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PAPER

Pro-inflammatory S100 proteins are associated with glomerulonephritis and anti-dsDNA antibodies in systemic lupus erythematosus

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Objectives: Systemic lupus erythematosus (SLE) is associated with elevated levels of S100A8/A9, pro-inflammatory proteins mainly secreted by activated polymorphonuclear neutrophils (PMNs). The underlying mechanisms for increased S100A8/A9 levels and their relation to the clinical phenotype have not been carefully investigated. We assessed S100A8/A9 and S100A12 levels in SLE patient sera in relation to disease activity, clinical phenotype, presence of anti-dsDNA antibodies and ability to promote phagocytosis of necrotic cells (NCs) by PMNs. **Methods:** Serum levels of S100A8/A9 and S100A12 were measured by ELISA in paired samples of 100 SLE patients at time points of higher and lower disease activity. Serum-mediated phagocytosis of NCs by PMNs was analysed by flow cytometry. Clinical data were recorded at time points of blood sampling. **Results:** Serum levels of S100A8/A9 and S100A12 were increased in SLE patients with high disease activity compared to paired samples at low disease activity ($p = 0.01$ and $p = 0.008$, respectively). Elevated levels of S100A8/A9 were particularly seen in patients with anti-dsDNA antibodies ($p = 0.01$) and glomerulonephritis before treatment ($p = 0.02$). Immunosuppressive therapy was associated with a reduction of S100A8/A9 serum levels ($p = 0.002$). The ability of serum to support phagocytosis of NCs by PMNs was related to increased S100A8/A9 levels ($p = 0.01$). **Conclusions:** Elevated serum levels of S100A8/A9 may be used to monitor disease activity and response to treatment in SLE patients, especially in patients with glomerulonephritis. S100A12 may be a marker of disease activity in SLE. Increased S100A8/A9 levels may reflect immune-pathological processes involving phagocytosis of immune complexes by PMNs. *Lupus* (2016) **0,** 1–11.

Key words: Anti-dsDNA antibodies; S100A12; S100A8/A9; SLE glomerulonephritis; systemic lupus erythematosus

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disorder characterized by inflammation in different organ systems and production of autoantibodies directed against nuclear antigens, double-stranded DNA (dsDNA) and histones proteins.[1]

Apoptotic and necrotic cells (NCs) are thought to be the main source of autoantigens in SLE. In the absence of appropriate clearance mechanisms, apoptotic cells will undergo secondary necrosis, a process in which nuclear proteins may become modified and such proteins can subsequently be presented to immune cells. In individuals with genetic predisposition and/or presence of infectious agents causing inflammation, modified self-antigens may be identified as non-self, which in turn might lead to loss of tolerance and production of autoantibodies.[2–4] Immune complexes (ICs) containing DNA or RNA are the predominant cause of inflammation and tissue damage in SLE through activation of complement and engagement of FcgR on immune effector cells, in particular neutrophils, leading to severe SLE manifestations including glomerulonephritis (GN).[5] Neutrophil activation is commonly observed in SLE and several neutrophil abnormalities have been

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demonstrated, one of the earliest being the lupus erythematosus (LE) cell, described in 1948 by Hargraves and colleagues.[6] An LE cell is a polymorphonuclear neutrophil (PMN) that has engulfed antibody- and complement-coated nuclear material and represents an autoimmune abnormality almost exclusively seen in SLE.[7,8] In vitro, the LE cell, or phagocytosis of NCs by PMNs, is dependent on antibodies directed against nuclear components, in particular anti-histone and anti-dsDNA antibodies and an intact classical complement pathway.[9] Even though not used frequently anymore, a positive LE cell test is suggested to indicate active disease, both clinically and serologically, with severe organ involvement.[10] LE cell assessment by light microscopy is not used anymore as a routine test because it is more laborious than new methods that are less complex and just as reliable. A method for quantification and assessment of LE cells by flow cytometry that is less complex than the earlier test has been described by Bohm.[11] In our study we have used a similar flow cytometry method (PNC assay) to detect and quantify PMNs containing NC material that is engulfed in the cytoplasm, where this engulfment has been mediated by SLE serum.[9]

The pro-inflammatory calcium-binding proteins S100A8, S100A9 and S100A12 (calgranulins) belong to the family of S100 proteins and are secreted mainly by activated PMNs and monocytes.[12,13] At inflammatory sites, S100A8 and S100A9 are released by PMNs as a heterodimeric complex, S100A8/A9 (calprotectin or MRP8/14), whereas S100A12 (EN-receptor for advanced glycation endproducts (RAGE)) is released as a homodimer or homohexamer.[12,14,15] S100A8/A9 can also be released during NETosis, in which PMNs extrude chromatin and antimicrobial molecules, including S100A8/A9 and S100A12, in an attempt to trap and kill pathogens.[16–18]

Once released, S100A8/A9 and S100A12 act as damage-associated molecular pattern molecules (DAMPs) and interact with several receptors, including Toll-like receptor (TLR) 4 and RAGE, to exert their pro-inflammatory effects.[19–22]

Elevated S100A8/A9 and S100A12 serum levels have previously been demonstrated in several autoimmune inflammatory disorders including inflammatory bowel disease, rheumatoid arthritis (RA), psoriatic arthritis and SLE[23–28] as well as in infection.[29] In SLE, we and others have demonstrated that S100A8/A9 serum levels are related to disease activity score (SLE Disease Activity Index (SLEDAI)), arthritis, GN and presence of anti-dsDNA antibodies.[26,28] Furthermore, we have demonstrated that S100A8/A9 and S100A12 serum levels are increased in inactive SLE patients with a history of cardiovascular disease compared to SLE patients with no cardiovascular morbidity.[30] To our knowledge, serum levels of S100A12 in active SLE patients have not been investigated.

The aims of this investigation were to assess serum levels of S100A8/A9 and S100A12 in relation to disease activity, and to assess serum levels of S100A8/A9 and S100A12 in relation to clinical phenotype with an emphasis on GN. We also wanted to study underlying pathogenetic mechanisms by investigating whether an increased ability of serum to support phagocytosis of NCs by PMNs could explain the elevated levels of S100A8/A9 seen in SLE patients.

Materials and methods

Patients and serum samples

Paired serum samples from each SLE patient were collected and had been drawn from the patients at two different occasions, at time points of clinically relatively higher and lower disease activity. Patients' disease activity had been assessed prospectively and verified by a medical records review. The patients had no apparent infection at time points of blood sampling, to avoid infection influencing the results. Other clinical and demographic data were also obtained from medical records. Disease activity was defined by physician assessment and scored according to the SLEDAI 2000 (SLEDAI-2K).[31] One hundred SLE patients from the research database were identified and 200 serum samples from the 100 SLE patients were collected from a local biobank and were included in the study. The levels of C1q, C3 and C4 as well as autoantibodies against dsDNA (*Crithidia luciliae* immunofluorescence test) were measured by standard routine laboratory tests. Informed consent was obtained from all participants and the study was approved by the Regional Ethics Board in Lund, Sweden (LU 378-02).

Renal outcome

To categorize the renal outcome, the SLE American College of Rheumatology Renal Response Criteria (ACR-RRC) were used.[32] The definitions of outcome according to these criteria are: complete renal response (CRR): estimated glomerular filtration rate (GFR) >90 ml/min/1.73 m^2 and spot urine protein/urine creatinine

(Up/Ucr) <0.2 and inactive urinary sediment, end-stage renal disease (ESRD): transplant or dialysis for at least three months, or nephrotic syndrome (NS): urinary protein >3.5 g/day or Up/Ucr > 3.0. Patients in between CRR and ESRD or NS were designated as partial renal responders (PRRs) if their Up/Ucr ratio was between 0.2 and 2.[33] We also used the Renal Disease Subcommittee's definitions of renal response for the dimensions renal function, urinary sediment, and urinary protein.[32] Estimated GFRs were constructed by using the modification of diet in renal disease (MDRD) equation.[32,34,35] In our cohort SLE GN was classified according to the World Health Organization (WHO) classification of renal biopsies in patients biopsied in 2006 or earlier. In the patients biopsied in 2007 or later, GN was classified according to the International Society of Nephrology and Renal Pathology Society (ISN/RPS) classification.[36]

S100A8/A9 and S100A12 enzyme-linked immunosorbent assay (ELISA)

Microtitre plates (Maxisorp, Nunc, Roskilde, Denmark) were coated with a monoclonal antibody against S100A8/A9 (27E10, BMA Biomedicals AB, August, Switzerland) at a concentration of 5 µg/ml in phosphate-buffered saline (PBS) pH 7.2 and incubated overnight at 4°C. The plates were then blocked by adding 150 µl of 1% bovine serum albumin (BSA) (ICN Biomedicals Inc, Aurora, OH, USA). Between every following step, the wells were washed three times with PBS containing 0.05% Tween 20 and the volume in each well was 100 µl unless otherwise stated. Serum samples, diluted in S100A8/A9 buffer containing 0.15 M NaCl, 10 mM HEPES (Invitrogen, Carlsbad, CA, USA), 1 mM $CaCl_2$, 0.02 mM $ZnCl_2$, 0.05% Tween 20 and 0.1% BSA were added and incubated for two hours before biotinylated polyclonal antibodies against S100A8/A9 (chicken polyclonal antibody MRP8/14 Abcam, Cambridge, UK) diluted 1/2000 in S100A8/A9 buffer were added and incubated overnight. This step was followed by adding alkaline-phosphatase-labelled streptavidin (Dako, Glostrup, Denmark) diluted 1/1000 in S100A8/A9 buffer. After incubation, bound streptavidin was visualized by adding disodium-*p*-nitrophenyl phosphate (Sigma, St. Louis, MO, USA) 1 mg/ml dissolved in 10% (w/v) diethanolamine pH 9.8 containing 50 mM $MgCl_2$, and the absorbance was measured at 405 nm. The values reported are means of duplicates with the background subtracted and the concentrations were calculated from titration curves obtained from a serum with known concentration. The lower detection level of S100A8/A9 was 3 ng/ml.

S100A12 serum levels were measured with a commercial ELISA (CycLex Corporation, Nagano, Japan) according to the manufacturer's instructions. The lower detection level of S100A12 was 78 pg/ml.

Preparation of phagocytic and NCs

To obtain PMNs and peripheral blood mononuclear cells (PBMCs), freshly heparinized blood from healthy donors was used. The blood cells were isolated by density gradient centrifugation on Polymorphprep™ (Axis-Shield Poc AS, Oslo, Norway) according to the manufacturer's protocol. To obtain NC material, PBMCs were incubated for 10 minutes (min) at 70°C and then stained with propidium iodide (PI) (BD Biosciences Pharmingen, San Diego, CA, USA). The PMNs were stained with anti-CD45-fluorescein isothiocyanate (FITC) (Dako A/S, Glostrup, Denmark).

Assay for phagocytosis of NC material (PNC assay)

Phagocytosis of NCs by PMNs was analysed by flow cytometry, as previously described.[9] PI-labelled NCs (4.5×10^5 cells) were incubated with 30 µl undiluted serum from the SLE patients at room temperature for 20 min, allowing antibodies to bind to the NCs. Anti-CD45-FITC-labelled PMNs purified from healthy individuals were added, at a concentration of 1.0×10^6 cells/ml in a total volume of 300 µl, followed by incubation at 37°C for 15 min. Normal human serum (NHS) from a healthy donor was used as a negative control as well as NHS supplemented with normal mouse immunoglobulin (Ig)G_1 and IgG_{2a} (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA). As a positive control NHS supplemented with mouse-anti-histone antibodies (H5300-10, US Biological, Swampscott, MA, USA) was used. Cells were washed with PBS containing 0.1% human serum albumin (Sigma-Aldrich, St. Louis, MO, USA) before analysis by flow cytometry. PMNs were identified based on forward and side scatter properties and by computerized gating. Phagocytosis was calculated from the percentage of cells positive both for CD45 and PI. The cut-off value for positivity in the phagocytosis assay was set to the mean value in the control group +2 standard deviations (SD). Based on the mean value from the control group +2 SD, the reference for positivity in phagocytosis assay was set to ≥4.6%.

Statistics

Nonparametric tests were used throughout the study because samples were not normally distributed. The comparisons between different groups were performed using the Mann-Whitney *U* test. Wilcoxon signed rank test was used for paired analyses. All *p* values were considered significant at $p < 0.05$. For the statistical analyses we used the following abbreviations: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Results

Clinical characteristics and disease activity of the SLE patients

An overview of ACR classification criteria[37] for the 100 SLE patients is presented in Table 1. All patients fulfilled ≥4 ACR criteria for SLE. Clinical and demographic characteristics of the SLE patients are shown in Table 2. Thus, Table 1 shows cumulative data: organ involvement and autoantibody profile ever seen in the SLE patients, and data in Table 2 demonstrates organ involvement and presence of autoantibodies at time points of blood sampling.

Thirty-five of the patients had active GN at the time point of active disease, of whom 15 had not started treatment with immunosuppressive drugs. In 13 of these patients, serum samples from the biobank were available from the follow-up visits after three to four and six to eight and in two cases 10 months after initiation of treatment with immunosuppressive drugs.

Active GN was found in 35 patients, in whom representative material on biopsy could be obtained in 31. In the majority of cases, biopsy was performed a few days before or after time point of blood sampling. A summary of GN classification is shown Table 3.

Increased serum levels of S100A8/A9 and S100A12 at higher disease activity

Serum levels of S100A8/A9 were increased at time points of higher as compared to lower disease

Table 1 Clinical characteristics of the SLE patients according to American College of Rheumatology (ACR) criteria

Characteristics	*SLE cohort* n = *100*
Malar rash	66
Discoid rash	30
Photosensitivity	62
Oral ulcers	29
Arthritis	83
Serositis	55
Renal disease	46
Neurological disorder	5
Haematological manifestations	53
Leucopenia	43
Lymphopenia	23
Thrombocytopenia	22
Immunology	75
Anti-dsDNA antibodies	63
ANA	98

SLE: systemic lupus erythematosus; Anti-dsDNA: anti-double-stranded DNA; ANA: antinuclear antibodies.

Table 2 Clinical characteristics and treatment at the time points of blood sampling for the 100 SLE patients. Items included in SLE Disease Activity Index 2000 (SLEDAI-2K) are shown

Characteristics	*SLE*[a] *Lower* n = *100*	*SLE*[b] *Higher* n = *100*
Age, median (range), years	43 (19–81)	41 (14–75)
Female, %	90	90
Disease duration, median (range), years	7 (0–43)	4 (0–40)
SLEDAI-2K score, median (range)	2 (0–6)	9 (2–28)
Seizure	0	2
Psychosis	0	0
Organic brain syndrome	0	0
Visual disturbance	0	8
Cranial nerve disorder	0	0
Lupus headache	0	2
Cerebrovascular accident	0	2
Vasculitis	0	12
Arthritis	2	30
Myositis	0	2
Kidney involvement (urinary cast, haematuria, proteinuria or pyuria)	0	35
Rash	5	31
Alopecia	0	7
Oral ulcers	0	7
Pleurisy	0	13
Pericarditis	0	7
Low complement (C3 or C4)	41	54
Anti-DNA antibodies	24	48
Fever	1	8
Thrombocytopenia	2	3
Leucopenia	6	10
Hydroxychloroquine	33	31
Chloroquine phosphate	4	6
Azathioprine	21	18
Mycophenolate mofetil	16	9
Corticosteroids	66	66
Methotrexate	0	2
Cyclophosphamide	1	5
Cyclosporine A	9	5

[a]Patients from time point with lower disease activity.
[b]Patients from time point with higher disease activity.
SLE: systemic lupus erythematosus.

activity (median 11,493 ng/ml vs 8778 ng/ml $p = 0.01$), Figure 1(a).

Similar results were noted for S100A12 (median 244 ng/ml vs 179 ng/ml $p = 0.008$), Figure 1(b). Altogether, elevated levels of S100A8/A9 and S100A12 were associated with disease activity in SLE patients.

Serum levels of S100A8/A9 and S100A12 in relation to clinical phenotype

Since GN represents a severe form of SLE, we were especially interested to see if elevated levels of S100A8/A9 could be found in this subgroup at the time point of active disease, but when comparing patients with GN to patients with non-renal flare no difference could be seen (median 12,177 ng/ml vs 10,757 ng/ml $p = 0.20$), Figure 2(a). However, several of the patients with active GN included in our cohort were already started on treatment with immunosuppressive drugs when the blood sample was taken. To investigate if therapy affected levels of S100A8/A9, patients were subdivided into groups with active glomerulonephritis with ($n = 20$) and without ($n = 15$) immunosuppressive treatment at time point of blood sampling. Patients with active GN but without immunosuppressive treatment had a markedly elevated level of S100A8/A9 compared to SLE patients with active GN treated with immunosuppressive therapy (median 39,982 ng/ml vs 9755 ng/ml $p = 0.04$), Figure 2(a). Furthermore, patients with active GN without immunosuppressive treatment had increased serum levels of S100A8/A9 compared with patients with active disease in general (median 39,982 ng/ml vs 10,757 ng/ml $p = 0.02$), Figure 2(a). The patients with GN without immunosuppressive therapy had increased serum levels of S100A8/A9 compared to patients with active disease in general who were not treated with immunosuppressive drugs (median 39,982 ng/ml vs 10,584 ng/ml $p = 0.02$), Figure 2 (a). For S100A12, no association was seen with GN, even when taking immunosuppressive therapy into account (median 423 ng/ml vs 265 ng/ml $p = 0.40$), Figure 2 (b).

Table 3 The WHO- and ISN/RPS classification in biopsied patients with glomerulonephritis

Nephritis type	*SLE patients* n = *35*
WHO II	3
WHO III	3
WHO IV	17
WHO V	2
ISN/RPS II	0
ISN/RPS III	0
ISN/RPS IV GA	3
ISN/RPS IV GAC	2
ISN/RPS IV GC	0
ISN/RPS IV + V	1
ISN/RPS V	0
No renal biopsy[a]	4

[a]No biopsy performed or no representative material in biopsy.
WHO: World Health Organization; ISN/RPS: International Society of Nephrology and Renal Pathology Society; SLE: systemic lupus erythematosus.

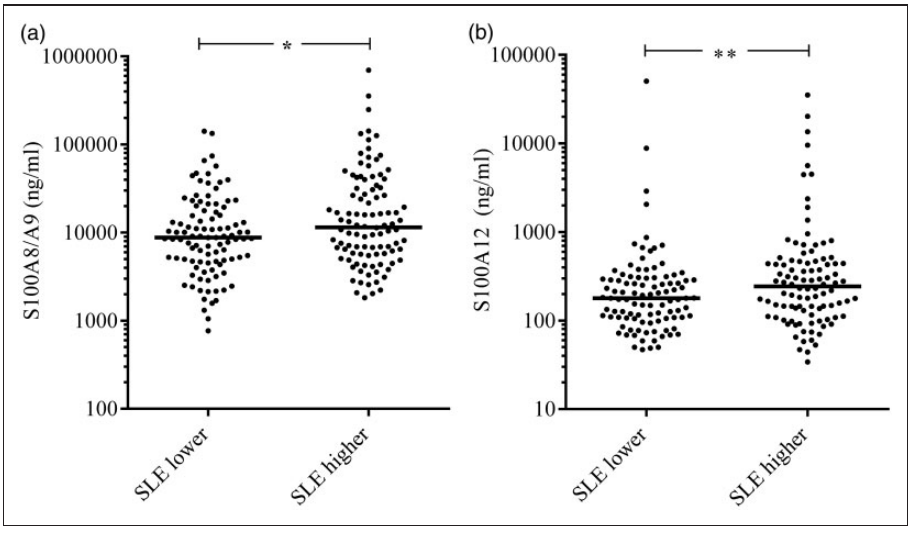

Figure 1 Increased serum levels of S100A8/A9 and S100A12 in SLE patients at higher disease activity. Serum levels of (a) S100A8/A9 and (b) S100A12 were analysed by ELISA in 100 SLE patients at time points of both relatively low and high disease activity. The line represents the median value in each group. For statistical analyses $*p < 0.05$, $**p < 0.01$.
SLE: systemic lupus erythematosus; ELISA: enzyme-linked immunosorbent assay.

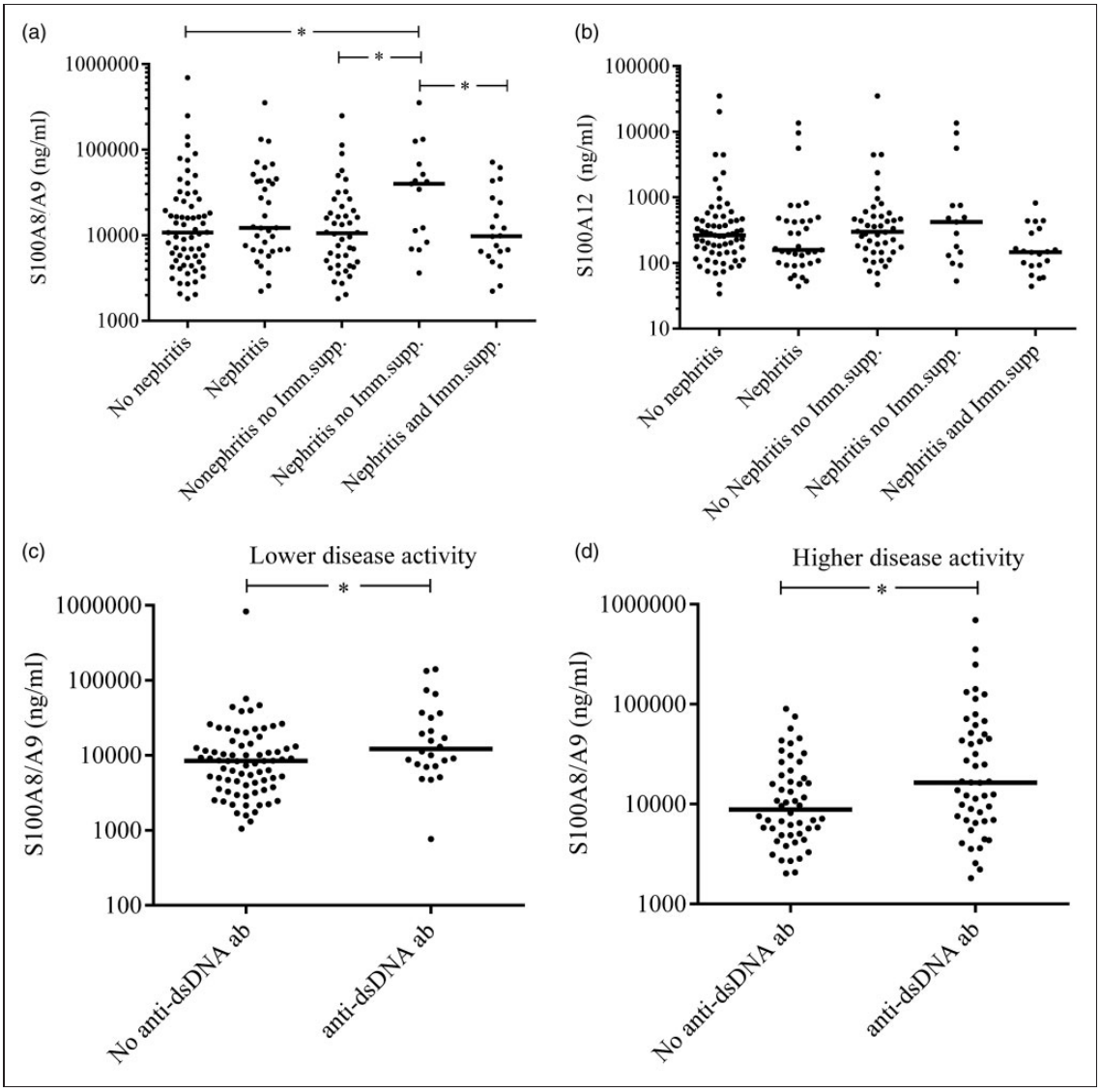

Figure 2 S100A8/A9 serum levels are increased in patients with active glomerulonephritis without immunosuppressive treatment. Serum levels of (a) S100A8/A9 and (b) S100A12 were analysed by ELISA in SLE patients with high disease activity. SLE patients were grouped based on absence ($n = 65$) or presence ($n = 35$) of ongoing glomerulonephritis, and treatment regimen based on presence ($n = 20$) or absence ($n = 15$) of immunosuppressive treatment in patients with active SLE. Serum levels of S100A8/A9 were related to presence of anti-dsDNA antibodies at time points of relatively (c) low and (d) high disease activity. For statistical analyses $*p < 0.05$.
SLE: systemic lupus erythematosus; ELISA: enzyme-linked immunosorbent assay; anti-dsDNA: anti-double-stranded DNA.

We could demonstrate that patients with anti-dsDNA antibodies, both at time point of lower as well as higher disease activity, had increased serum levels of S100A8/A9 compared to patients without anti-dsDNA antibodies (median 12,149 ng/ml vs 8390 ng/ml $p = 0.02$ and median 16,390 ng/ml vs 8861 ng/ml $p = 0.01$, respectively), Figures 2 (c) and (d). Presence of anti-dsDNA antibodies was not associated with serum S100A12 levels, either at lower or higher disease activity (median 242 ng/ml vs 161 ng/ml $p = 0.05$ and 282 ng/ml vs 230 ng/ml $p = 0.28$, respectively).

We found lower serum levels of S100A8/A9 in SLE patients with lupus-related skin rash, compared to patients with activity in other organ systems (median 5790 ng/ml vs 13,800 ng/ml $p = 0.006$), Figure 3. Corresponding results were not seen for levels of S100A12 (median 166 ng/ml vs 279 ng/ml $p = 0.07$).

In all, increased serum levels of S100A8/A9, but not S100A12, were seen in untreated GN. Furthermore, amongst patients with active disease, the lowest S100A8/A9 levels were found in patients with skin rash.

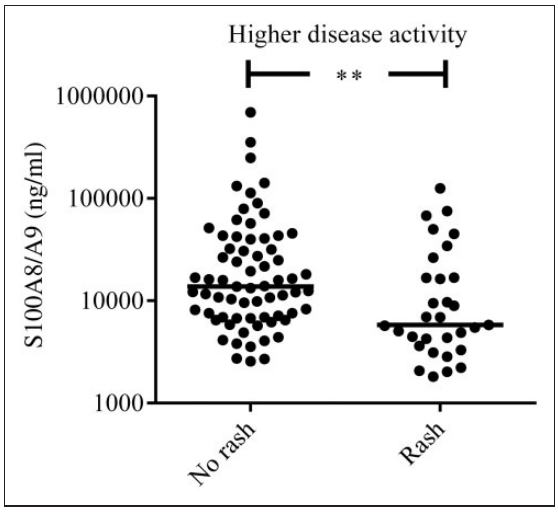

Figure 3 S100A8/A9 serum levels are decreased in patients with lupus-related skin rash compared to patients with activity in other organ systems. Serum levels of S100A8/A9 were analysed by ELISA in SLE patients with high disease activity. Serum levels were related to presence or absence of lupus-related skin rash. For statistical analyses $**p < 0.01$.
SLE: systemic lupus erythematosus; ELISA: enzyme-linked immunosorbent assay.

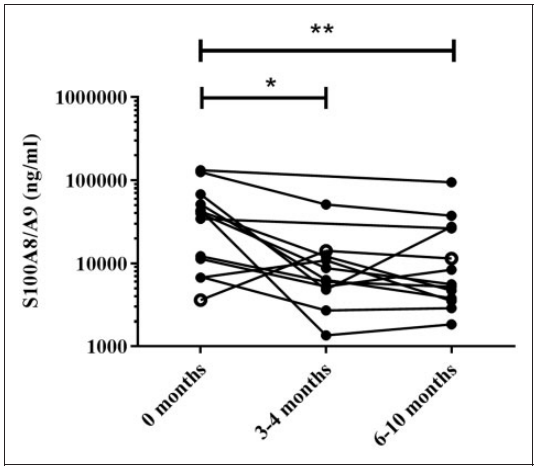

Figure 4 Serum S100A8/A9 levels decrease upon immunosuppressive treatment in patients with active glomerulonephritis. Serum, sampled at time point of active non-treated glomerulonephritis (0 months), three to four or six to 10 months after treatment initiation with immunosuppressive therapy, were analysed for S100A8/A9 levels by ELISA. The patient with cyclosporine treatment is displayed as open circles. For statistical analyses $*p < 0.05$, $**p < 0.01$.
ELISA: enzyme-linked immunosorbent assay.

Serum levels of S100A8/A9 as a marker to monitor response to treatment

To further explore an effect of immunosuppressive therapy on S100A8/A9 levels, we measured S100A8/A9 serum levels in samples collected longitudinally before and after treatment initiation in 13 patients with active GN for whom samples were available in our biobank as well as clinical data on renal response. Renal outcome was evaluated by the ACR-RRC.[32] CRR was achieved in 3/13 cases and PRR was seen in 9/13 cases. No cases of nephrotic syndrome (NS) or ESRD were seen although one patient was categorized as a non-responder.[32,33] Eleven of the patients were treated with cyclophosphamide, one patient was treated with azathioprine and one with cyclosporine. Serum concentrations of S100A8/A9 clearly decreased after three to four and six to 10 months (median 39,982 ng/ml vs 7560 ng/ml $p = 0.02$ and 39,982 ng/ml vs 5600 ng/ml $p = 0.002$, respectively), Figure 4. However, serum levels of S100A12 were not affected by treatment after three to four and six to 10 months (353 ng/ml vs 190 ng/ml $p = 0.11$ and 353 ng/ml vs 187 ng/ml $p = 0.30$, respectively).

In the cyclosporine-treated patient, serum levels of S100A8/A9 did not decrease after treatment. The patient treated with cyclosporine had remaining oedema and proteinuria at follow-up, but not NS by definition and was therefore classified as a PRR. This patient is displayed as open circles in Figure 4. Thus, upon administration with immunosuppressive treatment, serum levels of S100A8/A9, but not S100A12, decreased in SLE patients with active GN.

Phagocytosis of NC material by PMNs is associated with elevated serum levels of S100A8/A9

We performed a modified LE cell test in which phagocytosis of NCs by PMNs was analysed by flow cytometry.[9] Serum samples with increased ability to support phagocytosis of NCs to PMNs had elevated levels of S100A8/A9 at time points both of relatively lower and higher disease activity (median 10,825 ng/ml vs 8077 ng/ml $p = 0.008$ and 15,910 ng/ml vs 7575 ng/ml $p = 0.01$, respectively), Figures 5 (a) and (b). No associations were seen between S100A12 serum levels and phagocytosis of NC by PMNs at either lower or higher disease activity (median 213 ng/ml vs 161 ng/ml $p = 0.11$ and median 265 ng/ml vs 218 ng/ml $p = 0.33$, respectively). Thus, serum levels of S100A8/A9, but not S100A12, are associated with phagocytosis of NCs by PMNs.

Plasma levels of C-reactive protein (CRP), C3 levels in serum and leucocyte blood counts in relation to disease activity and clinical phenotype

We also wanted to know how serum levels of S100A8/A9 behave in comparison with established

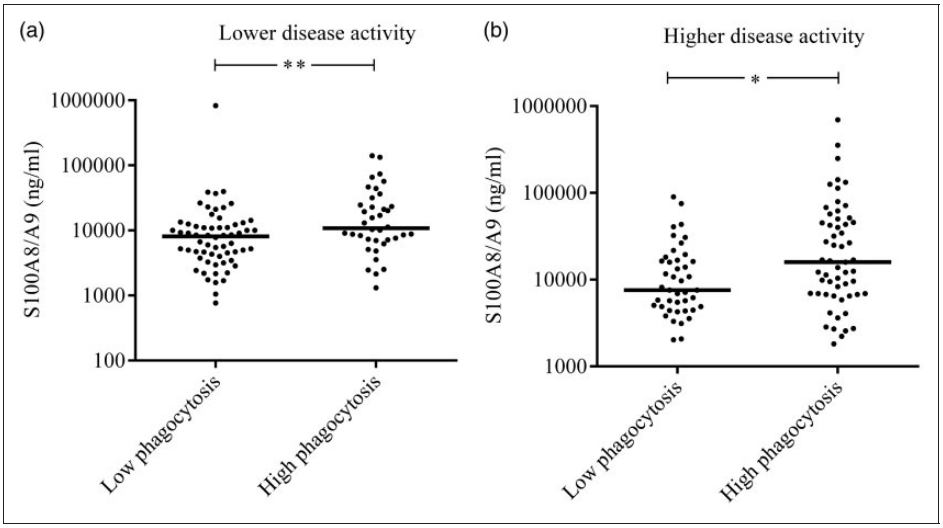

Figure 5 Increased serum-mediated phagocytosis of necrotic cells by neutrophils is related to elevated S100A8/A9 serum levels. Serum samples, both at time points of relatively low and high disease activity, were grouped based on ability to support phagocytosis of necrotic cells by neutrophils, and related to serum levels of S100A8/A9. For statistical analyses $*p < 0.05$, $**p < 0.01$.

biomarkers of SLE activity. We therefore investigated values of CRP, C3 and leucocyte blood counts obtained from medical records in relation to disease activity, clinical phenotype and serum levels of S100A8/A9. The results are shown in supplementary files.

Discussion

The pro-inflammatory protein complex S100A8/A9 has been described to be elevated in various diseases such as infections, cancer, cardiovascular disease as well as autoimmune disorders including RA and SLE in cross-sectional studies.[14,15,25,26] We have previously demonstrated that SLE patients with inactive disease have elevated serum levels of S100A8/A9 compared to healthy controls.[30] In this study we extend these observations and demonstrate increased serum levels of S100A8/A9 in SLE patients at time points of higher compared to lower disease activity. Furthermore, this is the first investigation demonstrating that also S100A12 is a marker of disease activity in SLE.

One of the main findings of this investigation was the markedly increased levels of S100A8/A9 observed in SLE patients with untreated GN. Also presence of anti-dsDNA antibodies, frequently found in patients with GN,[38,39] was associated with increased levels of S100A8/A9. In a recent study, elevated MRP-8 (S100A8) and MRP-14 (S100A9), messenger RNA (mRNA) expression was found in blood leucocytes as well as in renal tissue in patients with SLE GN, and mRNA levels were associated with severity and response to treatment.[40] These are new and important findings where responders and non-responders to treatment are compared. In the study serum levels of S100A8/A9 are not investigated.

It has previously been demonstrated that S100A8/A9 levels decrease after immunoglobulin (IvIg) treatment in patients with Kawasaki disease and in patients with RA treated with anti-tumour necrosis factor (TNF) alpha antibodies[41,42] but whether immunosuppressive treatment could also reduce serum S100A8/A9 levels in SLE GN is not known. Here we demonstrate that in long-term follow-up of patients with GN, S100A8/A9 levels decreased over time when renal activity was reduced by successful treatment, indicating that S100A8/A9 serum levels may be used to monitor treatment efficacy. Although the number of patients is small, this might be an important finding since there are no reliable markers available to diagnose or to monitor treatment in SLE GN, except for renal biopsy. Further studies with a larger number of patients with active GN are needed for comparison of S100A8/A9 serum levels in responders and non-responders to treatment. In our study we had only 3/13 complete renal responders and 1/13 non responders, and the majority of the patients for whom we had access to follow-up serum samples were PRRs. Therefore, it has not been possible to compare S100A8/A9 serum levels after treatment in the different response groups. However, our results show clearly decreasing S100A8/A9 serum levels after induction of

treatment and this indicates that measuring of S100A8/A9 serum levels might be an important tool in the management of lupus nephritis, and it would be important to evaluate this in prospective studies.

SLE patients with skin involvement at disease exacerbation are considered to have a milder disease phenotype compared to patients with kidney or other organ involvement.[43] We found lower S100A8/A9 levels in milder disease with skin rash at disease exacerbation and higher S100A8/A9 serum levels in more severe disease (active GN at disease exacerbation). This difference may be due to involvement of different autoantibodies in the different disease manifestations: Anti-Ro antibodies are often associated with skin rash and anti-dsDNA antibodies are associated with GN.[38,44] These autoantibodies might have a different ability to form ICs and thereby a different ability to activate S100A8/A9-producing cells such as neutrophils and therefore S100A8/A9 serum levels differ in different manifestations of disease. We have previously demonstrated high phagocytosis of NCs by PMNs in SLE patients with active GN and serositis, which is compatible with this hypothesis and with our findings in this study.[9]

Even though both S100A8/A9 and S100A12 were associated with disease activity, only S100A8/A9 seemed to be associated with disease severity and clinical phenotype, suggesting selective release of S100A8/A9 and not S100A12 by certain stimuli or cells, and/or differences in S100A8/A9 and S100A12 in promoting certain disease manifestations through their respective receptors. Whereas S100A8/A9 is produced by several cells, including neutrophil granulocytes, monocytes, plasmacytoid dendritic cells and endothelial cells, S100A12 expression seems to be almost completely restricted to neutrophil granulocytes, which may explain why elevation of S100A8/A9 and S100A12 in serum doesn't follow the same pattern.[12,13,28,45] Neutrophils were proposed to play a role in SLE pathogenesis and lupus nephritis in the 1980s[46] but also more recently neutrophil activation and release of neutrophil extracellular traps (NETs) have been implicated in the pathogenesis of SLE.[47–50] However, the underlying mechanism for the elevated serum levels of S100A8/A9 and S100A12 in SLE patients is not known.

As described by others, PMNs, as well as other phagocytes, release S100A8/A9 upon activation.[12] In SLE, PMN activation is commonly seen,[51,52] probably due to encounter with circulating or tissue-deposited ICs. Since in this study presence of anti-dsDNA antibodies was associated with increased S100A8/A9 levels, we hypothesized that autoantibodies targeting dead cell material may contribute to elevated serum levels of S100A8/A9 through enhancing phagocytosis and activation of PMNs, which is commonly seen in SLE.[52] Anti-dsDNA antibodies and NC material form ICs that are phagocytosed by PMNs. We hypothesize that this may lead to PMN activation with subsequent release of S100A8/A9. Thus, we believe that the elevated S100A8/A9 serum levels are, at least in part, due to such IC-mediated PMN activation resulting in S100A8/A9 release. In support of this theory we observed that the ability of serum from SLE patients to form ICs with NC remnants, subsequently phagocytosed by neutrophils, was highly associated with serum levels of S100A8/A9. Even though further mechanistic studies are warranted, our data are compatible with a central role of ICs in inducing neutrophil activation and release of calgranulins participating in inflammation and development of severe disease, including GN.

In conclusion, increased serum levels of S100A12 and S100A8/A9 are associated with disease activity in SLE and elevated serum levels of S100A8/A9 were seen especially in active untreated SLE GN, but further studies are needed to evaluate its role as a possible biomarker in SLE. Furthermore, elevated S100A8/A9 serum levels may reflect processes related to neutrophil activation that might play an important role in SLE pathogenesis.

Declaration of Conflicting Interests

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