

Paper II

Quantification of S100A12 (EN-RAGE) in Blood Varies with Sampling Method, Calcium and Heparin

A. Larsen*†, I. B. Bronstein‡, O. Dahl*†, T. Wentzel-Larsen§, E. K. Kristoffersen¶ & M. K. Fagerhol**

Abstract

*Section of Oncology, Institute of Internal Medicine, University of Bergen, Bergen; †Department of Oncology, Haukeland University Hospital, Bergen, Norway; ‡Physical Biochemistry Division, MRC National Institute for Medical Research, London, UK; §Centre for Clinical Research, Haukeland University Hospital, Bergen; ¶Department of Microbiology and Immunology, Haukeland University Hospital, Gades Institute, University of Bergen, Bergen; and **Department of Immunology and Transfusion Medicine, Ullevaal University Hospital, Oslo, Norway

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Correspondence to: Dr A. Larsen, Department of Oncology, Haukeland University Hospital, N-5021 Bergen, Norway. E-mail: annette.larsen@helse-bergen.no

S100A12 is a calcium-binding protein predominantly found in neutrophil granulocytes and monocytes. Its usefulness in monitoring inflammatory disease states depends on documentation that assay results are reliable. This study aimed at defining guidelines for blood sampling, selection of optimal material handling and reference intervals in healthy controls while taking into account the basic features of S100A12. An enzyme linked immunosorbent assay was developed based upon antibodies induced in rabbits by injection of recombinant S100A12. Our studies confirm that oligomers of S100A12 are generated in the presence of calcium. Structural changes in S100A12 mediated by calcium influence the interaction with antibody. This is proposed as the background for our very low readings of S100A12 in Ethylene Diamine Tetraacetic Acid (EDTA) plasma. Individual S100A12 levels did not change substantially over a 5-week sampling period. Based upon testing of 150 blood donors we suggest reference intervals of S100A12 in serum to be 49–1340 µg/l for women and 27–1750 µg/l for men. The estimated mean concentrations were 234 µg/l in serum samples (range 12–15791), 114 µg/l (range 3–17282) in re-calcified EDTA plasma and 48 µg/l (range 2–14843) in heparin plasma. Without adding calcium to EDTA plasma before running the assay, concentrations were around 2 µg/l (16 persons). S100A12 quantification is assumed to become relevant for diagnostic use in many disease states. The importance of the handling and analysing conditions for a reliable result was examined. We recommend serum collected in gel-containing tubes as the preferred sample material and have suggested reference intervals for healthy individuals.

Introduction

Several biomarkers of autoimmune and inflammatory disorders have been examined for their possible usefulness in the clinic. The two related S100-proteins calprotectin (heterotrimer of S100A8 and S100A9) and S100A12 were found to correlate with disease activity in rheumatoid arthritis and high concentrations were found in synovial fluid in inflamed joints [1–7]. Gene expression of S100A12 in peripheral blood mononuclear cells could discriminate between healthy controls and rheumatoid arthritis patients with or without positive rheumatoid factor [8]. In Kawasaki syndrome, an acute vasculitis in childhood, altered gene expression of calprotectin and S100A12 in peripheral blood mononuclear cells followed the clinical course of the disease activity [9, 10]. In patients with diabetes mellitus type 2, plasma concentra-

tions of S100A12 were higher than in healthy controls, and the mechanism was proposed to be related to sub-clinical vasculitis [11]. The clinical disease activity in patients with inflammatory bowel diseases correlated with serum concentrations of S100A12, and high expression of S100A12 were found in the inflamed intestinal mucosa [12]. The monoclonal enzyme linked immunosorbent assay (ELISA) used to study patients with diabetes mellitus type 2 [11] reported means of 10.7 µg/l in the healthy controls; patients' mean was 19.6 µg/l in Ethylene Diamine Tetraacetic Acid (EDTA) plasma. Serum samples of healthy controls had 52 µg/l and Kawasaki patients' had a mean of 453 µg/l [13]. The same polyclonal ELISA as in Ye *et al.* [13] showed means of 75 and 470 µg/l in serum from healthy controls and in patients with Crohn's disease [12]. The examined blood samples (plasma and serum) and the two ELISA yielded highly

different results with regard to S100A12 concentrations. No international standard exists for S100A12 measurements.

S100A12 is mainly found in neutrophil granulocytes and monocytes. The amount of S100A12 was found to constitute about 5% of the total cytosolic proteins in resting human neutrophil granulocytes [14], and up to 8% of cytosolic proteins in porcine and rabbit granulocytes [15, 16]. The systematic names S100A8, S100A9 and S100A12 were assigned according to the positions of their genes on chromosome 1q21 [17, 18]. Other names used are calgranulins A, B and C; and for S100A12 also: extracellular newly identified RAGE binding protein (EN-RAGE; receptor for advanced glycation end products: RAGE), calcium binding protein in amniotic fluid-1 (CAAF-1), p6, MRP-6 and CGRP [14, 17, 19–21].

Most of the S100-proteins have been shown in various conformational states depending on concentrations of calcium, copper and zinc [22–29]. Both calprotectin and S100A12 are calcium binding proteins, and *in vitro* experiments suggest that their ability to interact with their targets *in vitro* is markedly influenced by calcium [27, 28, 30]. S100A12 hexamers were proposed as ligands of RAGE providing a link to the inflammatory signalling pathways [21, 25].

The present study of S100A12 aimed at elucidating how calcium-induced conformational changes might influence quantitative assay results, and to define optimal materials for analysis in clinical settings. While promising results have been reported, improper sample handling may represent serious pitfalls as illustrated by our findings reported here.

Materials and methods

Development of the assay. Recombinant full-length human S100A12 was expressed in *Escherichia coli* from the pQE60 plasmid containing the S100A12 gene. The S100A12 DNA fragment with a stop codon had been inserted between promoter and His-tag. Although the pQE60 vector contains a 6×His sequence the recombinant protein was expressed without a histidine tag. Protein expression was induced with 1 mM Isopropyl- β -D-thiogalactosidase (IPTG) and the bacterial pellet was lysed by sonication. After sonication the insoluble material was removed by centrifugation. The supernatant was adjusted up to 10 mM CaCl₂ and after 15 min incubation at room temperature was clarified by centrifugation. Clear supernatant was applied onto Phenyl-Sepharose column equilibrated with 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM CaCl₂. The column was washed with the same buffer and subsequently eluted with 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA. S100A12-containing fractions were diluted with 50 mM Tris-HCl (pH 7.9) buffer (1:4) and applied to a ResQ column

equilibrated with 50 mM Tris-HCl (pH 7.9) buffer and eluted with 0–1M NaCl gradient using an AKTA Purifier chromatography system (Amersham, Bucks, UK). Protein concentration was determined by BSA protein assay (Bio-Rad, Bradford, UK, bovine gamma-globulin standard). After purification, a single 10 kDa band was observed on the SDS-PAGE. The molecular mass of the pure protein obtained by matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization mass spectrometry (ESI-MS) matched the calculated one demonstrating protein integrity and the absence of any modifications.

Polyclonal antibodies were obtained by immunization of rabbits with 0.1 mg of the recombinant S100A12 in complete Freund's adjuvant divided into six subcutaneous sites with repeated immunization every week for 5 weeks. Immunoaffinity-purified anti-S100A12 was prepared by use of a column where S100A12 had been covalently linked to agarose beads. These antibodies were conjugated with alkaline phosphatase (ALP). The IgG-fraction of rabbit anti-S100A12 serum was used for coating microwell plates (MaxiSorp, Nunc, Denmark). Dilutions of the recombinant S100A12 were used as standards. For the assay, standards and samples were diluted in TRIS buffered saline (TBS) with 10 g/l bovine serum albumin and 0.1 g/l sodium azide as an antimicrobial. The first incubation was for 2 h and the second, with ALP-conjugate, for 1 h. Between incubations wells were washed four times. Optical densities were read at 405 nm after reaction with substrate for about 30 min.

Double immunodiffusion testing in agarose gel containing TBS with 2 mM calcium chloride, and gel permeation chromatography (GPC) on a Hi Load 16/60 Superdex™ 75 column (Amersham Corp.) was performed to check the specificity of the antibodies compared with calprotectin. A previously described ELISA was used to quantify calprotectin [31]. Cross-ELISA with recombinant S100A12 standard analysed in the calprotectin ELISA [31] and the calprotectin standard (purified from human leucocytes) analysed in the S100A12 ELISA was performed. The GPC was also used to analyse the molecular size distributions of calprotectin and S100A12 in serum. Running buffers were TBS with 2 mM CaCl₂ or 2 mM EDTA. Total protein concentrations were determined by using the BSA protein assay (Bio-Rad).

Blood sampling to study the effects of sample handling. Testing the stability of S100A12 in blood. Venous blood was collected from 10 healthy volunteers to obtain serum, EDTA blood and heparin blood using BD Vacutainer © tubes (Becton Dickinson Corp., Franklin Lakes, NJ, USA): (i) empty vacuum tubes for 4 ml whole blood; (ii) 5.4 mg spray-coated K₂EDTA for 3 ml whole blood; (iii) spray-coated silica and polymer gel for 4 ml whole blood; (iv) sodium-heparin 17 IU/ml for 5 ml whole blood. Samples were centrifuged at 1600×g, 4 °C, for 10 min. To assess the stability during storage before

harvesting serum or plasma, samples were harvested after increasing delay; the time points before separation followed by freezing at -20°C were: 0.5, 1, 6, 24 and 48 h. All samples were first stored at room temperature; samples to be stored more than 6 h were then moved to 4°C until centrifugation. For this purpose, blood was collected in 20 different tubes during one venipuncture. EDTA blood drawn from the same venipuncture was analysed for leucocytes and blood platelets in an automatic analyser (Coulter GenS, Miami, FL, USA).

Serum and plasma from four individuals were handled in three different ways: exposure to 10 freeze/thaw cycles with 2 h at room temperature and frozen at -20°C ; 10 similar freeze/thaw cycles but freezing at -70°C ; storage at room temperature for 3 weeks.

Testing the influence of calcium and heparin on S100A12 estimates. Serum, EDTA plasma and heparin plasma were added to calcium chloride to give final concentrations of 1, 2, 4, 6 and 21 mM. The amount of EDTA in vacuum tubes used routinely is equivalent to 4 mM of calcium-binding capacity. In order to examine the influence of heparin as an anticoagulant in collection tubes, serum samples were added to either heparin-coated sepharose beads (HI Trap Heparin, Pharmacia AB, Uppsala, Sweden) or native sepharose beads and incubated overnight on a rotator. Supernatants were collected and assayed for S100A12.

Reference samples: influence of age, gender and individual variation over time. Samples of serum from gel tubes, EDTA plasma and heparin plasma from 150 healthy, regular, non-remunerated blood donors were analysed for S100A12 and calprotectin. Blood samples were centrifuged after about 1 h at room temperature. There were 69 women and 81 men, mean age 43.6 years (range 19–68). The donors reported good health and donated blood for transfusion. They were included sequentially after giving informed consent. Blood donors are no longer routinely examined for erythrocyte sedimentation rate, C-reactive protein or leucocyte count. To assess the intra-individual variation of S100A12 over time, 11 healthy volunteers were examined seven times during five consecutive weeks, with venipuncture once a week and every second day during one of the weeks. All samples were taken after breakfast and before noon (three men and eight women). None of the volunteers or blood donors in the study were fasting before examinations.

The study was approved by the Regional Ethical Committees for Medical Research and all subjects gave informed written consent to participation.

Statistics. Statistical analyses were performed with SPSS 13 (SPSS Inc., Chicago, IL, USA) and R (The R Foundation for Statistical Computing, Vienna, Austria). For the re-calcification data separate linear mixed effects models [R package nlme, (32)] were used for the three tube types, modelling the S100A12 concentration by cal-

cium concentration. For these analyses no log transform was used, as there was a noticeable non-linearity in log-transformed S100A12 by concentration of calcium added to EDTA plasma. For all other analyses of S100A12 and calprotectin concentrations the dependent variable was log-transformed and analysed using linear mixed effects models, Pearson correlations or paired-samples *t*-tests, as appropriate. Reference intervals for S100A12 and calprotectin for each of the three tube types were calculated separately for each gender as mean \pm 2 standard deviations on a log scale and transformed back. For the recommended sampling material the influence of age and gender was examined using a quadratic regression with possibly different relationships for women and men. $P < 0.05$ was considered significant.

Results

Experiments with varying calcium concentration

Specificity of the assay Double immunodiffusion in agarose gel gave a single precipitation line and no cross reaction with calprotectin (Fig. 1). As shown in Fig. 2, Gel permeation chromatography (GPC) of serum in a buffer containing EDTA demonstrated a distinct peak for calprotectin corresponding to a molecular weight of about 36 kDa as published previously [33]. S100A12 oligomers were at the threshold of detectability and almost all S100A12 was detected in fractions representing low molecular weight species (Fig. 2A). The EDTA fractions were assayed for S100A12 after adding dilution buffer resulting in a 2 mM calcium-containing sample (Fig. 2A). When serum was run on the GPC column in a buffer with 2 mM calcium, S100A12 was detected in several fractions representing different high molecular weight species (Fig. 2B).

Figure 3 shows the standard curve for the S100A12 ELISA. The assay range was 15–800 $\mu\text{g/l}$. To detect low concentrations additional standards with low concentration were included and the incubation time was increased. This was not sufficient to detect samples much below 1 $\mu\text{g/l}$, as found in some EDTA plasma samples before diluting with calcium. The coefficient variations were about 6% in the 15–800 range and about 20% in the standards below 10 $\mu\text{g/l}$, based on eight runs of the assay. The within and between variations were 8.4% and 31% respectively. There was no hook effect. Samples from a few individuals showed a three- to fivefold increase in concentrations of S100A12 during handling (storage at 4°C or freeze/thaw cycles) and were excluded from these calculations. There was no reactivity of the recombinant S100A12 standard in the calprotectin assay [31] and minimal reactivity of the calprotectin standard in the S100A12 assay.

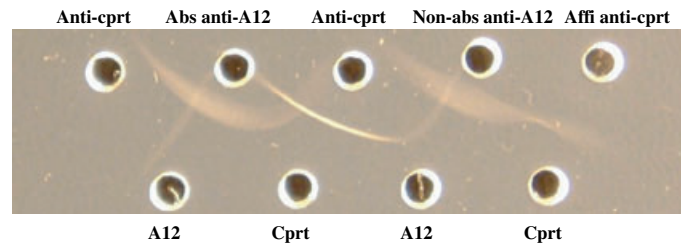


Figure 1 Double immunodiffusion in agarose gel. Ten-microlitre samples of reagents were added in wells in a 2-mm thick agarose gel on glass plate. Tris-buffered saline with 2 mM CaCl_2 , pH 7.5. S100A12: 0.9 mg/ml, Calprotectin: 1.1 mg/ml. Undiluted antisera. Two sets of immunoprecipitation are shown: one between anti-calprotectin and calprotectin, the other between anti-S100A12 and S100A12. The precipitation lines cross each other, showing non-identity. The wider precipitation line for S100A12 may suggest that S100A12 is present partly as complexes. Anti-cprt, polyclonal rabbit anti-calprotectin; Abs anti-A12, polyclonal rabbit anti-S100A12 passed through a column with calprotectin covalently bound to sepharose (Pharmacia); Non-abs anti-A12, neat polyclonal rabbit anti-S100A12; Affi anti-cprt, polyclonal rabbit anti-calprotectin purified on the calprotectin column mentioned above; A12, recombinant S100A12; Cprt, calprotectin purified from human leucocytes.

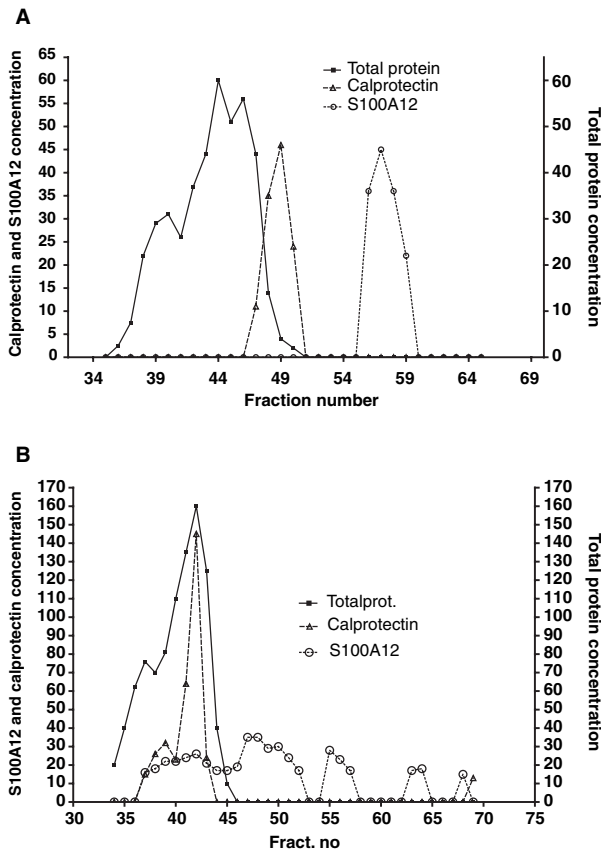


Figure 2 Gel permeation chromatography of serum. (A) Buffer: tris-buffered saline, 2 mM EDTA. Fractions were re-calcified to final 2 mM calcium chloride before detection in the two assays. The two proteins eluted in completely separated fractions. Concentrations: Total protein concentrations detected by BSA protein assay (Bio-Rad) and given as mg/100 ml. Calprotectin and S100A12 concentrations detected in their respective assays and given as $\mu\text{g/l}$. Re-calcified fractions for the estimation of S100A12. (B) Buffer: tris-buffered saline, 2 mM calcium chloride, pH 8.0. S100A12 detected in fractions corresponding to molecular sizes of about 5–360 kDa. Concentrations: total protein: mg/100 ml; calprotectin and S100A12: $\mu\text{g/l}$.

Figure 4 shows the influence of calcium added to the sample dilution buffer. Without any addition, the final calcium concentration was taken to be 1 mM, assuming normal serum calcium and using a typical sample dilution of 1:2. Addition of calcium to heparin plasma had no significant effect ($P = 0.44$) while S100A12 concentrations in serum increased by $67 \mu\text{g/l}$ per 10 mM calcium (95% CI 17–117, $P = 0.010$). In EDTA plasma there was a significant increase by $42 \mu\text{g/l}$ per 10 mM calcium (95% CI 41–58, $P < 0.001$). At 1 and 21 mM calcium concentrations, means were: 46 and 55 $\mu\text{g/l}$ in heparin plasma; 169 and 342 $\mu\text{g/l}$ in serum; 2 and 101 $\mu\text{g/l}$ in EDTA plasma. As a consequence of these results, all further EDTA plasma estimates are based upon analyses after adjusting the calcium concentration to 6 mM in the diluted sample; this is subsequently called re-calcified EDTA plasma.

Incubation of serum with heparin-coated sepharose beads reduced the S100A12 concentration significantly from 579 $\mu\text{g/l}$ (incubated with beads without heparin) to 15 $\mu\text{g/l}$ ($P < 0.001$).

Blood collection: stability before separation and during freeze–thaw–cycles

Freshly drawn blood left at room temperature before separation showed increased concentrations of S100A12 if collected in empty tubes, in tubes with EDTA or heparin (Fig. 5). Serum collected in gel tubes, however, showed a stable concentration of S100A12 when measured repeatedly up to 48 h before harvesting. The specific results were as follows: There was a significant increase in log S100A12 concentration for serum from empty tubes (by 0.018 per hour, 95% CI 0.010–0.026, $P < 0.001$), and also for re-calcified EDTA plasma (by 0.031 per hour, 95% CI 0.023–0.039, $P < 0.001$), but not for serum on gel tubes (0.001 per hour, 95% CI 0.006–0.009, $P = 0.738$). For heparin blood there was a significant increase

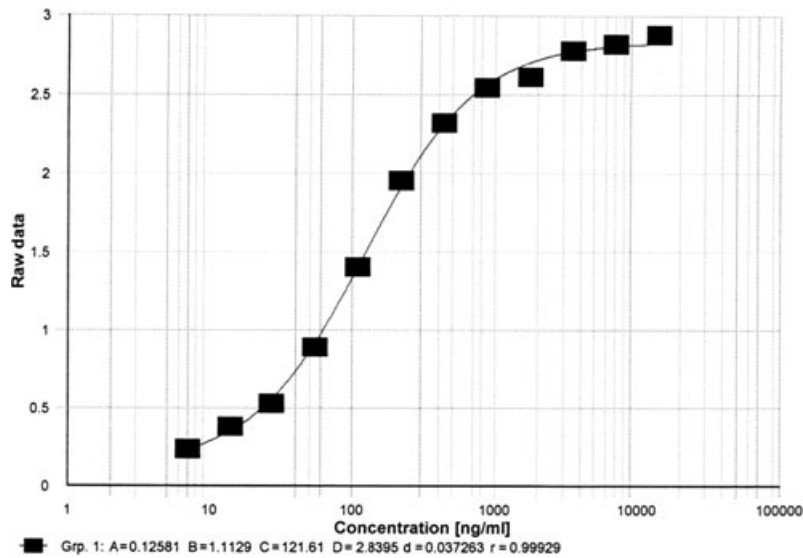


Figure 3 Standard curve. S100A12 standard curve for standards 7–14 000 $\mu\text{g/l}$. The coefficient variations were about 6% in the 15–500 $\mu\text{g/l}$ range, and about 20% if read from standards lower than 10 $\mu\text{g/l}$ (based on eight runs of the assay). *y*-axis: optical density.

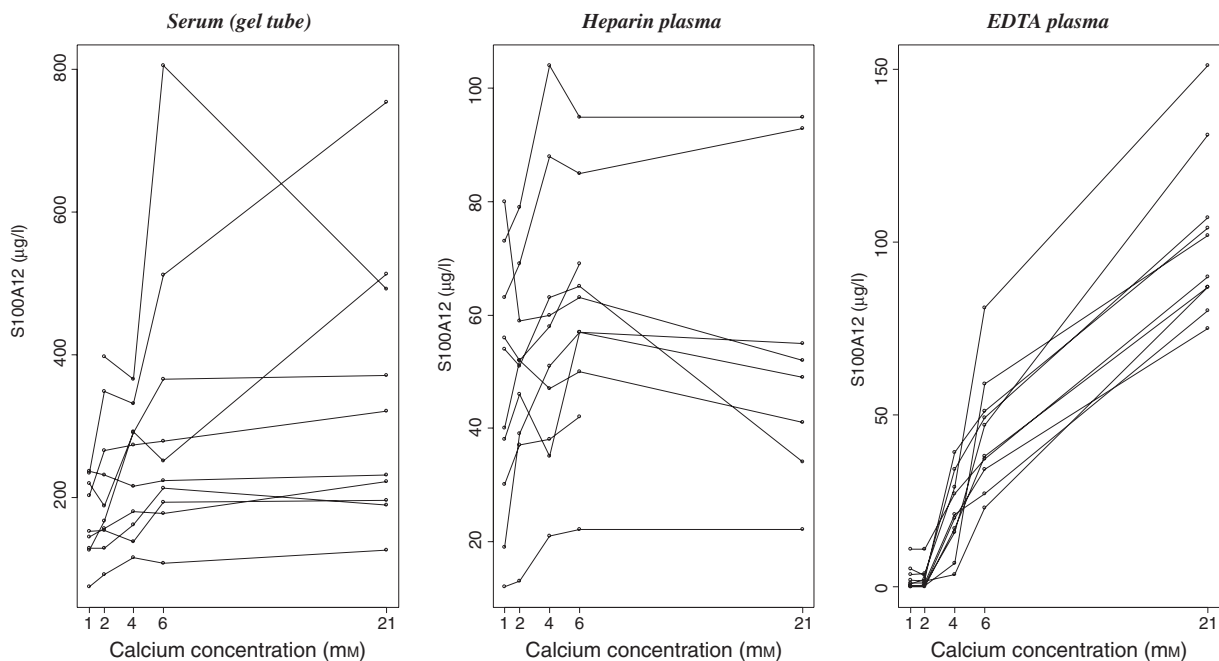


Figure 4 Calcium-induced analytical variances. Influence of calcium concentration in dilution buffer on S100A12 measured in blood collected in three different tube types. Samples were tested after addition of calcium-containing dilution buffers to give the theoretical final concentrations (*x*-axis) of 1–21 mM.

by time (by 0.016/h, log scale, 95% CI 0.006–0.027, $P = 0.003$) (Fig. 5). Expressed as mean concentrations at time 30 min and 48 h, S100A12 were: 72 and 173 $\mu\text{g/l}$ in serum from empty tubes; 197–2511 $\mu\text{g/l}$ in re-calcified EDTA plasma; and 139–286 $\mu\text{g/l}$ in plasma from heparin blood.

Concentration of S100A12 was not influenced by over-end turning the EDTA-coated tubes zero or eight

times ($n = 11$, $P = 0.82$). Leucocytes and blood platelets were within normal limits in all volunteers.

The concentration of S100A12 in serum was not significantly changed by 10 freeze/thaw cycles to $-20\text{ }^{\circ}\text{C}$ (means 573 and 743, range 401–964 $\mu\text{g/l}$, $P = 0.26$) or to $-70\text{ }^{\circ}\text{C}$ (means 1064 and 1129, range 718–1320 $\mu\text{g/l}$, $P = 0.30$). Concentrations increased when serum was left at room temperature for 3 weeks (means 119 and 787,

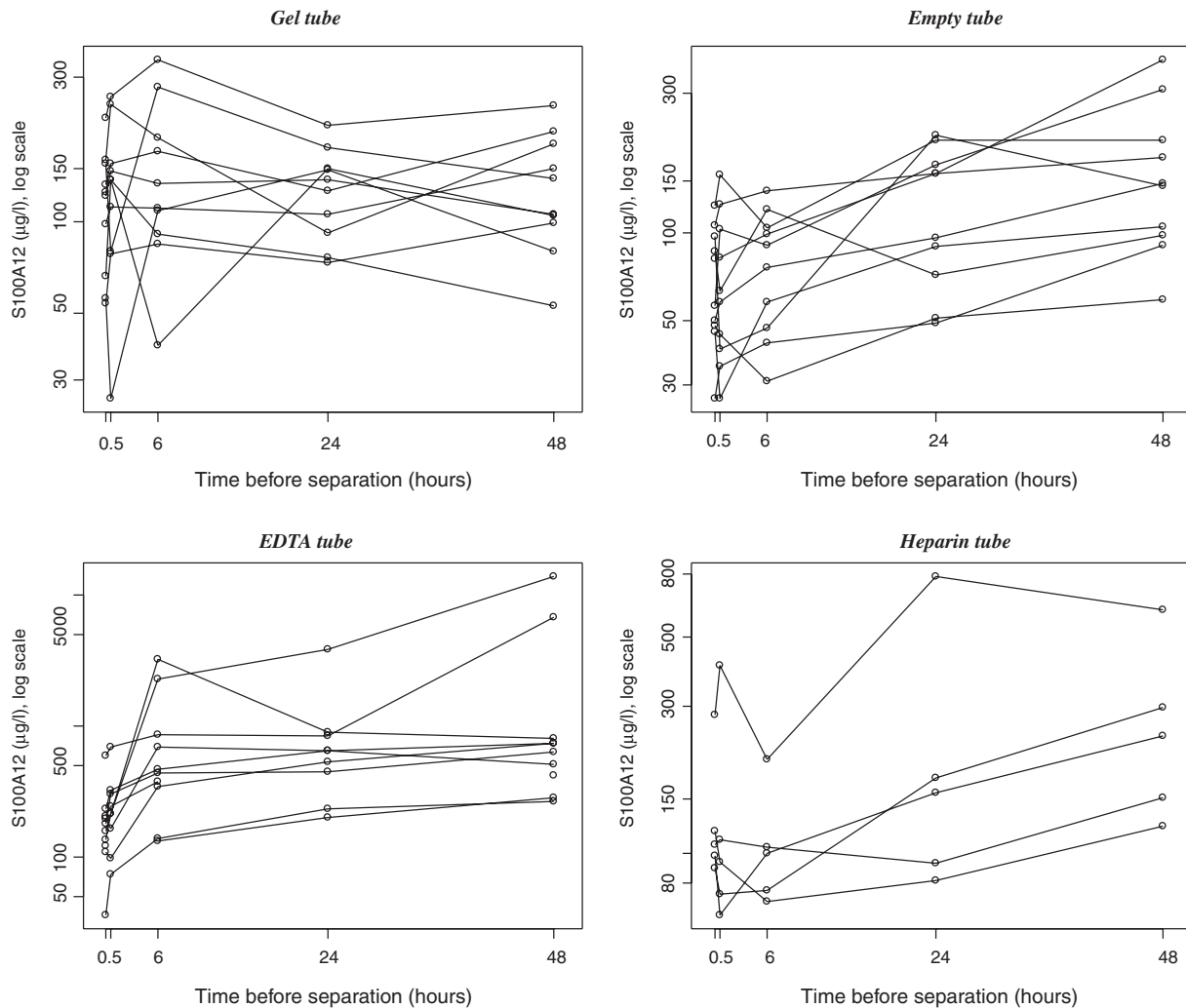


Figure 5 Time before separation of serum or plasma. Influence of storage medium and time before harvesting serum or plasma on S100A12 concentration. The time points before separation followed by freezing at -20°C were: 0.5, 1, 6, 24 and 48 h. All samples were first stored in room temperature; after 6 h at 4°C until centrifugation. Lines represent single samples. All EDTA plasmata analysed after re-calcification to 6 mM.

range 66–1320 $\mu\text{g/l}$, $P = 0.006$). The concentration in serum was stable for 6 months at -20°C . Individual variations in stability were observed: samples from few individuals showed three- to fivefold higher concentrations of S100A12 when analysed after a new freeze-thaw-cycle, e.g. during the inter-assay analysis.

Reference intervals, individual variation and influence of age and gender

Figure 6 shows the mean and ± 2 standard deviations of S100A12 on a log scale in serum from blood donors when gel tubes were used. S100A12 concentrations were higher in the middle age range for women, with the opposite results for men ($P = 0.005$), indicated by concave and convex curves. However, the deviations from the horizontal lines were minor. The moderate number

of blood donors in the younger and older age groups calls for careful interpretation of concentrations near the suggested upper reference limit in these age groups. Table 1 shows S100A12 and calprotectin concentrations tested in serum, re-calcified EDTA plasma and heparin plasma in 150 blood donors. The suggested reference intervals for S100A12 in serum are 49–1340 $\mu\text{g/l}$ for women and 27–1750 $\mu\text{g/l}$ for men (concentrations calculated from geometric mean ± 2 SD). Without gender-specific reference intervals, we suggest that S100A12 in serum should be 35–1570 $\mu\text{g/l}$, calprotectin in serum 0.51–4.10 mg/l and calprotectin in EDTA plasma 0.29–1.98 mg/l. Taken together, our results indicate that the concentration of calprotectin in serum is about six times higher than that of S100A12 (mean of S100A12 in serum: 0.234 mg/l, calprotectin in serum: 1.44 mg/l). There was a highly significant positive correlation between

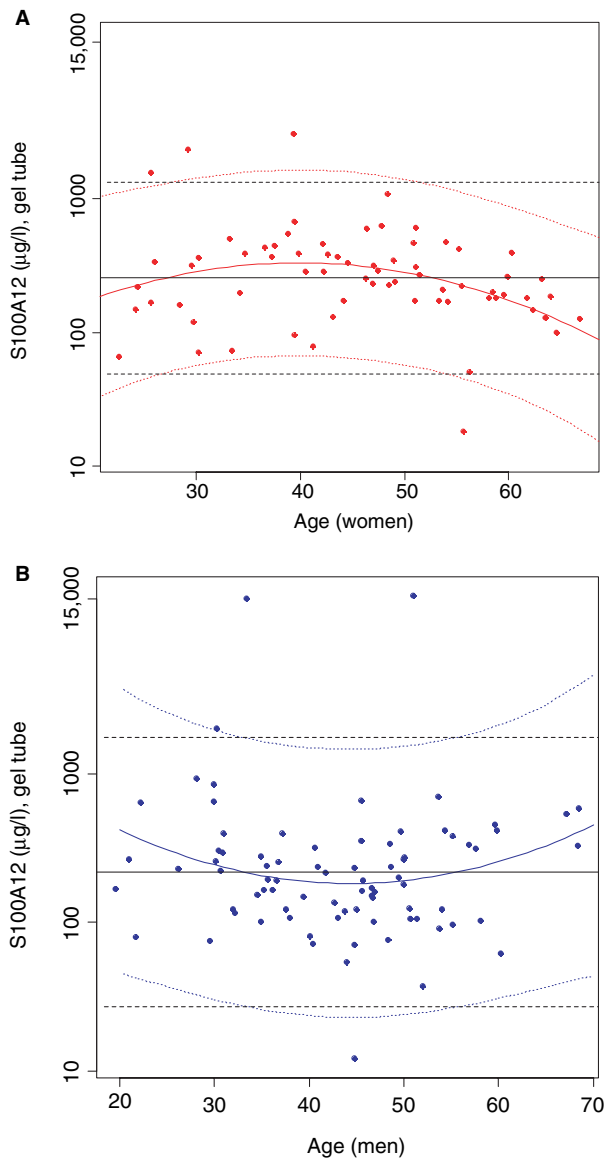


Figure 6 Reference intervals. Mean and reference intervals of S100A12 in serum; from women (A) and men (B) (black horizontal lines). Age-dependent means and prediction bands (coloured curves) are based on quadratic regressions by age for each gender are included.

S100A12 and calprotectin concentrations in serum ($P < 0.001$). Calprotectin concentrations in EDTA plasma decreased significantly with increasing age ($P = 0.036$), log concentrations fell by 0.08/10 years of age. Gender did not influence this reduction.

Figure 7 shows the variation of S100A12 in 11 individuals sampled repeatedly for 5 weeks. The standard deviation for within-individual variation was 0.38 (95% CI 0.34–0.43) for S100A12. The mean difference was a threefold higher maximum than minimum S100A12 concentration during the 5-week period in each individual.

Table 1 Reference intervals.

	Mean	Reference intervals
S100A12 ($\mu\text{g/l}$)		
Serum (gel tubes)		
Women	256	49–1343
Men	217	27–1746
Plasma (re-calcified) (EDTA tubes)		
Women	123	25–612
Men	107	11–1004
Plasma (heparin tubes)		
Women	48	9–257
Men	48	5–484
Calprotectin (mg/l)		
Serum (gel tubes)		
Women	1.56	0.57–4.27
Men	1.35	0.46–3.91
Plasma (EDTA tubes)		
Women	0.73	0.26–1.99
Men	0.80	0.32–1.97
Plasma (heparin tubes)		
Women	0.68	0.28–1.66
Men	0.72	0.29–1.80

Means in the table represent geometric means. Similarly, reference intervals were computed as mean \pm 2SD from the log-transformed concentrations, and transformed back.

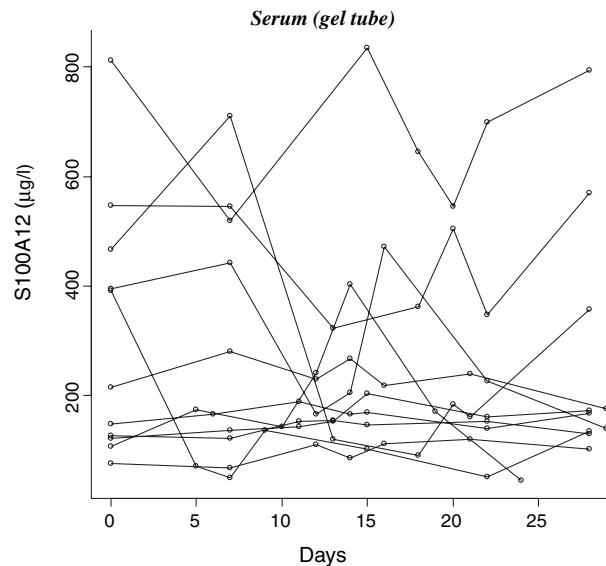


Figure 7 Concentrations of S100A12 over a period of 5 weeks. Variation in 11 healthy volunteers' samples (seven examinations in 5 weeks). All S100A12 samples are within suggested reference intervals. Lines represent separate volunteers.

Discussion

This study shows that S100A12 estimates vary with collection medium, time before separation, calcium in sample dilution buffer and storage conditions. We found a significant relationship with age and gender in the reference group: concentrations were higher in women

30–50 years old than in the younger and older individuals; for men the inverse was found. It is difficult to see any explanation for this. Re-calcification of EDTA plasma gave higher concentration estimates of S100A12, serum assayed with increasing concentrations of calcium also seemingly increased the S100A12 concentration, while this could not be observed in heparin plasma. Serum incubated with heparin-coated sepharose beads showed a significant decrease in S100A12 concentration, indicating that S100A12 is a heparin-binding protein. We speculate that this may interfere with calcium-induced conformational changes of S100A12.

X-ray analysis of the S100A12 three-dimensional structure has revealed that this protein does exist in several oligomeric forms [24, 25]. One of them has been described as dimers which assemble in hexamers [25]. While each monomer of S100A12 binds two calcium ions, the hexamer binds a total of 18 calcium ions. Oligomerization of S100A12 in solution in the presence of 5 mM calcium was confirmed by dynamic light-scattering analysis [25]. A fragment of the extracellular domain of RAGE was shown to bind to S100A12 [25]. The hexameric assembly of S100A12 has been suggested to be the extracellular form of S100A12 that binds to RAGE [25].

These facts must be taken into consideration in attempts at determining the true concentration of S100A12 in plasma and serum. A signal in an ELISA requires at least two antigenic epitopes on the protein, one for binding the antibody on plastic of the wells and one for binding the enzyme-labelled antibody. By having two different monoclonal antibodies, one of which reacts with S100A12 independently of calcium concentration [11], EDTA plasma could be used for the quantification of S100A12 in patients with diabetes mellitus type 2. Our assay could not detect S100A12 in EDTA plasma unless calcium was added to the samples (Fig. 4). The GPC fractions in EDTA buffer were also assayed after addition of calcium (Fig. 2A). In order to estimate S100A12 concentration in EDTA plasma, we considered the molarity of EDTA in the sampling tube (4 mM) and the calcium concentration in the blood sample (normal: 2 mM). To exclude influence from any low calcium concentrations in blood, we chose to analyse EDTA plasmata in 6 mM calcium in the final, diluted plasma sample. However, this is only a theoretical concentration, not a measured calcium concentration (Fig. 4). The calcium affinities of EDTA and S100A12 in blood are not known. From our observations in Fig. 4, we infer that S100A12 is influenced by the EDTA present in the blood sample and that calcium in the dilution buffers influences the quantification of S100A12 in EDTA plasma and serum, but not in heparin plasma. This may suggest that the rabbits have produced only one type of antibody against a single epitope on S100A12, and either (i) this epitope is not present in the conformational state that occurs

with EDTA competing with S100A12 for calcium ions, or (ii) there is only one such epitope on each S100A12 molecule, consequently the recognition of two epitopes requires dimers or oligomers to be present in the sample. The observed increase of S100A12 in serum when calcium was added to dilution buffers (Fig. 4) may also be explained by the formation of more (and larger) oligomers, as they may be expected to be captured firmly in the wells, and bind more conjugated antibodies than dimers would. The GPC (Fig. 2B) in 2 mM calcium buffer supports this hypothesis, as S100A12 was detected in fractions with many molecular weight species. In heparin plasma the influence of additional calcium did not lead to higher estimates of S100A12 (Fig. 4). Assuming that dimers are present, the excess calcium may have failed to induce formation of higher molecular weight complexes of S100A12. In addition to these interpretations based on the assumption of only one recognized epitope, there is also the possibility that a second, specific epitope present only in S100A12 complexes may have been produced by the rabbits. Such an antibody would only react when the relevant complex is formed in the samples, e.g. the hexamer. Thirdly, different antibodies may detect epitopes with varying affinities in a manner that might influence the quantification as calcium concentration increases (Fig. 4). The recombinant S100A12 was assayed as unknown samples after dilution in a calcium-containing buffer or an EDTA-containing buffer (data not shown). Dilution with calcium increased the expected concentrations whereas the protein could not be detected in EDTA-buffered samples. This confirms that the recombinant S100A12 has a structure and reactivity similar to the native protein.

We have not calibrated our S100A12 assay with the two groups that have reported data in patients and healthy controls [4, 11–13, 34]. One of them [4] used S100A12 purified from human granulocytes as standard; such materials may be less homogenous and reactive than recombinant protein. The latter may also be preferable because it can be produced in large quantities and used as an international standard. The research group that found higher EDTA plasma concentrations of S100A12 in patients with diabetes mellitus type 2 compared with healthy controls, reported means of 19.6 and 10.7 $\mu\text{g/l}$ respectively [11]. The means of S100A12 levels in serum varied more than 250-fold when comparing healthy controls and patients [4, 12, 13, 34]. Eight juvenile rheumatoid arthritis patients had a mean S100A12 concentration of 13 400 $\mu\text{g/l}$, compared with the healthy controls' mean of 50 $\mu\text{g/l}$ [5]. This indicates a very wide range of possible concentrations, and the variations we have shown related to age and gender (Fig. 6) are minor compared with differences reported for disease states. There is at present no international standard for S100A12 quantification, so comparisons of our concentrations with those

reported previously are difficult. For clinical purposes, the increase in concentrations that we found in EDTA blood if left for some time before separation of plasma (Fig. 5) may disfavour this as the collection medium. It is an unresolved question why calprotectin and S100A12 increase during coagulation *in vitro* (Table 1). Release of the proteins because of activation of neutrophils and monocytes is an attractive hypothesis, supported by data on *in vitro* secretion of calprotectin [35, 36].

It is not known whether oligomers of S100A12 molecules or complexes with other proteins/substances may be generated by handling of samples (storage or freeze/thaw cycles) and explain some of the between-assay variation we have found. There was some variation in the S100A12 concentration when seemingly healthy individuals were tested repeatedly for 5 weeks (Fig. 7), the reason for which is unknown. All S100A12 concentrations were, however, within the suggested reference intervals. Reference values from this group of healthy persons of a selected age (blood donors) are expected to be lower than those of a normal population, in which also persons with acute and chronic diseases, as well as all age groups, are represented. Studies using the described ELISA for quantification of S100A12 in serum samples in patient groups are underway.

For the time being, we suggest that for assays of S100A12 in blood, serum should be harvested from gel tubes in a strictly standardized way. If necessary, samples should be diluted in a buffer without calcium chelators and analysed as soon as possible. Samples can be stored frozen for at least 6 months and thawed for assays at a later time. Storage at room temperature may give a six-fold or greater increase which suggests that caution must be exercised when drawing conclusions from testing of old samples, in particular if samples from patients and controls have been treated differently.

In conclusion, we recommend serum harvested from gel tubes as the preferred sampling medium for analysis of S100A12. If the quantification can not be carried out within few days, samples should be frozen and thawed close to the time of analysis. Future studies aimed at examining the usefulness of S100A12 for diagnostic purposes would benefit from the results presented here.

Authors contributions

AL participated in writing the protocol and collecting blood samples, carried out the immunoassays, and drafted the manuscript. IBB provided the recombinant S100A12 and helped to draft the manuscript. OD and EKK wrote the study protocol and supervised the immunoassays. TW-L performed the statistical analysis. MKF conceived the study, carried out the immunization of rabbits and preparation of reagents, supervised the immunoassays and

helped to draft the manuscript. All authors read and approved the final manuscript.

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