

A panel of urinary proteins predicts active lupus nephritis and response to rituximab treatment

Short title: Urinary proteins predict renal involvement in SLE

Authors

Jennifer C. Davies¹, Emil Carlsson¹, Angela Midgley¹, Eve MD Smith^{1,2}, Ian N. Bruce^{3,4}, Michael W. Beresford^{1,2,§}, Christian M. Hedrich^{1,2,§} on behalf of the BILAG-BR and MRC MASTERPLANS Consortia

[§] authors contributed equally to the work presented

¹ Department of Women's and Children's Health, Institute of Life Course and Medical Sciences, University of Liverpool, Institute in the Park, Alder Hey Children's NHS Foundation Trust, Hospital, Eaton Road, Liverpool, L12 2AP, UK

² Department of Paediatric Rheumatology, Alder Hey Children's NHS Foundation Trust Hospital, member of Liverpool Health Partners, Liverpool, UK

³ Versus Arthritis Epidemiology Unit, Faculty of Biology Medicine and Health, Manchester Academic Health Sciences Centre, The University of Manchester

⁴ NIHR Manchester Musculoskeletal Biomedical Research Centre, Manchester University NHS Foundation Trust, Manchester Academic Health Science Centre Manchester, UK.

Corresponding author

Prof CM Hedrich, MD, PhD

Department of Women's & Children's Health, University of Liverpool,

Institute in the Park, Alder Hey Children's NHS Foundation Trust

Eaton Road, Liverpool, L12 2AP, UK.

Email: christian.hedrich@liverpool.ac.uk

Tel: +44 151 252 5849

Contributors

Clinical information and serum/urine samples from SLE patients were provided by the following BILAG-BR centres and local CIs: Dr Mohammed Akil, Royal Hallamshire Hospital, Sheffield; Prof Ian N Bruce, NIHR Manchester Musculoskeletal Biomedical Research Centre, Manchester University NHS Foundation Trust, Manchester; Dr Bhaskar Dasgupta, Southend University Hospital, Westcliff-on-Sea, Essex; Dr Shirish Dubey, University Hospital of Coventry and Warwickshire, UK; Dr Nicole Erb, Dudley Group of Hospitals NHS Foundation Trust, West Midlands; Dr Mary Gayed, Heart of England NHS Foundation Trust, Birmingham; Dr Patrick Gordon, King's College Hospital, London; Dr Bridget Griffiths, Freeman Hospital, Newcastle upon Tyne; Dr Peter Hewins, Queen Elizabeth Hospital, Birmingham; Dr Michelle Hui, Countess of Chester Hospital NHS Foundation Trust, Chester; Prof David Isenberg, University College Hospital, London; Dr David Jayne, Addenbrooke's Hospital, Cambridge; Dr Rachel Jeffrey, Northampton General Hospital, Northampton; Dr Munther Khamashta, St Thomas's Hospital, London; Dr Peter Lanyon, Nottingham University Hospitals NHS Trust, Nottingham; Dr Neil McHugh, Royal United Hospitals Bath NHS Foundation Trust, Bath; Dr Athiveer Prabu, Sandwell and West Birmingham Hospitals NHS Trust & Worcestershire Acute Hospitals NHS Trust; Dr Marian Regan, Royal Derby Hospital, Derby; Dr Shireen Shaffu, Leicester Royal Infirmary, Leicester; Dr Robert Stevens, Doncaster and Bassetlaw Hospitals NHS Foundation Trust, Doncaster; Dr Steven Young-Min, Portsmouth Hospitals National Health Service Trust, Portsmouth; Dr Asad Zoma, Hairmyres Hospital, Lanarkshire.

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Authors' contributions

The project was planned by CMH, MWB, EMDS and AM on behalf of the MRC MASTERPLANS Consortium. JCD, EC and AM, carried out the work presented in this article. All authors were involved in data analysis and interpretation, and provided valuable discussion. EC, JCD, AM and CMH wrote the manuscript, and all authors read and approved the final version.

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Conflicts of interest

The authors report no conflict of interest relevant for the study presented. There has not been any financial support or other benefits from commercial sources for the work reported in this manuscript. The authors do not have any financial interests that could create a potential conflict of interest or the appearance of a conflict of interest with regard to the work.

PPI Involvement

The MRC MASTERPLANS Consortium included PPI representatives from early stages of project planning and establishment of the wider consortium. This happened through focus group meetings and personal conversations which established that SLE Patients wish and require treatment that includes no or significantly lower steroid doses alongside target-directed and individualised approaches. Results and conclusions were shared with patient representatives during Consortium and Scientific Advisory meetings throughout the study. Patient representatives were actively included in discussions and had the opportunity to make suggestions. Dissemination of results from MASTERPLANS related projects are guaranteed through close links with the LUPUS UK charity (regular progress reports already publicised). Patients were involved in writing patient summaries.

Abstract

Background: Approximately 30% of patients with systemic lupus erythematosus (SLE) develop lupus nephritis (LN). Presence and/or severity of LN are currently assessed by renal biopsy, but biomarkers in serum or urine samples may provide an avenue for non-invasive routine testing.

Methods: 197 SLE patients and 48 healthy controls were recruited, and urine samples collected. 75 of the SLE patients had active LN and 104 had no or inactive renal disease. Concentrations of lipocalin-like prostaglandin D synthase (LPGDS), transferrin, alpha-1-acid glycoprotein (AGP-1), ceruloplasmin, monocyte chemoattractant protein 1 (MCP-1) and soluble vascular cell adhesion molecule-1 (sVCAM-1) were quantified by MILLIPLEX® Assays using the MAGPIX Luminex platform. Binary logistic regression was conducted to examine whether proteins levels associate with active renal involvement and/or response to rituximab treatment.

Results: Urine levels of transferrin ($p<0.005$), AGP-1 ($p<0.0001$), MCP-1 ($p<0.001$) and sVCAM-1 ($p<0.005$) were significantly higher in SLE patients when compared to healthy controls. Furthermore, levels of transferrin, AGP-1, ceruloplasmin, MCP-1 and sVCAM-1 (all $p<0.0001$) were higher in SLE patients with active LN when compared to patients without active LN. A combination of five urine proteins, namely LPGDS, transferrin, ceruloplasmin, MCP-1 and sVCAM-1 was a good predictor of active LN (AUC 0.898). A combined model of LPGDS, transferrin, AGP-1, ceruloplasmin, MCP-1 and sVCAM-1 predicted response to rituximab treatment at 12 months (AUC 0.818).

Conclusions: Findings support the use of a urinary protein panel to identify active LN and potentially predict response to treatment with rituximab in adult SLE patients. Prospective studies are required to confirm findings.

Introduction

Systemic lupus erythematosus (SLE) is a pathophysiologically complex systemic autoimmune/inflammatory disease that can affect any organ of the human body and cause severe morbidity and mortality (1). The disease predominantly affects females and is highly heterogenic in its clinical presentation, severity and outcomes. Approximately 30% of adult-onset SLE patients develop lupus nephritis (LN) (2-4). Disease presentation and activity varies between individuals, sexes, age groups, and races. However, across cohorts, renal involvement significantly contributes to mortality and morbidity (5, 6).

Proteinuria unreliably indicates active renal disease activity (particularly in children and young people) and can be the result of renal damage (7). Thus, the current gold standard for determining the presence, extent and severity of LN is renal biopsy (7). However, this is an invasive procedure with the potential for complications such as infections, thromboembolisms, and bleeds that sometimes require blood transfusions (8). In addition, renal biopsy only provides a single snapshot of LN at a specific area and time, and is therefore not suitable for routine on-going disease monitoring. Hence, there is an urgent need for identification and validation of reliable biomarkers predicting LN and measuring disease activity.

We previously validated a urinary protein panel in independent international cohorts of patients with juvenile-onset SLE (jSLE)-associated LN (9). The six urinary proteins studied included:

- the enzyme lipocalin-like prostaglandin D synthase (LPGDS) that is involved in prostaglandin production,
- the glycoprotein transferrin that is considered a “negative” acute phase reactant as it is reduced in systemic inflammation,
- the acute phase plasma alpha-1-acid glycoprotein (AGP-1),
- the iron and copper binding plasma protein ceruloplasmin,
- the soluble isoform of the adhesion protein vascular cell adhesion molecule-1 (sVCAM-1),
- and the chemokine monocyte chemoattractant protein 1 (MCP-1).

Our group previously reported that urinary proteins reliably distinguish between jSLE patients with active vs inactive LN, reaching an “excellent” area under the receiver operating characteristic (ROC) curve (AUC) predictive value of 0.949 (9). Whether this panel would also be predictive for LN in an adult cohort has not been investigated to date.

The MRC MASTERPLANS (Maximizing SLE therapeutic Potential by Application of Novel and Systemic Approaches) Consortium aims to identify subsets of SLE patients responding to certain treatment

strategies, as well as identifying biomarkers associated with treatment response to rituximab. In the study presented, we tested a urinary protein panel, including LPGDS, transferrin, AGP-1, ceruloplasmin, MCP-1 and sVCAM-1 for its ability to predict active renal involvement in SLE. Furthermore, associations of altered urine protein expression patterns with response to rituximab treatment were tested.

Patients and methods

Patients and healthy controls – Urine samples were collected from adult-onset SLE patients (n=197) in the UK through centres involved in the British Isles Lupus Assessment Group (BILAG) Biologics Register (BR). The BILAG BR received ethical approval from the Health Research Authority dated 11/09/2009 (IRAS ref. 24407) and written informed consent was obtained in accordance with the declaration of Helsinki. Urine samples were collected at the point of changing to rituximab (from other immunosuppressive/immunomodulatory treatments) for insufficiently controlled disease activity.

Classification of disease activity – Disease activity was classified using the BILAG2004 grading system as follows: A (severe disease), B (moderate disease), C (mild or improving disease), D (inactive disease but previous system involvement) and E (system has never been involved) (10). In this study, grades A or B were considered active involvement, while grades D or E were considered inactive involvement. In order to more reliably differentiate between disease states during data analysis, grade C cases were omitted from analysis. Letter gradings for each nine domains were converted to numerical scores as previously described (11). Additionally, SLE Disease Activity Index (SLEDAI)-2K scores (12) were used, with “Low” disease activity defined as scores between 0-4 and “significant disease activity” as scores ≥ 5 .

Definitions of response to treatment – Criteria for treatment response were defined using BILAG-BR, SLEDAI/Safety of Estrogens in Lupus National Assessment (SELENA SLEDAI) (13) or SLEDAI-2K, and steroid dose at 6- and 12-month endpoints. Patients identified as responders had at least one BILAG (or BILAG 2004) A and/or two or more B scores at baseline, exhibiting a major clinical response (MCR) when all domains reduced to a C or D score, and steroid dose was reduced to ≤ 7.5 mg daily. In addition, SLEDAI/SELENA SLEDAI or SLEDAI-2K score all reduced to ≤ 4 . Showing improvement (SI) was defined as a reduction to no more than one B score, no new organ domain involved, no increase in steroid dose from baseline, and no increase in total SLEDAI/SELENA SLEDAI or SLEDAI-2K score (14). No improvement was used as definition for patients that did not meet the requirements for either MCR or SI.

Detection and quantification of proteins by multiplex Luminex assay – An in-house assay to detect six proteins (LPGDS, transferrin, AGP-1, ceruloplasmin, MCP-1 and sVCAM-1) was designed by Smith et al. (9, 15). Here we analysed the concentrations of these proteins using MILLIPLEX® Multiplex Assays using MAGPIX Luminex xMAP technology. Urines were centrifuged at 2000 rpm for 10 minutes before aliquots made and stored at -80°C . Aliquots were further spun at 2000 rpm for 5 minutes before use in the assay. Each urine was diluted 1:400 for detection of LPGDS, transferrin and ceruloplasmin and used neat for AGP-1, MCP-1 and sVCAM-1. As total proteinuria levels were not available for many of

the samples, as well as proteinuria being an unreliable marker for active nephritis (7), analyte levels were normalised against creatinine (Cr) and are presented as ng/mmol Cr. Cr levels were determined by the routine clinical chemistry lab at Alder Hey Children's NHS Foundation Trust Hospital with the Abbott Enzymatic Creatinine assay using an Abbott Architect Ci8200.

Statistical analysis – Because urinary protein values did not follow a normal distribution non-parametric tests were used to test statistical significance. For cross-sectional analysis between two groups, Mann-Whitney U test was used, and across more than two groups Kruskal-Wallis test with Dunn's multiple comparison post hoc test was applied. Pearson's Chi-square test was used to assess for differences in demographic and clinical factors between binary data and Mann-Whitney U test for categorical data. Bonferroni adjustment was applied to account for multiple testing (e.g. 16 comparisons per cohort if 16 tests done). Spearman's rank correlation coefficient was used to test proteins against continuous clinical variables and between protein levels in urine.

Binary logistic regression was conducted to examine whether protein levels (all log transformed) could predict renal involvement status (outcome: active LN=1; inactive/no LN=0) and response to RTX treatment (outcome: responder=1; non-responder=0) at time x and y. For renal involvement forwards stepwise approach was used with proteins added in order of statistical significance. 'stepAIC' function in R (16) was also used to determine the relative quality of models against each other, and choosing the model with minimum Akaike information criterion (AIC) value. AUC analysis was calculated for individual proteins and models, using their predicted probabilities, with outcome ARI or responder to RTX. Statistical analysis was performed in R version 3.6.0, SPSS (SPSS; IBM Corp., Armonk, NY) version 24 or Graphpad Prism version 8. All corrected p values were considered significant at $p_c < 0.05$. Boxplots show median, IQR and extremes in grey with individual results as black dots.

Results

Clinical and demographic data – The study cohort included a total of 197 adult-onset SLE patients and 48 healthy controls (HCs). While patients were matched for age, women were slightly over-represented in the SLE cohort when compared to the control cohort (91% vs 75%). Furthermore, White Caucasians were over-represented in the control group as compared to the SLE cohort (96% vs 58%). Clinical and demographic information are summarised in Table 1.

Urine protein levels are elevated in SLE patients independent of global disease activity – Individual levels of the six proteins of interest (LPGDS, transferrin, AGP-1, ceruloplasmin, MCP-1, and sVCAM-1) were analysed in urine samples from SLE patients and healthy controls (HC). In relation to HCs, levels of transferrin ($p_c < 0.005$), AGP-1 ($p_c < 0.0001$), MCP-1 ($p_c < 0.001$) and sVCAM-1 ($p_c < 0.005$) were significantly higher in urine samples of SLE patients (Fig. 1). No differences were seen for LPGDS and ceruloplasmin. As a majority of individuals within the control cohort were Caucasians, we performed analyses on white SLE patients only, showing elevated levels of AGP-1 ($p_c < 0.005$) and sVCAM-1 ($p_c < 0.05$) (Fig. S1A). When comparing urine protein expression across ethnicities within the SLE cohort, patients of Asian ethnicity showed higher levels of transferrin, ceruloplasmin, and sVCAM-1 compared with White Caucasians (Fig. S1B). Notably, Asian patients also exhibited a trend to increased renal disease activity (BILAG) when compared to the remaining cohort (Fig. S1C).

Next, we tested whether urinary protein concentrations correlate with global disease activity. Indeed, ceruloplasmin and MCP-1 levels were significantly elevated in patients with “high” (SLEDAI ≥ 15) disease activity when compared individuals with “no/low” or “moderate” (SLEDAI 0-4 and 5-14, respectively) disease activities (all $p_c < 0.05$) (Fig. 2). **When not grouped into these categories, but only looking at calculated composite SLEDAI scores, this was reflected by Pearson’s correlation analysis, however, only resulted in relatively low correlation coefficients (Fig. S2). Gross proteinuria did not correlate with overall disease activity (Fig. 2, S2).**

Urinary protein patterns associate with active renal and musculoskeletal involvement – Next, urinary protein levels were tested for possible associations with disease activity in individual BILAG-BR organ system domains. Activity in two organ domains correlated with altered urine protein levels: renal and musculoskeletal. In patients with active musculoskeletal involvement (BILAG scores A/B), urine levels of transferrin ($p_c < 0.005$), AGP-1 ($p_c < 0.005$) and ceruloplasmin ($p_c < 0.05$) levels were significantly lower when compared to patients with inactive/no musculoskeletal disease (BILAG scores D/E) (Fig. S3). Patients with active renal disease (BILAG A/B; $n = 75$) had significantly higher urine levels of transferrin, AGP-1, ceruloplasmin, MCP-1 and sVCAM-1 (all $p_c < 0.0001$) compared to those with inactive or no LN (BILAG D/E; $n = 104$). Of note, gross proteinuria also delivered statistical significance, but to a lower level as compared to individual proteins ($p_c < 0.005$) (Fig. 3)). As above for systemic disease activity (SLEDAI), **when not stratified by renal disease activity scores (BILAG A-E), and applying Pearson’s correlation analysis, low correlation coefficients resulted for individual proteins as well as gross proteinuria (Fig. S4). Indeed, gross proteinuria and individual (not grouped, see below) panel proteins transferrin, AGP-1 and ceruloplasmin performed comparably.**

Levels of transferrin, AGP-1 and ceruloplasmin were significantly higher in patients with active LN, but lower in those with musculoskeletal disease, we wanted to see if these inverse relationships meant

that patients with renal disease in general would not also have musculoskeletal involvement. However, among patients where active (BILAG A/B) or inactive/no (BILAG D/E) scores for both active renal and musculoskeletal involvement were available, 53 had only musculoskeletal involvement, 25 had only renal involvement, 26 had both, and 19 had neither. Remaining patients had either a BILAG score of C or no score recorded in one or both categories.

Urinary protein patterns, global disease activity and clinical markers of disease -- SLE patients with active LN also had significantly higher SLEDAI-2K and Global BILAG scores (both $p < 0.001$), and were more frequently anti-dsDNA positive ($p < 0.001$) and/or exhibited pathologically low serum C3 and/or C4 levels when compared to SLE patients with inactive/no LN ($p < 0.01$) (Table 1). Urinary protein levels were therefore compared between patients positive vs negative for anti-dsDNA antibodies (Fig. S5,S6), or patients with pathologically low vs normal serum levels of complement C3/C4 (Fig. S7,S8), demonstrating that dsDNA positive patients had higher transferrin ($p_c < 0.005$), AGP-1 ($p_c < 0.01$), ceruloplasmin ($p_c < 0.0001$), MCP-1 ($p_c < 0.0005$) and sVCAM-1 ($p_c < 0.01$) levels. Patients with low C3/C4 had increased urine transferrin ($p_c > 0.05$) and MCP-1 ($p_c > 0.005$).

Assessment of combinations of urinary proteins as predictors of active LN – Combinations of the six urinary proteins detailed above and previously validated within a jSLE cohort (9), were assessed as predictors of LN in the BILAG-BR adult-onset SLE cohort. Using the StepAIC function in R, the optimal protein panel in this analysis was found to be comprised of transferrin, LPGDS, ceruloplasmin, sVCAM-1 and MCP-1, achieving an AUC of 0.898 (Table 2). Inclusion of transferrin, LPGDS and ceruloplasmin in the panel still resulted in an AUC of 0.881, with addition of sVCAM-1 and MCP-1 to the panel resulting in only minor improvements (to 0.895 and subsequently 0.898), while addition of AGP-1 had no further positive effect.

Urinary proteins associate with response to treatment with rituximab in a combined model – In addition to using the protein panel to predict renal involvement in SLE, we aimed to evaluate whether the same panel could be used for predicting future clinical response to rituximab treatment at 6 and 12 months. Potential outcomes at 6/12 months were classified as no improvement, SI, or MCR as defined previously (14). Urinary proteins were included in binary logistic models either alone (crude) or adjusted for confounders known to affect disease outcomes (17) in two models: age, disease duration, renal disease and disease activity, anti-dsDNA antibody positivity, and low complement C3 and/or C4 (model A), or age, disease duration, renal disease and disease activity, and oral steroid dose (model B). Results for individual proteins and combined model are presented in Table 3 and Table S1. Significant differences were seen at 12 months for MCP-1 and ceruloplasmin (SI) in both crude and adjusted models, and transferrin and MCP-1 (MCR) in adjusted model A, while only transferrin reached significance in the crude model and none in adjusted model B. The combined model of proteins achieved AUCs of 0.818 and 0.759 for predicting MCR and SI, respectively, at 12 months, while the corresponding AUCs at 6 months were 0.69 and 0.621, respectively. **Of note, when testing models applying gross proteinuria as single measure, response to RTX treatment cannot be predicted (Table S2).**

Discussion

Effective clinical management of LN is highly dependent on an accurate, timely diagnosis and treatment initiation. While renal biopsy will likely continue to comprise the gold standard method for LN diagnosis and classification in the immediate future, biomarker testing in more easily accessible biological samples, such as urine or serum, provides a complementary method that promises potential for future diagnostic application. To some extent, monitoring of gross urine protein levels together with erythrocyte and leukocyte counts already are used for routine disease monitoring and analysis of treatment response. However, results can be difficult to interpret, and proteinuria only unreliably reflects active renal involvement (LN) (7).

The increased predictive power of protein biomarker panels over any single biomarker has been discussed in the context of LN (18, 19). Our group previously reported that urinary proteins reliably distinguish between jSLE patients with active vs inactive LN, reaching an “excellent” area under the receiver operating characteristic (ROC) curve (AUC) predictive value of 0.949 (9). Selection of the panel components was based on previous studies testing individual urine proteins in patients with or without active LN (20-26). While none of the individual components reached an ‘excellent’ predictive score (defined as AUC>0.9) the combination of at least 2 of these (AGP-1 and CP) performed as a strong predictive test, demonstrating the value of urinary protein panels in LN. As part of the MRC MASTERPLANS Consortium, the aim of this study was to validate a urinary protein panel previously tested in jSLE (9) in adult-onset SLE cohorts and to test associations with response to treatment with rituximab. **This study demonstrated that, while individual proteins included in the panel may perform comparably to gross proteinuria, their combination strongly associates with renal involvement in SLE, therefore promising added value as a non-invasive diagnostic test.**

Proteins tested in this study included LPGDS, transferrin, AGP-1, ceruloplasmin, MCP-1 and sVCAM-1.

LPGDS is a secreted enzyme of the lipocalin family responsible for production of prostaglandin D2. In diabetes mellitus, elevated urinary levels were detected in early stages of kidney injury (27). Of note, in contrast to reports in pediatric cohorts (9, 28), LPGDS was not elevated in the urine of adult-onset SLE patients analysed here. Furthermore, it did not correlate with disease activity or active organ involvement.

Blood plasma proteins transferrin and ceruloplasmin are involved in transfer of iron and copper, respectively. Elevated urinary levels of both proteins have been detected in hypertensive diabetic patients when compared with normotensive diabetics (29), suggesting a potential role detection of diabetic nephropathy. Transferrin levels are increased in individuals with inflammation and associated anaemia, including some SLE patients (30). In this study, transferrin, but not ceruloplasmin levels were increased in the urine of SLE patients when compared to controls. While urine transferrin did not

correlate with global disease activity in SLE, ceruloplasmin levels were (only) increased in SLE patients with high disease activity. Both proteins were elevated in the urine of patients with active LN when compared to SLE patients with inactive/no renal disease. The relatively high molecular weights of transferrin and ceruloplasmin (76 and 151 kDa, respectively) may in part explain why urine levels associate with kidney damage.

AGP-1 is released mainly from the liver in response to acute inflammation and was suggested to be an early marker of LN (23). Here, AGP-1 was elevated in the urine of SLE patients as compared to controls independent of disease activity, and, within the SLE cohort, correlated with active LN. This observation may be in part due to increased overall AGP-1 production, but also a result of AGP-1 producing immune cells infiltrating the kidneys during LN.

MCP-1 is a member of the C-C chemokine family that regulates migration and infiltration of monocytes during states of inflammation (31). In the context of LN, increased glomerular expression of MCP-1 correlates with poor renal prognosis in paediatric patients (32), and members of our group previously reported urinary levels of the protein to be elevated with active LN in jSLE (24). In adult-onset SLE patients included in this study, urinary levels of MCP-1 were elevated when compared to controls and reflected disease activity as measured by SLEDAI scored. Furthermore, MCP-1 was elevated in SLE patients with active LN as compared to patients with inactive/no renal disease. MCP-1 expression and secretion from renal cells are upregulated in response to inflammatory cytokines, and as MCP-1 facilitates recruitment of monocytes into kidneys during inflammation (33), which may contribute to renal damage.

VCAM-1 is an adhesion molecule that interacts with integrins to modulate intracellular contact between leukocytes and endothelial cells (34). In a mouse lupus model, glomerular expression of VCAM-1 correlated with severity of LN (35). Serum (36) and urinary (21) sVCAM-1 correlates with overall SLE disease activity and has been discussed to be involved with increased immune cell infiltration to inflamed kidneys. In this study, sVCAM-1 was elevated in the urine of SLE patients, did not correlate with disease activity, but correlated with active renal disease (LN). Membrane-bound VCAM-1 is converted into its soluble form by metalloproteinase-mediated cleavage, a process that in vitro can be accelerated by PMA-induced inflammation (37). Thus, shedding of VCAM-1 in inflamed kidneys may result in increased urine levels.

Consistent with previous reports from our group, the panel format of urinary proteins performed noticeably better when compared to any individual marker protein tested. **Notably, individual proteins within the panel performed only slightly better when compared to gross proteinuria, while their combination promises significant added value as a non-invasive test.** The optimal panel in this study

comprised transferrin, LPGDS, ceruloplasmin, sVCAM-1 and MCP-1, achieving an AUC of 0.898, which is considered “good”. In contrast to previous results in jSLE cohorts (9), the addition of AGP-1 in the panel had no significant effect on the panel accuracy. This may reflect biological differences between jSLE and adult-onset SLE, or possibly differences in the prevalence of relevant comorbidities in adults, such as increased levels of cardiovascular disease, metabolic conditions, etc., and demonstrates the need for independent validation and optimisation of any proposed biomarker panel in relevant cohorts.

As ethnicity may affect all disease severity, gross proteinuria and/or expression of individual proteins (38, 39), we compared urine protein expression across ethnicities. Indeed, Asian patients exhibited higher urine levels of transferrin, ceruloplasmin and sVCAM-1 when compared to the remaining SLE cohort. Whether this is due to ethnicity rather than LN activity remains somewhat unclear. However, Asian SLE patients also exhibited a trend towards higher renal disease activity (BILAG) when compared to the remaining LN cohort, suggesting that this may (at least partially) be responsible for differences in protein concentrations.

The MRC MASTERPLANS Consortium aims at patient stratification to improve personalized approached to treatment. Thus, the predictive value of urinary proteins was tested for response to treatment with rituximab. Binary regression models adjusted for confounders (age, disease duration, renal disease and disease activity, anti-dsDNA antibody positivity, and low complement C3 and/or C4 (model A), or age, disease duration, renal disease and disease activity, and oral steroid dose (model B)) suggested predictive value for MCR to rituximab treatment at 12 months (AUC 0.818). However, findings must be confirmed in larger, independent studies with a randomised and prospective design that includes longitudinal sampling.

While delivering promising results, limitations of the study also have to be considered. The control group is smaller than the SLE cohort and lacks diversity with 96% White Caucasian individuals as compared to 58% in the SLE patient group, but as the majority of analyses were performed between subgroups of SLE patients, this would not impact the main findings of this study. Though accessing a large national cohort study (BILAG-BR), subgroup analyses are somewhat limited by a relatively small sample size. Though reliable in nature, some datasets were incomplete, which required us to exclude individual samples/data from analyses. The focus of this study was to identify active LN in SLE patients using a single patient sample at baseline. While the panel was shown to be effective in this regard, it is not clear whether it reflects disease activity in individual patients over time and in response to treatment. This needs to be validated in longitudinal follow up studies, which is an area of ongoing research.

The SLE cohort tested here contained a relatively large proportion of patients negative for anti-dsDNA antibodies. The presence of anti-dsDNA antibodies is widely regarded as a marker for “classic” SLE (40), but the proportion of patients without these antibodies vary significantly between studies. Although anti-dsDNA positive patients displayed elevated levels of proteins tested here, subgroup analysis performed on anti-dsDNA positive vs anti-dsDNA negative patients showed that both groups largely maintained comparable urinary protein expression patterns segregating between patients with active vs inactive/no LN. Significant differences between patients with active LN vs patients without active LN were only seen in urinary transferrin, AGP-1, ceruloplasmin and MCP-1 among anti-dsDNA positive patients, while significant differences were seen in urinary ceruloplasmin, MCP-1 and sVCAM-1 among anti-dsDNA negative patients. For subgroup analysis on patients displaying low or normal levels of C3 and/or C4, significant differences between LN and non-LN patients were seen in urinary transferrin, AGP-1, ceruloplasmin and MCP-1 among patients low in C3/C4, while significant differences in transferrin, ceruloplasmin, MCP-1 and sVCAM-1 were seen in patients with normal C3/C4. Why these small differences occur remain unclear.

While the main objective of this study was to evaluate urinary levels of proteins in the context of LN, associations between altered protein levels with musculoskeletal disease were noted. Transferrin, AGP-1, and ceruloplasmin all showed significantly lower urine levels in SLE patients with musculoskeletal manifestations as compared to patients without active musculoskeletal disease. Of note, this was the opposite pattern observed in patients with LN. Of the 123 patients with clearly defined presence or absence of both renal and musculoskeletal involvement, 51 (41%) had renal involvement, but among the 79 that had musculoskeletal involvement, only 26 (33%) also had renal involvement. The precise mechanisms behind these differences are not yet known and warrants further study. As this study was designed to test urinary proteins in the context of LN and their predictive value for treatment response to rituximab, only urine samples were assayed and, while interesting, the question of why patients with musculoskeletal involvement may exhibit altered urinary protein expression was beyond the scope of this project and remains to be addressed in the future.

Conclusions

A urinary protein panel comprised of LPGDS, transferrin, AGP-1, ceruloplasmin, MCP-1 and sVCAM-1 predicts active LN in a large national cohort of patients with adult-onset SLE. Binary logistic regression models adjusted for confounders predict response to treatment with rituximab. Findings must be

confirmed in independent large, prospectively designed longitudinal studies before clinical application can be recommended.

Figure Legends

Figure 1: Comparison of protein levels in the urine of SLE patients and healthy controls. Analysis of concentrations of LPGDS (A), transferrin (B), AGP-1 (C), ceruloplasmin (D), MCP-1 (E) and sVCAM-1 (F) in SLE patients and healthy controls. Boxplots show median, IQR and extremes in grey with individual results as black dots. Mann-Whitney U tests with Bonferroni adjustment to account for multiple testing were performed to compare differences in urinary protein levels between groups. Corrected p-values (p_c) are reported.

Figure 2: Comparison of protein levels in the urine of SLE patients grouped by disease severity. Analysis of concentrations of LPGDS (A), transferrin (B), AGP-1 (C), ceruloplasmin (D), MCP-1 (E), sVCAM-1 (F) and proteinuria (G) in SLE patients grouped by disease severity according to SLEDAI score. No-mild was defined as a score of 0-4, Moderate as a score of 5-14, and High-very high as a score of 15 and above. Boxplots show median, IQR and extremes in grey with individual results as black dots. Kruskal-Wallis test with Dunn's multiple comparison post hoc test was used to compare differences in urinary protein levels between groups. Corrected p-values (p_c) are reported.

Figure 3: Protein levels in the urine of SLE patients with active renal disease. Analysis of concentrations of LPGDS (A), transferrin (B), AGP-1 (C), ceruloplasmin (D), MCP-1 (E) and sVCAM-1 (F) in SLE patients based on presence of active renal disease. Boxplots show median, IQR and extremes in grey with individual results as black dots. Mann-Whitney U tests with Bonferroni adjustment to account for multiple testing were performed to compare differences in urinary protein levels between groups. Corrected p-values (p_c) are reported.

Figure S1: Comparison of urine protein levels in SLE patients of different ethnicities. (A) Analysis of concentrations of LPGDS, transferrin, AGP-1, ceruloplasmin, MCP-1 and sVCAM-1 in Caucasian SLE patients and healthy controls. Boxplots show median, IQR with individual values in black dots. Mann-Whitney U tests with Bonferroni adjustment to account for multiple testing were performed to compare differences in urinary protein levels between groups. (B) Analysis of concentrations of LPGDS, transferrin, AGP-1, ceruloplasmin, MCP-1 and sVCAM-1 in SLE patients grouped by ethnicity (White Caucasian, Asian, Black, "Other"). Boxplots show median, IQR with individual values in black dots. Kruskal-Wallis test with Dunn's multiple comparison post hoc test was used to compare differences in urinary protein levels between groups. Corrected p-values (p_c) are reported. (C) Comparison of renal BILAG scores between ethnicities with median scores marked.

Figure S2: Correlations between urinary proteins and global disease severity (SLEDAI). Correlation between SLEDAI disease activity scores and LPGDS, transferrin, AGP-1, ceruloplasmin, MCP-1, sVCAM-1 and gross proteinuria. Pearson's correlation coefficients (r) are shown above each graph.

Figure S3: Protein levels in the urine of SLE patients with musculoskeletal involvement. Analysis of concentrations of LPGDS (A), transferrin (B), AGP-1 (C), ceruloplasmin (D), MCP-1 (E) and sVCAM-1 (F) in SLE patients based on musculoskeletal involvement. Boxplots show median, IQR and extremes in

grey with individual results as black dots. Mann-Whitney U tests with Bonferroni adjustment to account for multiple testing were performed to compare differences in urinary protein levels between groups. Corrected p-values (p_c) are reported.

Figure S4: Correlations between urinary proteins and renal disease severity (BILAG). Correlations between BILAG renal score and LPGDS, transferrin, AGP-1, ceruloplasmin, MCP-1, sVCAM-1 and proteinuria. Pearson's correlation coefficients (r) shown above each graph.

Figure S5: Comparison of protein levels in the urine of SLE patients segregated for presence of anti-dsDNA. Analysis of concentrations of LPGDS (A), transferrin (B), AGP-1 (C), ceruloplasmin (D), MCP-1 (E) and sVCAM-1 (F) in SLE patients divided between patients with or without the presence of anti-dsDNA. Boxplots show median, IQR and extremes in grey with individual results as black dots. Mann-Whitney U tests with Bonferroni adjustment to account for multiple testing were performed to compare differences in urinary protein levels between groups. Corrected p-values (p_c) are reported.

Figure S6: Subgroup comparison of protein levels in the urine of SLE patients segregated for renal disease and anti-dsDNA positivity. Analysis of concentrations of LPGDS, transferrin, AGP-1, ceruloplasmin, MCP-1 and sVCAM-1 in SLE patients positive (A) or negative (B) for anti-dsDNA antibodies based on active renal involvement. Boxplots show median, IQR with individual values in black dots. Mann-Whitney U tests with Bonferroni adjustment to account for multiple testing were performed to compare differences in urinary protein levels between groups. Corrected p-values (p_c) are reported.

Figure S7: Comparison of protein levels in the urine of SLE patients segregated for low levels of C3/C4. Analysis of concentrations of LPGDS (A), transferrin (B), AGP-1 (C), ceruloplasmin (D), MCP-1 (E) and sVCAM-1 (F) in SLE patients based on low levels of C3/C4. Boxplots show median, IQR and extremes in grey with individual results as black dots. Mann-Whitney U tests with Bonferroni adjustment to account for multiple testing were performed to compare differences in urinary protein levels between groups. Corrected p-values (p_c) are reported.

Figure S8: Subgroup comparison of biomarker levels in the urine of SLE patients segregated for renal disease and low C3/C4 levels. Analysis of concentrations of LPGDS, transferrin, AGP-1, ceruloplasmin, MCP-1 and sVCAM-1 in SLE patients with low (A) or normal (B) levels of C3 and/or C4 based on active renal involvement. Boxplots show median, IQR with individual values in black dots. Mann-Whitney U tests with Bonferroni adjustment to account for multiple testing were performed to compare differences in urinary protein levels between groups. Corrected p-values (p_c) are reported.

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