; P: ethylenediaminetetraacetic acid plasma; PD: power Doppler; S: serum, US: ultrasound; VAS: visual analogue scale.
[#]$n = 137$,
*$= p \leq .05$,
**$= p \leq .01$,
***$= p \leq .001$,
[a]Data are partly published earlier, ref. [28] (but with $n = 141$), under the Creative Commons Attribution License 4.0.

released from monocytes after activation of a protein kinase C (PKC) [38], and conventional isoforms of PKC enzymes require calcium for activation. Conventional isoforms of PKC are found in monocytes [39]. EDTA blocks coagulation by binding of calcium. This may lead to less intracellular calcium, less PKC activation and less release of calprotectin from monocytes.

EDTA binds calcium ions necessary for coagulation, thus inhibiting the coagulation process in plasma. On the other hand, the coagulation process is necessary to obtain serum. Coagulation involves activation of platelets, and platelet activation via Toll-Like receptor 4 induces platelets binding to neutrophils. This results in strong neutrophil activation and production of neutrophil extracellular traps (NETs) [40].

NETs are fibres of chromatin and proteins released from activated neutrophils, with the ability to kill bacteria [41]. An *in vitro* study has shown that calprotectin is one of the cytosolic proteins localizing to NETs, and that calprotectin is released from neutrophils in NETs [42]. The cell membrane ruptures and neutrophils die in this specialized process; a type of cell death named NETosis [43]. Coagulation and activated platelets may thus induce release of calprotectin from neutrophils bound to NETs and unbound via NETosis and may thereby contribute to the higher levels of calprotectin in serum. NETosis is increased in neutrophils in peripheral blood of RA patients compared with healthy controls [44], and thus, this mechanism could be of greater importance in RA patients than healthy controls.

In contrast to EDTA-plasma, serum has calcium freely available. In the presence of calcium, calprotectin dimers and oligomers are generated, causing significant alterations in the protein structure [45]. In a sandwich ELISA assay, more antigenic epitopes per antibody in the coat may be available for reaction with the enzyme-conjugated antibody when calprotectin is present in dimers or oligomers, and this may cause increased serum levels of calprotectin.

In previous studies, blood samples have been stored in the freezer for many years before analysing calprotectin [17,24,31,32]. Presently, we showed stability of EDTA plasma levels of calprotectin assessed two years apart. We are not aware of similar studies for serum samples, but the present finding supports a high degree of stability of plasma calprotectin during long-time frozen conditions, which may be of importance for clinical trials. In future studies, stability after centrifugation and before freezing or during refrigerating conditions should also be explored.

Strengths of this study are the rather large group of patients studied during follow-up, and inclusion of many clinical and US measures of disease activity. The choice of only assessing plasma from EDTA tubes and no other anticoagulants was based on previous studies of healthy blood donors [34], showing that the level of calprotectin increased dramatically with storage time when measured in acid–citrate–dextrose solution and citrate–phosphate–dextrose solution, but not in EDTA–plasma. Citrate and EDTA are calcium chelators that will inhibit the coagulation cascade. The binding of calcium of the former is so weak that a ten-fold excess is used; still, some clotting and platelet activation may occur if citrated blood is not handled properly. EDTA is much more efficient, and may give more reliable calprotectin estimates. Heparin is a substance that will bind to antithrombin and thereby increase the affinity and inhibition of thrombin by a factor of several thousand. In addition, heparin can dissociate histone from DNA in the chromatin network NETs where calprotectin is a major component [42]. Whether this can influence the assayed calprotectin levels in plasma is unknown but should be explored in future studies. Care was taken to use only the upper part of the plasma to avoid any cells from the buffy coat. EDTA gel tubes are now available, and should be tested if they have an advantage to ordinary EDTA tubes. A limitation of this study is that gel tubes were not used for obtaining serum. Neutrophils and monocytes could therefore be mixed with serum, causing increased levels of calprotectin. On the other hand, one could speculate if gel could activate neutrophils and give rise to more NETosis and accordingly higher serum levels. The optimal way of obtaining plasma and serum samples should be explored in future studies.

Conclusions

In RA patients, calprotectin in EDTA-plasma was found to have stronger associations with assessments of disease activity than calprotectin measured in serum, and EDTA–plasma is therefore suggested to be the optimal medium for the quantification of calprotectin as a biomarker in these patients. Plasma had lower values of calprotectin compared with serum, which may reflect the increased in vitro release of calprotectin from neutrophils activated during handling of blood for serum sampling.

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Disclosure statement

Hilde Berner Hammer has received fees for speaking and/or consulting from AbbVie, Pfizer, UCB, Roche, MSD, BMS and Novartis.

The other authors declare no conflicts of interest.

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