**Differential Analysis of Serum and Urine S100 Proteins in juvenile-onset Systemic Lupus Erythematosus (jSLE)**

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**Abstract**

Up to 80% of juvenile-onset systemic lupus erythematosus (jSLE) patients develop lupus nephritis (LN) that affects treatment and prognosis. Easily accessible biomarkers do not exist to reliably diagnose LN, leaving kidney biopsies as the gold-standard. Calcium-binding S100 proteins are expressed by innate immune cells and epithelia and may act as biomarkers in systemic inflammatory conditions.

We quantified S100 proteins in the serum and urine of jSLE patients, matched healthy and inflammatory (IgA vasculitis) controls. Serum S100A8/A9, and serum and urine S100A12 are increased in jSLE patients when compared to controls. Furthermore, serum S100A8/A9, and serum and urine S100A12 are increased in jSLE patients with active as compared to patients with inactive/no LN. No differences in S100A4 levels were seen between groups.

This study demonstrates potential promise for S100A8/A9 and S100A12 as biomarkers for jSLE and active LN. Findings require to be confirmed and tested prospectively in independent and larger multi-ethnic cohorts.

**Keywords:** S100, juvenile-onset, SLE, nephritis, biomarker

**Introduction**

Systemic Lupus Erythematosus (SLE) is a complex multi-system autoimmune/inflammatory condition that can affect any organ of the human body and result in significant tissue and organ damage[1]. Up to 20% of SLE patients develop disease before their 16th birthday and are therefore, diagnosed with juvenile-onset SLE (jSLE)[2-4]. Juvenile-onset SLE presents with a more variable clinical picture, higher disease activity and increased mortality when compared to adult-onset disease (aSLE)[2, 5]. More severe phenotypes in jSLE may be caused by different pathomechanisms between age groups. This is suggested by variable sex distributions between jSLE (female:male = 4-5:1) and aSLE (9:1) cohorts and the fact that particularly young jSLE patients less frequently exhibit “classical” autoantibody patterns [4, 6, 7].

While the exact pathophysiology of SLE (across age groups) is unknown, a complex network of genetic predispositions, environmental and hormonal influences mediate altered immunological functions[8, 9]. Molecular pathomechanisms include the presence of autoantibodies directed against nuclear antigens that result in the generation of immune complexes and their deposition in tissues and organs, self-directed effector lymphocytes and altered cytokine responses[2]. The Resulting chronic inflammation contributes to organ damage that defines long-term outcomes and prognosis[10-12].

Renal involvement in SLE (lupus nephritis; LN) contributes significantly to mortality and morbidity. It affects almost 50% of jSLE patients at diagnosis and up to 80% of jSLE patients within a 5-year period from diagnosis[2, 13]. When compared to aSLE, LN in jSLE correlates less closely with proteinuria. Thus, kidney biopsies resemble the gold-standard to confirm renal involvement and its severity[14]. However, kidney biopsies are invasive and carry several risks, including bleeding and infection which are both further increased in SLE making them less practical for disease monitoring, especially in children[15]. Therefore, easily accessible biomarkers for the diagnosis and/or monitoring of jSLE-associated LN are needed to reduce the number of biopsies required and to measure disease activity during clinical follow-up, allowing the prediction of disease outcomes.

A group of pro-inflammatory calcium-binding proteins known as the S100 proteins have recently emerged as markers of disease activity in several paediatric systemic inflammatory conditions[16-18]. S100 proteins are mainly expressed in granulocytes and mononuclear blood cells such as neutrophils and macrophages[19, 20]. More recently, expression of these S100 proteins has been reported in renal endothelia[21]. Once released from cells at sites of inflammation, S100 proteins act in an autocrine and paracrine manner and bind to pattern recognition receptors, propagating inflammatory responses[21, 22].

The Medical Research Council’s MASTERPLANS (Maximizing SLE therapeutic Potential by Application of Novel and Systemic Approaches) Consortium aims to stratify adult and paediatric lupus patients based on molecular patterns to predict treatment responses. Here, we determined serum and urine concentrations of S100 proteins (S100A4, S100A8/A9 and S100A12) in jSLE with or without active renal disease and tested their applicability as markers for global and renal disease activity.

**Methods**

**Study cohort --** Serum and urine samples were collected from jSLE patients within the UK JSLE Cohort Study and Repository (REC: 6/Q1502/77). Written informed patient/parental assent/consent was obtained in accordance with the declaration of Helsinki. All patients involved in the study met the 1997 American College of Rheumatology (ACR) Lupus Classification Criteria. For all samples, access was available to both clinical and demographic datasets (**Supplementary Table 1**). Serum and urine samples were also collected from healthy paediatric control individuals attending hospital appointments (Alder Hey Children’s NHS Foundation Trust) for surgery unrelated to inflammatory conditions. Patients with Immunoglobulin (Ig)A vasculitis (IgAV) who had serum and urine samples collected as part of ongoing renal research at Alder Hey were used as inflammatory disease controls in this study.

**S100 protein quantification --** Both S100A8/A9 and S100A12 proteins in the serum and urine of jSLE patients, healthy paediatric controls and IgAV inflammatory controls were quantified using the Meso Scale Discovery Electrochemiluminescence R-PLEX Singleplex Immunoassay (MSD, Maryland, USA). All assays were performed as per the manufacturer’s instructions using MSD GOLD 96-well Small Spot Streptavidin plates and assay diluents. All serum and urine samples were vortexed thoroughly before use. All plates were read using the MSD QuickPlex SQ 120 platform. S100A4 proteins in serum and urine were measured in duplicate with a commercially available enzyme-linked immunosorbent assay (CircuLex S100A4 ELISA Kit Ver.2 (MBL International, Woburn, Massachusetts, USA) as per the manufacturer’s instructions.

Urine S100 protein concentrations were normalised to urine creatinine. Urine creatinine levels were measured by the Clinical Chemistry Laboratory at Alder Hey Children’s NHS Foundation Trust using the Abbott Enzymatic Creatinine assay on the Abbott Architect Ci8200 (Abbott, Illinois, USA). Data expressed as (median [range]).

**Disease activity --** For the data within this study, the paediatric version BILAG2004 grading system was used to measure disease activity (pBILAG)[23, 24]. pBILAG scores of A, B and C were classified as active disease and pBILAG scores of D and E were classified as inactive disease. pBILAG scores of C were grouped with active disease as although disease activity is mild or improving at this grade, these patients cannot be classified as having no disease activity. SLE Disease Activity Index (SLEDAI)-2K score were also used in this study to assess disease activity. SLEDAI scores of 0-4 were classified no to mild disease activity, SLEDAI scores of 5-10 were classified as moderate disease activity and scores 11+ were high disease activity.

**Statistical Analysis --** Figures were generated and statistical analysis performed using GraphPad Prism version 8.2.1 (GraphPad Software, California, USA). All values at p<0.05 were considered significant. All S100 protein datasets in the study did not follow a Gaussian distribution and therefore, data was analysed using non-parametric tests. For analysis between all three study populations a Kruskal-Wallis test with Dunn’s multiple comparison post hoc test was used and for analysis between two groups a Mann-Whitney U test was used. Non-parametric Spearman’s Rho correlation coefficient was used to assess S100 protein concentration against continuous clinical variables.

**Results**

**Demographic and clinical information --** A total of 64 jSLE patients (60 serum and 59 urine samples), 53 race- and age-matched healthy controls and 9 IgAV patients were included in this study. Clinical and demographic data are summarised in **Supplementary table 1**. Juvenile-onset SLE patients were further categorised into active renal disease patients (renal pBILAG scores of A, B or C (serum; n=18, [30%], urine; n=18 [30.5%])) and those with currently inactive or no renal disease (renal pBILAG scores of D or E (serum; n=39 [65%], urine; n=35 [59.32%])). Three patients in the serum group (n=3 [5%]) and 6 patients in the urine group (n=6 [10.17%]) had unknown renal pBILAG scores. These samples were therefore excluded from some analyses.

**S100 protein concentrations discriminate jSLE patients from controls --** Serum S100A8/A9 levels were significantly increased in the serum of jSLE patients (1,577.80ng/mL [34.5-13,417]) when compared to healthy paediatric controls (732.53ng/mL [34.5-5,685.6]; p<0.001) and patients with IgAV (958.17ng/mL [234.17-3,417.1]; p=0.0019) (**Figure 1A**). No differences were seen between S100A8/A9 urine levels in jSLE patients, healthy paediatric controls and IgAV patients (**Figure 1B**). S100A12 concentrations were significantly increased in both the serum (41.12 ng/mL [1.41-4,080.40]; p=0.035) and urine (90.12ng/mmol creatinine [0.71-8,047.8]; p=0.029) of jSLE patients when compared to healthy paediatric controls (serum; 17.75ng/mL [2.10-586.62], urine; 14.88ng/mmol creatinine [0.44-6,276.5]) (**Figures 1C and 1D**). Increased S100A12 protein levels in the serum and particularly the urine was observed in jSLE patients (serum; p=0.999, urine; p=0.553) when compared to patients with IgAV, however, these data did not reach statistical significance (**Figures 1C and 1D**). No differences were seen the S100A4 concentrations in the serum or urine of jSLE patients when compared to controls or patients with IgAV (**Figures 1E and 1F**). It is important to note that for a large subset of jSLE and IgAV patients as well as healthy control, S100A4 levels were below the level of detection for the assay and thus were recorded as 265 pg/mL (lowest level of detection/√2).

Following this, we assessed possible relationships between clinical characteristics and S100 concentrations. No significant differences were observed in S100 protein concentrations and serum complement C3 levels (data not shown). A trend to increased S100A12 urine protein concentrations in patients positive for anti-dsDNA antibodies was seen. However, this did not reach statistical significance (**Supplementary table 2).** Similarly, no correlation between S100 protein serum and urine levels and disease activity as measured by SLEDAI-2K score were seen (data not shown). Incomplete SLEDAI-2K data for our patient cohort may have affected the outcome of this analysis.

**S100 proteins correlate with active renal involvement in jSLE --** Serum and urine S100 protein concentrations were assessed in relation to disease activity in various organs/ organ system pBILAG domains (**Supplementary table 1**). Of all domains tested, statistical significance was only seen in the renal pBILAG domain; S100A8/A9 levels were significantly increased in the serum of jSLE patients with active renal disease (renal pBILAG A, B or C) (2,113ng/mL [813.45-8,286.2]) as compared to patients with inactive or no renal disease (renal pBILAG D and E) (1,348.69 ng/mL [34.5-12,417]; p=0.043; **Figure 2A**). Of note, this was not reflected in the urine of these patients (**Figure 2B**). S100A12 concentrations were also significantly increased in the serum (116.03ng/mL [6.19-316.10]; p=0.021) and urine (353.11pg/mmol creatinine [3.91-8,047.8]; p=0.0095) of jSLE patients with active renal disease compared to those with inactive or no renal disease (serum; 32.84ng/mL [4.06-4,080], urine; 52.02pg/mmol creatinine [0.71-849.08]) (**Figures 2C and 2D**). No differences were seen in S100A4 serum or urine proteins levels between jSLE patients with active renal disease and those with inactive or no renal disease activity **(Figures 2E and 2F**).

**Discussion**

Juvenile-onset SLE presents with a more severe phenotype and greater disease-related morbidity and mortality when compared to aSLE. Yet, reliable and widely-accepted biomarkers for assessing disease activity and progression are not available[2, 5].

Calcium-binding S100 proteins, specifically S100A8/A9 and S100A12 have recently emerged as biomarkers to monitor disease activity in a number of paediatric inflammatory conditions including juvenile idiopathic arthritis[16-18]. More recently, S100 proteins have been suggested as biomarkers for disease activity in aSLE[25] and in a paediatric cohort of LN patients[17]. The S100 family member A4 is expressed in highly motile cells, including macrophages[19] and neutrophils[20] where it interacts with cytoskeletal proteins, having a prominent effect on cell migration[21]. A role for S100A4 in fibrosis[26] has also been suggested which may be important in patients with LN[17], particularly since it is expressed in renal endothelia[27]. The S100A8 and S100A9 proteins are usually found in a heterodimeric complex (S100A8/A9) and, along with S100A12, are mainly expressed in phagocytes where S100A8/A9 can comprise up to 45% of the cytosolic proteins of neutrophils[21]. S100A4, S100A8/A9 and S100A12 can be secreted from innate immune cells at sites of inflammation and function as damage-associated molecular patterns, further perpetuating the inflammatory process[22]. Once released from cells, S100 proteins bind to pattern recognition receptors, such as toll-like receptor 4 (TLR4; S100A8/A9 and S100A12), receptor for advanced glycation end products (RAGE; S100A8/A9, S100A12 and S100A4) and ErbB4 (S100A4). However, extracellular signalling by S100A4 remains largely unexplored[28-31]. Receptor recruitment subsequently leads to downstream activation of NF-κB and MAPK pathways inducing the expression of a variety of proinflammatory cytokines including IFN-γ and TNF-α[18, 32-34].

We found significantly elevated levels of S100A8/A9 protein in the serum and S100A12 protein in the serum and urine of jSLE patients when compared to healthy paediatric controls. We did not see statistically significant differences in S100A4 protein levels in serum or urine of jSLE patients when compared to healthy controls or patients with IgAV. These findings correlate with a previously published study[19] which showed increased plasma S100A8/A9 and S100A12 protein concentrations in aSLE patients when compared to healthy controls. This study investigated plasma protein concentrations alone, while our study analysed serum and urine, giving a broader picture of S100 proteins in different bodily fluids.

Studies in jSLE have mainly focused on organ-specific disease. Tunier *et al.* reported elevated S100A4, S100A8/A9 and S100A12 protein levels in urine from active LN patients as compared to jSLE patients with active extra-renal disease without renal involvement or healthy controls[17]. This study used SLEDAI-2K scores as a measure of disease activity, this provides an overall score for disease activity. The study presented here applied the pBILAG 2004 scoring system instead of SLEDAI-2K scoring as this allows for assessment of S100 proteins concentrations in relation in individual organ domains as well as collectively to give an overall view of correlations between disease activity and S100 protein concentrations. This discrepancy in clinical data may identify why we were unable to replicate some of the findings in our cohort (differences in S100A4 expression between groups, see below).

Increased levels of S100A8/A9 in the serum of jSLE patients as compared to IgAV patients was also observed in this study. Furthermore, we observed elevated levels of S100A8/A9 in the serum of jSLE patients with active renal disease when compared to patients with inactive renal disease, but not in the urine of these patients. Elevated S100A12 protein levels were seen in the serum and urine of jSLE patients with active renal disease compared to patients with inactive or no renal disease.

Of note, S100A4 protein levels did not correlate with renal disease activity in this study. This reflects findings from a recent study that delivered increased S100A4, S100A8/A9 and S100A12 protein concentrations in the serum and urine of jSLE patients with LN when compared to healthy paediatric controls[17]. Why S100A4 levels vary between studies remains unclear, but may be caused by variable race distribution and/or renal disease activity between cohorts. Another explanation is based on the small molecular weight of S100 proteins. Data pertaining to urine S100 protein concentrations in the urine may (partially) be a affected by renal loss of (elevated) serum S100 proteins through the kidneys (S100A4: 11.7 kDa; S100A8: ~11 kDa, S100A9: ~13 kDa; S100A8/A9: 23.9 kDa; S100A12: 21kDa) rather than being a result of kidney inflammation or damage alone. This may explain why we failed to demonstrate elevated urine S100A4 levels in jSLE patients with active renal disease as well as overlap in urine S100A8/A9 and S100A12 levels between patients with active vs inactive renal disease.

To test associations between S100 protein levels and certain disease subgroups, we assessed the relationship between S100A4, S100A8/A9 and S100A12 proteins and clinical characteristics such as ESR, Complement C3, and the presence of anti-dsDNA autoantibodies. A trend towards increased S100A12 protein levels in the urine of jSLE patients positive for anti-dsDNA autoantibodies (as compared to anti-dsDNA negative patients) was observed in our study. This relates to previous data in an aSLE cohort which found raised serum S100A8/A9 levels in patients positive for anti-dsDNA autoantibodies independent of other markers disease activity[35]. In the context of jSLE, this observation is particularly interesting, since jSLE patients more frequently remain negative for anti-dsDNA antibodies. Interestingly, autoantibody negative jSLE patients may experience alternative pathomechanisms. For example, data from the UK JSLE Cohort Study indicate that “autoantibody negative” jSLE patients less frequently develop renal disease [5] (and unpublished data, in revision process).

Inclusion of an inflammatory disease control group (IgAV) is unique among studies of S100 proteins to date. It allows evaluation of whether differences observed in urine and/or serum S100 protein concentrations in jSLE patients are due to the presence of immune complexes with relatively low systemic inflammatory activity or whether these changes may be specific to SLE. Indeed, serum S100A8/A9 and (though not reaching statistical significance in a small cohort) urine S100A12 differentiate between jSLE and IgAV. This is particularly interesting since, based on clinical and available laboratory findings, IgAV can be difficult to differentiate from jSLE in early disease stages. Juvenile-onset SLE can be associated with cutaneous immune complex vasculitis and some patients with IgAV can be positive for antinuclear (and even anti-dsDNA) antibodies, which may be caused by associations with recent infections in children and young people[5].

While demonstrating promising potential for S100 family proteins as biomarkers for overall disease activity and renal involvement in jSLE, this study is limited by relatively small patient numbers particularly in the IgAV group and the need for longitudinal data. Furthermore, despite the differences seen between active and inactive renal BILAG groups, numbers of patients per group, namely the “active renal disease” group are low and overlap between the study populations is seen. Significant differences do, nonetheless suggest increased serum and urine S100A8/A9 and S100A12 protein levels in jSLE patients with active LN.

**Conclusions**

Data from this study suggest S100 proteins may be suitable, non- or minimally invasive biomarkers for the assessment of overall disease activity or specific organ involvement (renal) in jSLE patients. Further studies are required to see how the levels of S100 proteins change over time in relation to disease activity, on an individual patient basis and assessment of S100 protein levels may be more suited as part of a biomarkers panel, including currently used markers of renal disease such as proteinuria. Such a study would also allow the evaluation of the usefulness of S100 proteins in predicting disease flares as well as what role these proteins play in the pathophysiology of j/SLE

**Figure Legends**

**Figure 1: S100A4, S100A8/A9 and S100A12 concentration in jSLE, Control and IgAV patient serum and urine.** (A) Serum S100A8/A9 levels, (B) urine S100A8/A9 levels, (C) serum S100A12 levels, (D) urine S100A12 levels, (E) serum S100A4 levels and (E) urine S100A4 levels. Data are expressed as the median and are analysed using Kruskal-Wallis test with Dunn’s multiple comparisons test; logarithmic (Log10) Y axis. \*P<0.05 and \*\*\*P<0.001.

**Figure 2: S100A4, S100A8/A9 and S100A12 concentration in jSLE patients in relation to renal disease activity.** Levels of S100 proteins were assessed in renally active (renal BILAG A, B and C) and renally inactive/no renal activity (renal BILAG D and E) for (A) Serum S100A8/A9, (B) Urine S100A8/A9, (C) Serum S100A12, (D) urine S100A12, (E) Serum S100A4 and (F) urine S100A4. Data are expressed as the median and are analysed using Mann Whitney U-test; logarithmic (Log10) Y axis. \*P<0.05 and \*\*P<0.01.

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