International validation of a urinary biomarker panel for identification of active lupus nephritis in children

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Abstract

Introduction: Conventional markers of Juvenile-onset Systemic Lupus Erythematosus (JSLE) disease activity fail to adequately identify Lupus Nephritis (LN). While individual novel urine biomarkers are good at detecting LN flares, biomarker panels may improve diagnostic accuracy. The aim of this study was to assess the performance of a biomarker panel to identify active LN in two international JSLE Cohorts. **Methods:** Novel urinary biomarkers; vascular cell adhesion molecule-1 (VCAM-1), monocyte chemoattractant protein 1 (MCP-1), lipocalin like prostaglandin D synthase (LPGDS), transferrin, ceruloplasmin, alpha-1-acid glycoprotein (AGP) and neutrophil gelatinase associated lipocalin (NGAL) were quantified in in a cross-sectional study including participants of the UK JSLE Cohort Study (Cohort 1) and validated within the Einstein Lupus Cohort (Cohort 2). Binary logistic regression modeling and receiver operating curve analysis were used to identify and assess combinations of biomarkers for diagnostic accuracy. **Results:** 91 JSLE patients were recruited across both cohorts, 31 (34%) had active LN and 60 (66%) had no LN. Urinary AGP, ceruloplasmin, VCAM-1, MCP-1 and LPGDS levels were significantly higher in active LN compared to non-LN patients (all corrected p-value (p_c)<0.05) across both cohorts. Urinary transferrin also differed between patient groups in Cohort 2 ($p_c=0.001$). Within Cohort 1, the optimal biomarker panel included AGP, ceruloplasmin, LPGDS and transferrin (AUC 0.920 for active LN identification). These results were validated in Cohort 2, with the same markers resulting in the optimal urine biomarker panel (AUC 0.991). **Conclusion:** In two international JSLE Cohorts, urinary AGP, ceruloplasmin, LPGDS and TF demonstrate an 'excellent' ability for accurately identifying active LN in children.

Key works: Lupus Nephritis, urine biomarkers, glomerulonephritis, BILAG, systemic lupus erythematosus

Introduction

Juvenile-onset systemic lupus erythematosus (JSLE) is a life-threatening multi-system autoimmune disease that displays a more aggressive course than adult onset SLE [1-3]. More renal manifestations occur in childhood, with up to 80% of JSLE patients developing lupus nephritis (LN) within the first 5 years from diagnosis [1,4-9]. LN is characterised by a relapsing and remitting course, requiring close surveillance and prompt treatment to prevent renal damage. Worldwide, the 5-year renal survival rate in children with LN has been shown to vary between 44-94% [10-13].

Renal histology is the gold standard for diagnosing and predicating renal prognosis in LN, but only provides a snapshot of a discrete area of the kidney, and is rarely repeated for monitoring purposes due to its invasive nature [14,15]. Composite disease activity scores such as the British Isles Lupus Assessment Group (BILAG) score or the Systemic Lupus Erythematosus Disease Activity Index (SELENA SLEDAI), and a number of traditional clinical biomarkers can be used to assess JSLE disease activity; however their role in monitoring LN within the clinic is limited [16-19].

Over recent years, numerous individual novel urinary biomarkers have been investigated for monitoring LN disease activity, outperforming both traditional and novel serum biomarkers, including monocyte chemoattractant protein-1 (MCP-1), neutrophil gelatinase associated lipocalin 1 (NGAL), vascular cell adhesion molecule-

1 (VCAM-1) and tumour necrosis like weak inducer of apoptosis (TWEAK) [20-26]. Using a proteomic approach, urinary transferrin, ceruloplasmin, lipocalin-type prostaglandin D synthase (LPGDS), AGP, albumin and albumin fragments have been shown to differentiate between children with active LN and no LN [27]. When assessed longitudinally, LPGDS, AGP and transferrin were all elevated up to 3 months before LN flare [27].

No individual urine biomarker has achieved an 'excellent' predictive value (AUC>0.9) to date. Combining urinary biomarkers in a 'biomarker panel' has been shown to improve the ability to predict renal function loss in a combined pediatric / adult SLE cohort LN [28] and relate to LN histological features [29] and activity [30]. This study therefore aimed to build on previous work [22,25-27,31-33] by exploring the most promising candidate urinary biomarkers to date used in combination, namely VCAM-1, MCP-1, NGAL, ceruloplasmin, transferrin, LPGDS, and AGP in a pediatric cohort from the UK (UK JSLE Cohort Study), to assess which novel biomarker combinations can improve the identification of active LN. Since the JSLE phenotype and disease severity varies by ethnicity and race [2,4,34], we sought to confirm our results in a validation cohort from the US (Einstein Lupus Cohort, ELC) [35] to identify a urinary biomarker panel which is internationally applicable. Such a transatlantic comparison of a biomarker panel provides considerable strength to this study and the validation of this panel.

Methods

Patients

This study was based on two cross-sectional JSLE Cohorts: the exploratory UK JSLE Cohort [1], which included all recruited patients from Alder Hey Children's NHS Foundation Trust, Liverpool, and Great Ormond Street NHS Hospital for Children, London, UK. The validation cohort included ELC patients who were followed regularly at lupus clinics at the Children's Hospital at Montefiore, Bronx, NY, USA [35]. In both cohorts, urine samples were collected during routine clinical care together with detailed demographic data, self reported ethnicity / race data, clinical laboratory results and medication information. Disease activity data was determined using the BILAG2004 disease activity score [36,37]. Eligible patients were diagnosed with JSLE prior to 16 years of age and met ≥4 of the revised American College of Rheumatology (ACR) SLE classification criteria [38]. Patients were excluded if they had a urinary tract infection or if no urine samples had been collected.

Compliance with Ethical Standards

The research was carried out in accordance with the declaration of Helsinki. Patient assent / consent and parental consent was obtained to participate in the studies, and full ethical approvals were in place from the National Research Ethics Service North West, Liverpool East, UK (reference 06/Q1502/77) and the Institutional Review Board at Einstein-Montefiore (IRB 2000-154). 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.

Renal disease activity classification

Patients were categorized according to the renal domain of BILAG2004 disease activity score, defined as follows; BILAG2004 grade A / B: severe, moderate disease respectively, grade D: inactive disease but previous system involvement, grade E: system has never been involved [37]. The composite renal BILAG score consisted of six items, including proteinuria (defined in terms of urine dipstick or urine protein / albumin creatinine ratio or 24 hour protein levels), deteriorating renal function (based on plasma creatinine and GFR), presence of active urinary sediment, hypertension, nephrotic syndrome and histological evidence of active nephritis in the previous three months, with different test score cut-offs relating to the different disease activity categories. In both cohorts, all active LN patients had biopsy-proven LN during their disease course. Renal disease activity was therefore defined as having a renal BILAG2004 score of A or B with previous histological confirmation of LN. Non-LN was defined by a renal BILAG2004 score of D or E. This study sought to identify biomarkers that differentiate between the binary outcome of active vs. no LN, therefore renal BILAG2004 C patients (where a patient had mild or improving renal disease) were excluded.

Urine sample selection

In Cohort 1, when more than one patient urine sample had been collected, urine biomarkers were quantified in a single sample for inclusion within this study (cross-sectional approach). An active LN sample was chosen for inclusion where available, to allow as many active LN patients as possible to contribute to the study. If a patient contributed an in-active LN sample, then the first sample collected with adequate aliquots for quantification of the whole biomarker panel was included. In Cohort 2, 23/30 study patients had an active LN sample available and 14/30 had in-active LN

(non-LN) sample available. Urine biomarker levels were quantified in all samples, however, 16 of these active-LN and all 14 non-LN samples contributed to the cross-sectional analysis, to provide similar patient numbers per group. The other 7 active-LN samples were subsequently included in analyses comparing urine biomarker concentrations in biopsy vs renal BILAG defined active LN.

Extra-renal disease activity classification

To allow assessment of biomarker levels according to whether extra-renal JSLE disease activity was present or not, patients were subdivided further as having 'any active extra-renal involvement' if they had a BILAG2004 of A or B in any of the remaining domains (constitutional, mucocutaneous, neuropsychiatric, musculoskeletal, cardiorespiratory, gastrointestinal, ophthalmic or haematological) or 'no extra-renal involvement' if they had a BILAG2004 score of D or E in all extra-renal domains. Biomarker levels were therefore compared in active / non-LN patients with and without extra-renal involvement.

Laboratory techniques

Urine dipstick and / or microscopy and culture excluded infection. Samples were centrifuged at 2000 rpm for 10 minutes. Urine supernatant was aliquoted and stored at -80^{o}C. Pre-coated enzyme-linked immunosorbent assay (ELISA) kits were used for quantification of urinary Ceruloplasmin (St Charles, Assay Pro, USA), transferrin (GenWay, San Diego, USA), LPGDS (BioVendor, Brno, Czech Republic), AGP and MCP-1 (R&D Systems Ltd, Minneapolis, USA). An R&D systems duo-kit (R&D Systems Ltd, Minneapolis, USA) was used to quantify urinary VCAM-1 following internal validation (95% spike recovery, 104% linearity of dilution, co-efficients of

inter / intra-assay variability 5.1 and 7.5% respectively). The Ceruloplasmin, LPGDS, MCP-1 and AGP assays are commercially validated for use in urine and used as per manufacturers instructions. Urinary NGAL and creatinine concentrations were measured using Abbott Architect assays (Abbott Laboratories, Texas, USA). All biomarker results were standardised for urinary creatinine (Cr) concentration and presented in units per milligram creatinine (mgCr).

Statistical analysis

Summary statistics for demographics (age at diagnosis, current age, gender, ethnicity), baseline clinical data (medication use and laboratory parameters) and biomarker data (Ceruloplasmin, Transferrin, LPGDS, MCP-1, VCAM-1, AGP and NGAL) were provided in terms of median values and interquartile ranges (IQR). Univariate logistic regression (quantitative data) and Pearson's chi-square test (binary data) were used to assess for differences in demographic and clinical factors between different patient groups. Due to the number of factors explored, a Bonferroni adjustment was applied to account for multiple testing (16 comparisons per cohort).

Mann Whitney U tests with Bonferroni adjustments were used to compare biomarker concentrations between active and non-LN patients (7 comparisons). Correlation between the individual urine biomarkers was assessed using Spearman's rank correlation tests. Grading of correlataion co-efficients (r) can vary but for the purposes of this study was defined as 0.2-0.3 = weak / little correlation, 0.3-0.7 = moderate, 0.7-1.0 = strong correlation [39]. A binary logistic regression model was fitted to assess for association between a combination of biomarkers and LN status (outcome: LN active=1; non-LN JSLE=0). All novel biomarkers (log-transformed)

were included in an initial model and the ‘stepAIC’ function in R [40] applied to select a final model. This function compares models based on all possible combinations of biomarkers and chooses the model with the minimum AIC (Akaike Information Criterion) value. The AIC is a measure of the relative quality of a model relative to each of the other models, with a lower value meaning better quality.The AUC for the final model was calculated. Each of the remaining novel biomarkers were then added back into the final model in turn, in order of statistical significance according to the original model including all novel biomarkers, and the AUC for each updated model calculated. This allowed exploration of the effect of each biomarker on the model’s AUC, as well as an assessment of which combination of biomarkers led to the optimal AUC. This final process was repeated in the ELC validation cohort in order to determine whether the findings could be replicated. The data was then pooled to identify the optimal combined model. AUC values of 1.0–0.9, 0.9–0.8, 0.8–0.7, 0.7–0.6, 0.6–0.5 were considered “excellent, good, fair, poor and fail” respectively [41].

To assess the renal specificity of the urine biomarkers and whether biomarker levels vary according to whether extra-renal JSLE disease activity is present, biomarker levels in patients with ‘any active extra-renal involvement’ were compared to those with ‘no extra-renal involvement’ (Mann Whitney U tests with a Bonferroni adjustment for the 7 biomarkers examined). Similarly, when comparing urinary biomarker levels in patients where a diagnosis of LN was made on the basis of recent renal biopsy results versus BILAG defined nephritis alone, Bonferroni adjusted Mann Whitney U tests were also used. The ability of traditional biomarkers to identify active LN was investigated using binary logistic regression models for each / a

combination of biomarkers (log-transformed) and LN status, and the AUC calculated.

Data analysis was undertaken using Statistics Package for Social Sciences (SPSS Ltd, USA) version 21.0 and R version 3.1.1 [40]. Graphical illustrations were generated using GraphPad Prism version 6.0. Where Bonferroni adjustment was made to account for multiple testing, the Bonferroni corrected p-value, p_c is reported.

Results

Cohort 1 - Exploratory Cohort (UK JSLE Cohort Study)

Clinical and demographic data

The UK JSLE study cohort consisted of 61 JSLE patients, 15 (25%) were classed as JSLE active LN (2/15 renal BILAG score=A, 13/15=B) and 46 (75%) as JSLE non-LN patients (27/46 renal BILAG score=D, 19/46=E). Active and non-LN JSLE patients had a median age of 15.8 [14.8-17.1] and 15.4 [13.8-17.5] years respectively, with disease duration of 2.8 [0.7-3.9] and 2.4 [0.8-4.8] years at the time of biomarker analysis. Females comprised 86.7% of the active LN patients and 62.5% of the non-LN patients. There was no difference in ethnicity between patient groups. All JSLE patients had a median of 5 ACR classification criteria at diagnosis [IQR 4-7]. All active LN patients had biopsy proven LN during their disease course, with the majority having International Society of Nephrology/Renal Pathology Society 2003 (ISN/RPS) class III (59%) or IV (27%) LN. Class II (7%) and mixed class II/V (7%) LN was seen in the remaining patients (see Table 1).

More active LN patients had received Rituximab ($p_c<0.05$) but use of other medications did not differ significantly between the patient groups. Of the laboratory parameters investigated, urine albumin-to-creatinine ratio (UACR) and erythrocyte sedimentation rate (ESR) were significantly higher in the active LN patients (all $p_c<0.05$) (see Table 1).

Novel urinary biomarkers

Figure 1 depicts the distribution of novel urinary biomarker concentrations standardized to urinary creatinine in patients with active LN and no LN. Patients with active LN had significantly higher urinary concentrations of AGP, ceruloplasmin, VCAM-1, MCP-1, and LPGDS than non-LN patients (all $p_c<0.05$, see Figure 1 and Online Resource 1). Urinary Transferrin and NGAL concentrations did not differ significantly between the patient groups (p_c = 0.06 and *1.0* respectively, see Figure 1 and Online Resource 1). LPGDS and AGP were strongly correlated ($r = 0.71$). All other biomarker combinations were moderately correlated ($r = 0.3$-0.7) except LPGDS + TF and MCP-1 + TF which were weakly correlated ($r < 0.3$, see on-line resource 2 for further details).

Urine biomarker levels did not differ between non-LN patients who had previous LN (renal-BILAG score D) and those with no previous renal involvement (renal-BILAG score E, all $p_c>0.05$). Similarly, there was no difference between patients with severe or moderate active LN (renal-BILAG score A / B respectively, all $p_c>0.05$, see on-line resource 3). There was also no significant difference in urinary biomarker levels depending on the presence or absence of extra-renal involvement (see Figure 2).

On fitting a binary logistic regression model including all novel biomarkers, and applying the 'stepAIC' function in R [40], the final model included both AGP and Ceruoplasmin (see Table 2). AUC for this final model was 0.88. On addition of LPGDS, the AUC increased to 0.90, increasing further to 0.92 on addition of transferrin. Addition of VCAM-1 and MCP-1 into the model however, did not increase the AUC (see Table 3).

Cohort 2 - Validation Cohort (Einstein Lupus Cohort)

Clinical and demographic data

The validation cohort consisted of 30 JSLE patients, 16 (53%) were classed as active LN (11/16 renal BILAG score=A, 5/16=B) and 14 (47%) as non-LN JSLE patients (6/16 renal BILAG score=D, 8/16=E). Active and non-LN JSLE patients had a median age of 15 and 18 years respectively, with a disease duration of 3.1 and 1.7 years at the time of biomarker analysis. One hundred percent of the active LN and 71% of the non-LN patients were female. Both JSLE patient groups had a median of 5 ACR classification criteria at diagnosis. ELC patients were largely African / African American (53%) and Hispanic (43%), wheras UK JSLE Cohort patients were predominatly Caucasian (41%) and Indian (23%). All active LN patients had biopsy proven LN during their disease course, with the ISN/RPS 2003 classess as follows; class III = 19%, class IV = 19%, class V = 31%, mixed class III/V = 31%. Both groups of patients had a median of 5 ACR classification criteria at diagnosis. Active LN and non-LN patients differed significantly in terms of their UACR and use of angiotensin converting enzyme inhibitors (ACEi) / angiotensin 2 blockers (AT2) (both $p<0.05$, see Table 1).

Novel urine biomarkers

Figure 1 shows the distribution of novel urinary biomarker concentrations in Cohort 2, relative to Cohort 1 patients. Patients with active LN had significantly higher urinary concentrations of AGP, ceruloplasmin, LPGDS, TF, MCP-1 and VCAM-1 than non-LN patients (all $p_c<0.05$). NGAL levels did not differ between patient groups in either cohort ($p_c=1.0$). CP and MCP-1, AGP, TF were all strongly correlated. LPGDS was also strongly correlated with AGP and VCAM-1. AGP was strongly correlated with VCAM-1 and TF (all $r > 0.7$). All other biomarker combinations were moderately correlated ($r = 0.3$-0.7, see on-line resource 2 for further details).

A binary logistic regression model was fitted with Cohort 2 data, adding them in a stepwise manner one at a time in the same order as was done for Cohort 1. The model including AGP, ceruoplasmin, LPGDS and transferrin again produced the optimal AUC (0.991). As a combination of biomarkers led to excellent identification of active LN in both cohorts, AUCs were also calculated for both cohort datasets combined (see Table 3). A combined Cohort 1 and Cohort 2 model, including AGP, Ceruoplasmin, LPGDS and Transferrin again gave excellent AUC (0.949), however adding VCAM-1 slightly improved the AUC further (0.952). The receiver-operating curve (ROC) generated by this optimal Cohort 1 and 2 model is shown in Figure 3.

Urine biomarker concentrations in biopsy vs renal BILAG defined active LN

Urine biomarker levels from twelve samples from Cohort 2 patient which were taken at the time of or within 6 weeks of renal biopsy, were compared with eleven patient

samples with a current composite renal BILAG score-based diagnosis of active LN (but a previous history of having had biopsy defined active LN). Urinary AGP, CP, LPGDS, TF, MCP-1 and VCAM-1 levels did not differ significantly between the two groups of active LN patients (all *pc = 1.0*; see Figure 4). Cohort 1 urine samples were not available close to the time of renal biopsy, therefore comparable groups were not available for inclusion in these analyses. The study was underpowered to assess for differences in any of the urinary biomarkers according to ISN/RPS 2003 sub-class.

Ability of traditional biomarkers to identify active LN

Traditional biomarkers which do not contribute to the composite renal BILAG score were assessed for their ability to identify active LN. ESR was the best traditonal biomarker with a fair AUC of 0.796 (ESR only measured routinely within cohort 1). C3 and dsDNA showed a poor ability to identify active LN in both cohorts (AUC's from 0.617-0.645). C4 performed worst with an AUC or 0.593 and 0.482 in cohort 1 and 2 respectively. Inclusion of all traditional biomarkers together in a regression model did not improve the AUC. Addition of ESR - the best traditional biomarker - to the optimal UK novel biomarker combination, including AGP, LPGDS, Transferrin and Ceruloplasmin, did not improve the AUC further (AUC 0.910, see Table 4).

Discussion

To optimise effective management of LN, readily available and easily measured biomarkers are urgently needed within clinical practice. Early diagnosis and prompt treatment of LN can improve long-term renal survival [18]. The invasive nature of renal biopsy limits its clinical utility, especially in childhood. By simultaneously measuring urinary AGP, ceruloplasmin, VCAM-1, transferrin, LPGDS, MCP-1 and

NGAL at a single patient visit in two ethnicially diverse cohorts of JSLE patients, the aim of this study was to derive and internationally validate a biomarker panel which could improve identification of active LN, over and above individual biomarkers. Across both cohorts we have demonstrated an optimal urine biomarker combination including AGP, Ceruoplasmin, LPGDS and Transferrin with excellent AUC values for active LN identification (0.920 and 0.991 for Cohorts 1 and 2 respectively). Furthermore, the presence of extra-renal disease activity does not appear to influence the accuracy of these urine biomarkers. This is therefore the first LN urine biomarker panel study to include a exploratory and validation cohort, providing a firm foundation for future development of a clinical urine biomarker panel test.

Previous studies complementing our work have focused on identification of biomarker combinations reflective of LN histological subtypes in patients with biopsy proven LN. Brunner et al investigated 28 childhood onset and 48 adult-onset SLE patients, assessing biomarker combinations differentiating biopsy defined activity, chronicity or membranous LN in samples taken within 2 months of biopsy. The best predictive ability detected was for LN activity, when MCP-1, AGP, ceruloplasmin and urine protein to creatinine ratio were considered together (AUC 0.850) [29]. Within the UK JSLE Cohort and the ELC, we have demonstrated stronger AUC values (0.920 and 0.991) for identification of active LN with the combination of urinary AGP, ceruloplasmin, LPGDS and transferrin. This supports the importance of a combination-approach to urinary biomarkers in LN, in these JSLE cohorts. In our present study, when the results from both the UK and ELC are pooled, VCAM-1 adds to the diagnostic ability of the above biomarker panel. This indicates that further investigation of the role of VCAM-1 in combination with other biomarkers for discriminating active LN in children is

required. The UK JSLE Cohort was predominately Caucasian and Indian, whereas the ELC cohort was mainly African American and Hispanic. Notably, African and African American patients often have more severe kidney involvement in SLE [4,34,42]. Interestingly within our study, the optimal biomarker panel performed even better in the validation ELC than the exploratory UK JSLE Cohort.

More recently, Brunner et al have looked at additional biomarkers in samples taken at the time of biopsy from 47 children with with ISN/RPS class II-V LN. They demonstrated NGAL, MCP-1, ceruloplasmin, adiponectin, hematopexin and KIM-1 to be the best predictors of LN activity status as assessed by the National Institute for Health Activity Index (NIH-AI), proposing a biomarker based Renal Activity Index for Lupus (RAIL) algorithm [30]. Our current study looked at a sub-set of these markers looking at their ability to identify BILAG defined active LN rather than NIH-AI status. These promising results of Brunner et al require further validation in larger prospective, multi-ethnic cohorts. In contrast to the markers validated in our current study, it remains unclear whether these biomarkers would be able to differentiate patients with active LN versus in-active LN, as all patients in the above study had definite biopsy-defined LN.

Our data demonstrate the key utility of urinary biomarkers in monitoring of LN. We have demonstrated and validated an excellent panel of biomarkers which differentiate JSLE patients with active LN and no current LN. As discussed above, Brunner et al have also proposed a distinct biomarker panel which accurately correlates with NIH-AI status. A large international prospective study or clinical trial is therefore warranted. This would longitudinally assess the biomarkers validated in the current

study for initial indentification of active LN, followed by assement of LN severity using the additional markers included in the RAIL as a proxy for histological changes. An international collaborative study will most probably be needed to be sufficiently powered given the multiplicity of biomarkers studied, distinct kidney biopsy features seen and the ethnic differences seen in JSLE severity.

In our current study we could not demonstrate a significant difference in urinary NGAL levels between those with active LN / non-LN patients in either the UK or ELC. This is in contrast with previous work which has shown NGAL to be highly sensitive / specific for identificantion of biopsy proven LN in children [26]. These results may be explained by differences in the timing of the sample and the outcome measures used. Urinary NGAL has previuosly been shown to be a useful predictor of impending flare in both the UK JSLE Cohort [26], and an adult SLE study of the ELC which included a University College London validation cohort [43]. Kiani et al were also unable to detect an association between urinary NGAL and LN in a prospective study including 107 adult SLE patients [44]. These observations may be due to urinary NGAL levels peaking before flares, and receeding before it becomes clinically detectable [45]. Urinary NGAL has also been demonstrated as a marker of renal damage in LN [46], which may also explain why patients with a history of biopsy proven LN have higher urinary NGAL levels. These observations suggest that NGAL requires further testing longitudinally as part of a urine biomarker panel despite the results seen in the current study, as it may able to predict active nephrits and in-active nephritis occurence.

It is interesting to consider the origin and renal-specificity of the novel biomarkers.

AGP belongs to the immunocalin family, a group of immunomodulatory binding proteins. AGP is mainly produced by the liver, but has also been reported in other cell types (macrophages [47], endothelial cells [48], and monocytes [49]). In active LN, increased production as part of the acute phase response, coupled with production by cells infiltrating the kidney, may be responsible for the high urinary levels demonstrated. Transferrin and ceruloplasmin are plasma proteins, primarily responsible for carrying iron and copper respectively. Differing from albumin in terms of their molecular radii and isoelectric points, urinary ceruloplasmin and transferrin have been shown to predict the onset of microalbuminuria in diabetic nephropathy [50]. LPGDS, a member of the lipocalin superfamily responsible for prostaglandin D2 production, is similar to albumin in chemical properties but it is also much smaller [51]. In type-2 diabetes, urinary LPGDS has been shown to increase in the early stages of kidney injury [52]. Urinary VCAM-1 levels have previously been shown to be higher than blood levels, suggesting that the inflamed kidney may represent an important source of urinary VCAM-1 [33].

Certain limitations of our study warrant recognition and should be addressed in future work. As our definition of active LN was based on the composite renal BILAG score, calculated from proteinuria, GFR, blood pressure, active urine sediment, plasma creatinine and recent biopsy findings, we could not directly compare such traditional markers with the novel urinary biomarkers studied. Due to the cross-sectional nature of this study we are unable to comment on the relationship of such biomarkers with other stages of the fluctuating LN disease course (e.g. prediction of flare / remission). Validation in a larger, longitudinal, prospectively collected study is therefore necessary, including children and young people with the full range of mild, severe and

in-active disease phenotypes from a range of patient cohorts (including Asian and African cohorts). With further prospective validation, it may become apparent that fewer biomarkers together can produce acceptable accuracy for active LN identification (e.g. AGP and CP) due to the level of correlation seen between biomarkers (especially for Cohort 2). This would potentially make it a simpler point of care testing device for biomarker quantification. Concurrent investigation of the role of such biomarkers *in vitro* or in LN mouse models, will also help to improve understand of LN pathophysiology.

Conclusions

JSLE patients have significant renal involvement and the potential to develop irreversible renal damage as the result of LN relapses that are either un-recognized, not identified early enough, or treated sufficiently [4,53]. This study has demonstrated and validated, a renal-specific excellent novel urine biomarker panel for recognition of active LN in two ethnically diverse JSLE populations, providing considerable strength to these findings. Further validation in larger, longitudinal, prospectively collected studies is required to define biomarker profiles that predict LN relapses and response to treatment. It is anticipated that a future urinary biomarker point of care testing device will help to improve the renal outcomes for JSLE patients through biomarker led renal monitoring in routine clinical practice.

Figure Legends

Fig 1 Distribution of biomarker concentrations in active / non-LN patients from Cohorts 1 & 2. Median value for each group shown by horizontal line. Mann Whitney-U tests used to compare distribution of biomarker concentrations between

patient groups within each cohort. A Bonferroni adjustment was applied to account for multiple testing, corrected p-values are reported (p_c). VCAM-1 biomarker data not available from 1 active-LN patient from Cohort 1. NGAL data not available from 3 active LN and 15 non-LN patients from Cohort 1

Fig 2 Urine biomarker concentrations in patients with / without extra-renal JSLE activity. Biomarker concentrations standardised to urinary creatinine and expressed as median values. Mann Whitney U tests used to compare biomarker concentrations between patient groups. A Bonferroni adjustment was applied to account for multiple testing, corrected p-values are reported (p_c). VCAM-1 measurement missing from 1 patient. NGAL data not available from 3 active LN and 15 non-LN patients

Fig 3 ROC generated from the optimal binary logistic regression model when data from both cohorts combined. Optimal model includes AGP, Ceruloplasmin, LPGDS, TF and VCAM-1 (AUC = 0.952)

Fig 4 Urine biomarker concentrations in cohort 2 patients with LN and no recent biopsy (BILAG defined active LN) versus biopsy defined active-LN. LN & biopsy patients, n=12. LN no biopsy, n=11

On-line resource 1 Urine biomarker concentrations standardised to urinary creatinine in active and non-LN patients from both cohorts

On-line resource 2 Correlation between urine biomarkers in cohorts 1 and 2

On-line resource 3 Urine biomarker concentrations (standardised to urinary creatinine) according to renal BILAG score in patients from the UK exploratory cohort.

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