

# Urinary markers differentially associate with kidney inflammatory activity and chronicity measures in patients with lupus nephritis

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## ABSTRACT

**Objective** Lupus nephritis (LN) is diagnosed by biopsy, but longitudinal monitoring assessment methods are needed. Here, in this preliminary and hypothesis-generating study, we evaluate the potential for using urine proteomics as a non-invasive method to monitor disease activity and damage. Urinary biomarkers were identified and used to develop two novel algorithms that were used to predict LN activity and chronicity.

**Methods** Baseline urine samples were collected for four cohorts (healthy donors (HDs, n=18), LN (n=42), SLE (n=17) or non-LN kidney disease biopsy control (n=9)), and over 1 year for patients with LN (n=42). Baseline kidney biopsies were available for the LN (n=46) and biopsy control groups (n=9). High-throughput proteomics platforms were used to identify urinary analytes  $\geq 1.5$  SD from HD means, which were subjected to stepwise, univariate and multivariate logistic regression modelling to develop predictive algorithms for National Institutes of Health Activity Index (NIH-AI)/National Institutes of Health Chronicity Index (NIH-CI) scores. Kidney biopsies were analysed for macrophage and neutrophil markers using immunohistochemistry (IHC).

**Results** In total, 112 urine analytes were identified from LN, SLE and biopsy control patients as both quantifiable and overexpressed compared with HDs. Regression analysis identified proteins associated with the NIH-AI (n=30) and NIH-CI (n=26), with four analytes common to both groups, demonstrating a difference in the mechanisms associated with NIH-AI and NIH-CI. Pathway analysis of the NIH-AI and NIH-CI analytes identified granulocyte-associated and macrophage-associated pathways, and the presence of these cells was confirmed by IHC in kidney biopsies. Four markers each for the NIH-AI and NIH-CI were identified and used in the predictive algorithms. The NIH-AI algorithm sensitivity and specificity were both 93% with a false-positive rate (FPR) of 7%. The NIH-CI algorithm sensitivity was 88%, specificity 96% and FPR 4%. The accuracy for both models was 93%.

**Conclusions** Longitudinal predictions suggested that patients with baseline NIH-AI scores of  $\geq 8$  were most sensitive to improvement over 6–12 months. Viable approaches such as this may enable the use of urine samples to monitor LN over time.

## WHAT IS ALREADY KNOWN ON THIS TOPIC

- ⇒ Diagnosis of lupus nephritis (LN) through a kidney biopsy often occurs after the accrual of irreversible kidney damage.
- ⇒ Non-invasive testing methods to accurately and safely evaluate renal involvement earlier in patients with SLE are needed.

## WHAT THIS STUDY ADDS

- ⇒ Proteomics screening revealed a set of proteins that were different in both SLE and LN compared with healthy donors, and that are associated with inflammation, renal dysfunction and angiogenesis. In addition, a set of unique proteins was identified that were associated with LN but were distinct from both SLE and non-SLE kidney disease.
- ⇒ A stepwise proteomics-based approach identified urinary biomarkers correlating to the National Institutes of Health Activity Index (NIH-AI) and the National Institutes of Health Chronicity Index (NIH-CI).
- ⇒ Two novel algorithms were developed that predicted NIH-AI/NIH-CI scores with  $\geq 93\%$  specificity and 93% accuracy.

## HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- ⇒ Urinary protein markers that correlate with pathology findings in LN would provide valuable information regarding kidney status beyond standard clinical laboratory tests.

## INTRODUCTION

SLE is an autoimmune disease with heterogeneous manifestations that can cause inflammation and irreversible damage to multiple organs, including the kidneys.<sup>1</sup> Lupus nephritis (LN) results, in part, from the accumulation of autoantibody deposits in the kidney glomerular basement membrane.<sup>2</sup> Up to half of patients with SLE develop LN,<sup>3</sup> with as many as 30% of these patients progressing to end-stage kidney disease.<sup>4</sup> LN and other

severe SLE manifestations disproportionately affect black patients with SLE.<sup>5,6</sup>

LN diagnosis is often made after the accrual of irreversible kidney damage because of the insensitivity of current clinical laboratory assessments (eg, glomerular filtration rate, microalbuminuria and proteinuria).<sup>1,7,8</sup> Kidney biopsy is the gold standard for clinical diagnosis of LN.<sup>1</sup> However, as an invasive procedure, it presents risks to the patient.<sup>9,10</sup> In addition, a biopsy is subject to sampling error.<sup>11</sup>

Non-invasive testing methods to accurately and safely evaluate renal involvement in patients with SLE are needed to facilitate early diagnosis, monitor for disease activity/progression and guide treatment plans. Specifically, the identification of markers in urine that detect inflammation and kidney damage may reduce the need for invasive procedures and allow treatment to be initiated earlier and altered over time before the onset of irreversible kidney damage.<sup>12</sup> As treatment guidelines seek to reduce disease activity and prevent loss of kidney function in patients with LN,<sup>9</sup> non-invasive methods that monitor kidney health are urgently needed and would provide a substantial benefit for managing patients with LN.<sup>13</sup>

Disease activity in patients with LN can be measured using histopathology-based scoring systems, known as the National Institutes of Health Activity Index (NIH-AI) and the National Institutes of Health Chronicity Index (NIH-CI). These are specifically used to determine the extent of activity/inflammation and damage in biopsied kidney tissue, respectively.<sup>14</sup> Identifying urine-based biomarkers correlating with these indices could provide easily accessible markers to monitor disease activity/inflammation in the kidney. We assessed the utility of high-throughput proteomics platforms (Luminex and SIMOA) to identify urine-based markers differentially expressed in patients with LN compared with healthy donors (HDs) and to understand the overlap of the identified proteins with those found in patients with SLE and non-LN kidney diseases. The association of the identified proteins with the NIH-AI and NIH-CI scores was performed along with pathway analyses. Histopathological assessments were used to confirm the presence of cell types indicated in the pathway results. We developed novel algorithms with these selected markers and used them to predict patient NIH-AI and NIH-CI scores. The performance of the models was evaluated using receiver operating characteristic (ROC) analysis.

## METHODS

### Participant inclusion criteria and study design

Patients  $\geq 18$  years old were enrolled in three groups (LN, SLE without active LN and non-SLE kidney disease) from three hospitals in Atlanta, Georgia (Emory University Hospital, Grady Memorial Hospital, and Emory University Hospital Midtown). Patients newly diagnosed with LN were defined by the treating physician, supported by the study rheumatologist (SSL) and a kidney biopsy analysis

consistent with LN. Patients with SLE met the 1997 American College of Rheumatology updated classification criteria for SLE,<sup>15,16</sup> had  $< 500$  mg proteinuria, and had no prior history or suspicion of LN. Patients with non-SLE kidney disease (including human immunodeficiency-associated nephropathy, minimal-change disease, thrombotic microangiopathy, immunoglobulin A nephropathy, secondary focal segmental glomerulosclerosis and diabetic nephropathy) were selected to represent a kidney disease biopsy control group. No specific treatment plan was implemented, but patients with SLE, LN and other non-LN kidney diseases maintained individual treatments and medications at their respective physician's discretion. HD controls, from participants with no evidence of kidney or immunological disease, were matched demographically to patients with LN. The biopsy control group was not demographically matched due to small group sizes.

### Patient and public involvement

The study was developed to specifically address the unmet need for a method to monitor kidney health in patients with SLE and LN over time using non-invasive techniques. Participants provided samples and medical data but were not directly involved in development or conduct of the study, nor the dissemination of the study results.

### Sample collection and handling

#### Kidney biopsy and NIH-AI and NIH-CI score generation

Baseline needle biopsies were collected from kidneys of patients with LN and the biopsy control group. Biopsy tissue, collected for medical purposes, was processed into formalin-fixed, paraffin-embedded blocks using standard methods<sup>17</sup> and was subjected to H&E and immunohistochemistry (IHC) staining and analysis. NIH-AI and NIH-CI scores were generated on LN samples based on histopathological analysis of H&E-stained kidney biopsy tissue by a board-certified renal pathologist (ABF) using a previously described multivariable semiquantitative system where total possible scores for NIH-AI and NIH-CI were 24 and 12, respectively.<sup>18</sup> The distribution and mean NIH-AI and NIH-CI scores for all patients were examined. For investigational purposes and to enable patient grouping, a designated threshold for the lowest 33% of possible scores was set for each index: NIH-AI score of  $\leq 8$  and NIH-CI score of  $\leq 4$ . Patients were then classified as having NIH-AI scores of  $\leq 8$  vs  $> 8$  and NIH-CI scores of  $\leq 4$  vs  $> 4$ .

### Urine sample collection

Baseline urine samples were collected for all participants evaluated for proteomics analysis (LN, SLE, biopsy control, HDs and longitudinally from patients with LN at months 1, 3, 6 and 12 when possible). At the time of sample collection, none of the patients with SLE had active or known urinary tract infections, and the female patients were not undergoing menses. Urine from patients with LN was collected either on the day of or within 1 week prior to the biopsy procedure and was quantified for proteinuria as

measured by protein:creatinine ratios. Immediately after collection, urine samples were centrifuged (400×g), and supernatant was collected and stored at −80°C. All urinalysis results were normalised to creatinine concentrations with the MicroVue Creatinine colorimetric assay (Quidel, catalogue number 8009).

### Analyte screening

Preliminary analyte testing was performed on nine commercially available LN urine samples (BioIVT, Westbury, New York, USA) using the Human Discovery Multi Analyte Panel (Discovery MAP V.3.3) of 272 analytes (Myriad Rules-Based Medicine (MRBM), Austin, Texas, USA) and run on the Luminex bead-based multiplex instrument platform. Additionally, the Quanterix ultra-sensitive SIMOA (MRBM) panel was used to test the LN urine samples for eight disease marker analytes expected to be present in low abundance, namely, interferon (IFN)- $\alpha$ , IFN- $\beta$ , IFN- $\gamma$ , interleukin (IL)-1 $\beta$ , IL-2, IL-6, IL-10 and IL-17A.

Assay suitability and accuracy were evaluated through dilutional linearity testing of nine LN urine samples as described (online supplemental methods). Analytes were evaluated for detectability above the lower limit of quantification (LLOQ) of each assay and excluded if >50% of the samples were below the LLOQ. Analytes where fewer than three participants passed linearity testing were also excluded from further consideration. Urine from all study participants was tested for passing analytes from the initial investigation of the Discovery MAP V.3.3 and SIMOA panels. Analytes were further excluded if the resulting concentrations were <1.5 SD of the mean of the HDs. The resulting analytes were labelled as the 'primary analyte pool'.

A Wilcoxon test ( $p < 0.5$ ) using Benjamini-Yekutieli false discovery rate adjustment<sup>19</sup> was used to identify analytes from the primary analyte pool that were statistically different in each cohort (LN, SLE and biopsy control) relative to HD controls.

### Narrowed analyte pools

The primary analyte pool was narrowed to analytes strongly and independently associated with either NIH-AI or NIH-CI by using stepwise and univariate regression analyses with NIH-AI and NIH-CI as both a continuous and categorical dependent parameter. P value cut-offs, <0.1 for categorical regression and <0.05 for continuous regression, were used.

### Pathway analysis of narrowed analyte pools

Pathway analysis was performed on the NIH-AI and NIH-CI narrowed analyte pools using Ingenuity Core Analysis against all genes in the Ingenuity Knowledge Base (Qiagen, Redwood City, California, USA). Top disease, functional and upstream regulator categories were selected using the p value from Fisher's exact test.

### Final analyte selection and predictive algorithm development

To develop predictive algorithms for NIH-AI and NIH-CI activity, multivariate logistic regression models were

evaluated for all possible analyte combinations from the narrowed analyte pools. Models where all markers and the entire model were statistically significant ( $p < 0.05$ ) were evaluated for misclassification rates and area under the curve (AUC) for the ROC curves. To be considered for final selection, all parameter estimates within the multivariate logistic model were required to have a p value of <0.05. Final predictive models for NIH-AI and NIH-CI were selected empirically by using a combination of the best AUC of the ROC curve and lowest misclassification rate. Models with the lowest sum of misclassification rate and AUC-1 were selected. Intercepts and weighting of analytes in the final model were derived from parameter estimates of the nominal logistic model. Thresholds were determined using the prediction probability by maximising Youden's statistic (sensitivity+specificity-1) in the ROC curve of the corresponding model. True positives were defined as NIH-AI or NIH-CI classification scores that were accurately predicted by the developed algorithm compared with observed histology-based NIH-AI and NIH-CI scores determined from baseline kidney biopsy results.

The algorithms were used to predict NIH-AI and NIH-CI scores at each time point for patients with LN where urine samples were available.

### IHC staining for macrophage-associated and neutrophil-associated markers

Kidney biopsy tissue samples were stained for macrophage-associated and neutrophil-associated markers as described in the online supplemental methods.

## RESULTS

### Participant demographics and characteristics

A total of 92 participants were enrolled in the study, of whom 47 (51%) had LN; 17 (18%) had SLE (without active LN); 9 (10%) were non-LN kidney disease biopsy controls; and 19 (21%) were HDs. Of the 47 patients with LN, 46 had kidney biopsies and 42 had urine analysis performed at baseline. Baseline participant demographics and characteristics are shown in [table 1](#). Among patients with LN, 35 (83%) were female and 37 (88%) were black. A total of 22 (52%) participants were newly diagnosed with LN. The mean SLE Disease Activity Index 2000 (SLEDAI-2K) score of the full LN group was 8.46 (SD 5.15). The mean proteinuria measured by spot urine protein:creatinine ratio was 2.66 (SD 2.11) g/g. Based on histopathology of kidney biopsies at baseline, 18/46 (39%) patients with LN were classified as having NIH-AI scores of >8 and 17/46 (37%) as having NIH-CI scores of >4.

### Analyte screening

The analyte selection workflow to identify LN-specific protein markers associated with NIH-AI and NIH-CI scores is shown in online supplemental figure S1. The nine commercially available LN urine samples were tested against the panel of 280 protein analytes for dilutional

**Table 1** Participant demographics and clinical characteristics

		LN	SLE	Biopsy control	HD
		n=47*	n=17	n=9	n=19†
Demographics		n (%)	n (%)	n (%)	n (%)
Age	<20	0 (0)	0 (0)	1 (11)	1 (6)
	20–29	15 (36)	9 (53)	2 (22)	7 (39)
	30–39	12 (29)	3 (18)	2 (22)	4 (22)
	40–49	9 (21)	1 (6)	1 (11)	4 (22)
	50–59	5 (12)	3 (18)	0 (0)	2 (11)
	60+	1 (2)	1 (6)	3 (33)	0 (0)
Gender	Female	35 (83)	15 (88)	5 (56)	16 (89)
	Male	7 (17)	2 (12)	4 (44)	2 (11)
Race	Black	37 (88)	17 (100)	3 (33)	15 (83)
	White	3 (7)	0 (0)	6 (67)	3 (17)
	Other	2 (5)	0 (0)	0 (0)	0 (0)
Clinical characteristics and LN therapies					
Newly diagnosed LN‡ (%)		52			
SLEDAI-2K Global score, mean (SD)		8.46 (5.15)			
Proteinuria§ (g/g)		2.66 (2.11)			
Elevated anti-dsDNA antibodies (%)		67			
NIH-AI and NIH-CI indices¶					
NIH-AI score, mean (SD)		7.0 (4.88)			
NIH-CI score, mean (SD)		5.2 (2.30)			
Abnormal complement concentration (%)					
C3 low		62			
C4 low		48			
Baseline treatments (%)					
Azathioprine		5			
Belimumab		2			
Cyclophosphamide		10			
Cyclosporine		0			
Dapsone		0			
Hydroxychloroquine		74			
Methotrexate		2			
Mycophenolate mofetil		50			
Prednisone		71			

Clinical characteristics, abnormal complement concentrations and baseline treatments were not available for the SLE, biopsy control and HD control groups.

\*Data available for 42 of the 47 patients with LN. Five patients with LN did not provide urine at baseline. Percentages are based on the data available for 42 patients with LN.

†Data available for 18 of the 19 HD controls. Percentages are based on the data available for 18 participants.

‡Diagnosed at first study visit following evaluation of the kidney biopsy.

§Measured as spot urine protein/spot urine creatinine.

¶Data available for 43 of the 47 patients with LN.

C3, complement 3; C4, complement 4; dsDNA, double-stranded DNA; HD, healthy donor; LN, lupus nephritis; NIH-AI, National Institutes of Health Activity Index; NIH-CI, National Institutes of Health Chronicity Index; SLEDAI-2K, SLE Disease Activity Index 2000.

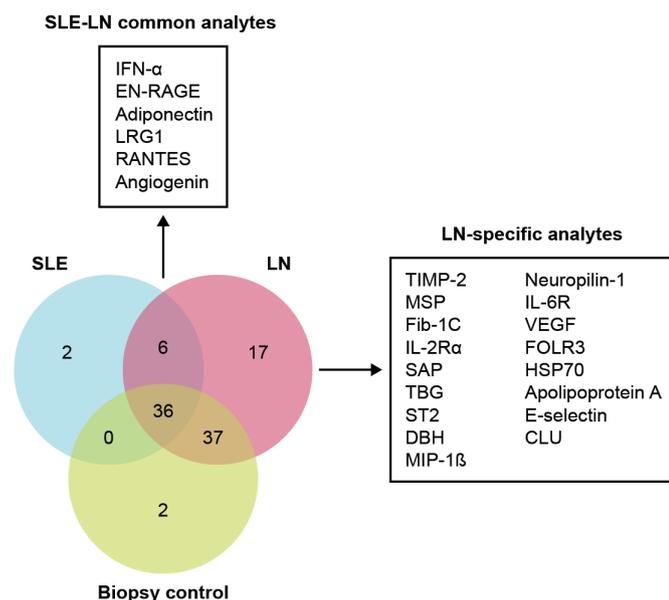
linearity and detectability. Of these analytes, 79 failed dilutional linearity and 57 were below the LLOQ in ≥50%

of samples and were therefore removed from further evaluation. The remaining 144 protein analytes in the

panel were used for the assessment of the study cohort samples. Of the 144 analytes, 112 had mean concentrations that were  $\geq 1.5$ SD higher in samples from patients with LN than HDs and were selected for further analysis. This group was labelled the primary analyte pool (online supplemental table S1). There were no analytes that were significantly decreased relative to HDs. The remaining 112 analytes in the primary analyte pool were therefore both quantifiable and overexpressed in samples from the LN, SLE and biopsy control patients compared with HDs. Of these, 96, 44, and 75 were statistically different ( $p < 0.05$ ) from HDs in the LN, SLE and biopsy control cohorts, respectively (online supplemental table S2 and figure 1). This analysis revealed 36 protein analytes common to all three cohorts. Six analytes were common to the SLE and LN cohorts, and 17 analytes were specific to patients with LN.

### Narrowed analyte pools associated with NIH-AI and NIH-CI

Stepwise and univariate regression analyses of the primary analyte pool of 112 proteins were used to further narrow



**Figure 1** Venn diagram of the number of creatinine-normalised urine analytes from the primary analyte pool that were significantly different with a  $\geq 2$ -fold cutoff in patients with SLE, LN and biopsy controls relative to HDs ( $p$  value  $< 0.05$  by Wilcoxon test using Benjamini-Yekutieli false discovery rate adjustment). CLU, clusterin; DBH, dopamine beta-hydroxylase; EN-RAGE, extracellular newly identified receptor for advanced glycation end products binding protein; Fib-1C, fibulin-1C; FOLR3, folate receptor gamma; HD, healthy donor; HSP, heat shock protein; IFN- $\alpha$ , interferon alpha; IL-2R $\alpha$ , interleukin-2 receptor alpha; IL-6R, interleukin-6 receptor; LN, lupus nephritis; LRG1, leucine-rich alpha-2-glycoprotein; MIP-1 $\beta$ , macrophage inflammatory protein-1 beta; MSP, macrophage-stimulating protein; RANTES, regulated on activation, normal T cell expressed and presumably secreted; SAP, serum amyloid P-component; ST2, suppression of tumorigenicity 2; TBG, thyroxine-binding globulin; TIMP-2, tissue inhibitor of metalloproteinases 2; VEGF, vascular endothelial growth factor.

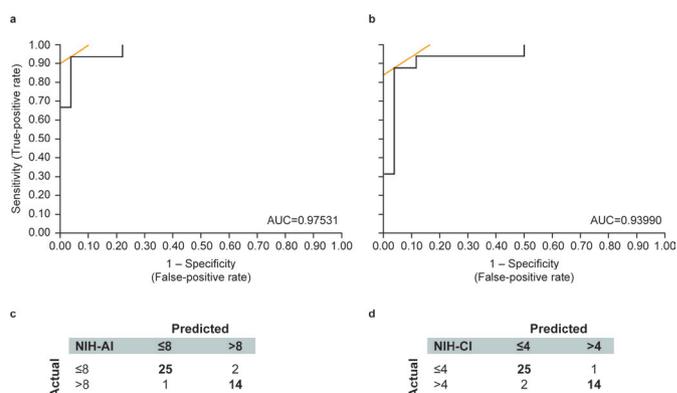
the pool of urine analytes and to identify those strongly associated with NIH-AI or NIH-CI. These analyses identified two separate narrowed pools, with 30 analytes associated with NIH-AI and 26 analytes associated with NIH-CI (online supplemental table S3). Only four analytes (B-cell-activating factor (BAFF), dopamine beta-hydroxylase (DBH), interleukin-6 receptor subunit beta (IL-6R $\beta$ ) and osteoprotegerin) were common to both NIH groups, demonstrating a difference in the mechanisms associated with activity and chronicity.

### Pathway analysis of narrowed analyte pools associated with NIH-AI and NIH-CI

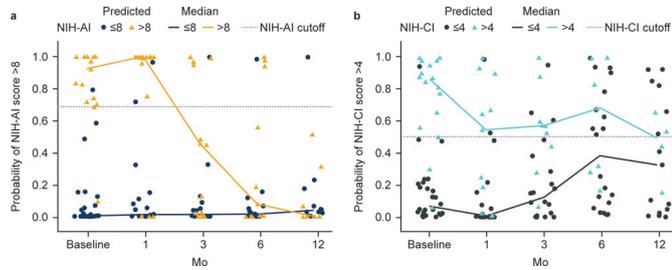
Analysis of the narrowed analyte pools revealed three unique pathways, each associated with NIH-AI and NIH-CI (online supplemental table S4). The two pathways common to both NIH-AI and NIH-CI were role of macrophages, fibroblasts and endothelial cells in rheumatoid arthritis and hepatic fibrosis/hepatic stellate cell activation.

### Final analyte selection and predictive algorithm development

Four markers were identified for each of the NIH-AI and NIH-CI predictive algorithms using multivariate logistic regression models (online supplemental table S5). The NIH-AI algorithm markers were apolipoprotein A-II (ApoA-II), von Willebrand factor (vWF), interleukin-1 alpha (IL-1 $\alpha$ ) and insulin-like growth factor-binding protein 2 (IGFBP2). The NIH-CI algorithm markers were IL-6R $\beta$ , kidney injury molecule 1 (KIM-1), DBH and fetuin A. Models where all four markers were statistically significant ( $p < 0.05$ ) were evaluated for predictive accuracy using AUC for the ROC curves based on observed kidney biopsy NIH-AI and NIH-CI scores at baseline (figure 2). The AUCs for the NIH-AI and NIH-CI algorithms were 0.97531 and 0.93990, respectively (figure 2A,B). Confusion matrices representing the true and predicted



**Figure 2** ROC curves and confusion matrices for NIH-AI and NIH-CI predictive algorithms. (A) NIH-AI predictive algorithm ROC curve, (B) NIH-CI predictive algorithm ROC curve, (C) NIH-AI confusion matrix and (D) NIH-CI confusion matrix. AUC, area under the curve; CI, Chronicity Index; NIH-AI, National Institutes of Health Activity Index; NIH-CI, National Institutes of Health Chronicity Index; ROC, receiver operating characteristic.



**Figure 3** NIH-AI and NIH-CI longitudinal predictions for patients with LN classified by index score. The solid lines represent median values over time. Dotted lines are the determined cut-off values using the predictive algorithm (NIH-AI cut-off=0.69, NIH-CI cut-off=0.5). (A) Predicted NIH-AI and (B) NIH-CI classifications using the predictive algorithm at various time points. CI, Chronicity Index; LN, lupus nephritis; NIH-AI, National Institutes of Health Activity Index; NIH-CI, National Institutes of Health Chronicity Index.

positives and negatives for each NIH index are shown in figure 2C,D. Final algorithms for NIH-AI and NIH-CI were selected based on the lowest score of misclassification rate, as outlined further.

**NIH-AI and NIH-CI predictive algorithms**

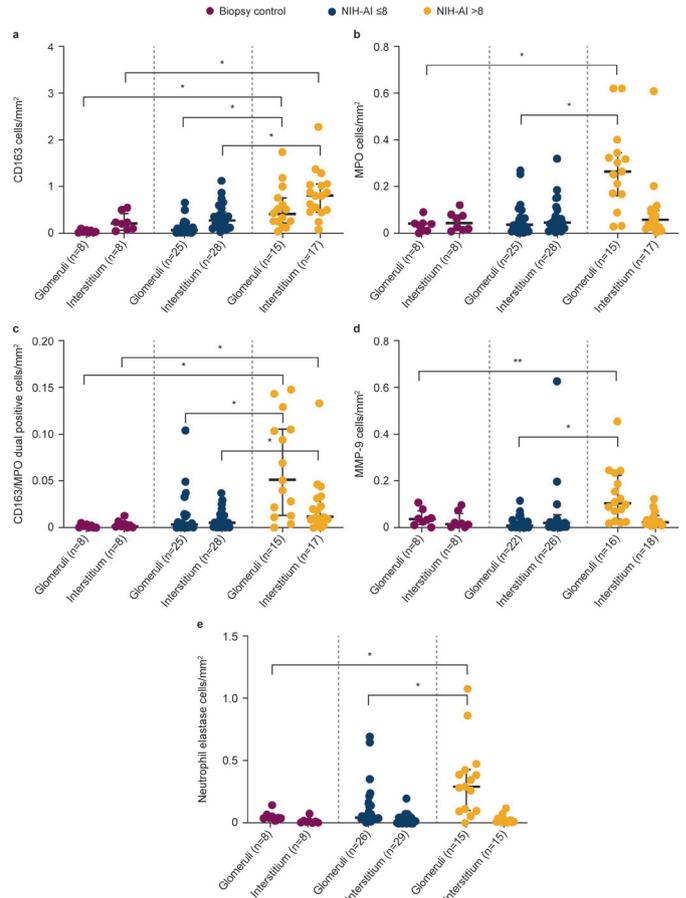
$$\begin{aligned} \text{NIH-AI} &= -4.396 - 31.476 \times \text{ApoA-II} + 76.47 \times \\ &\quad \sqrt{\text{WF}} - 1004.63 \times \text{IL-}\alpha + 0.1313 \times \text{IGFBP2} \\ \text{NIH-CI} &= 3.47 - 1.469 \times \text{IL-6R}\beta + 1.858 \times \\ &\quad \text{KIM-1} + 0.0854 \times \text{DBH} + 0.0068 \times \text{fetuin A} \end{aligned}$$

The NIH-AI algorithm sensitivity and specificity were 93% with a false-positive rate (FPR) of 7%. The NIH-CI algorithm sensitivity was 88% and specificity was 96% with an FPR of 4%. The accuracy for both models was 93%.

NIH-AI and NIH-CI longitudinal predictions over the course of 12 months are shown for patients with LN classified by observed NIH index scores at baseline (figure 3). Most of the patients with NIH-AI baseline scores of >8 were predicted to transition to NIH-AI scores of ≤8 by month 3. The NIH-AI ≤8 group predicted medians remained below the algorithm-predicted NIH-AI cut-off of 0.69 for the duration of the study, whereas the NIH-CI >4 group predicted medians remained above the algorithm predicted NIH-CI cut-off of 0.5 for most of the study. NIH-CI ≤4 group predicted medians remained below the predicted cut-off for the entirety of the study except in month 12, when the median narrowly crossed the predicted cut-off, possibly due to the smaller sample size at later time points.

**IHC staining of macrophage and neutrophil markers**

To confirm pathway analysis results, which identified granulocyte-associated and macrophage-associated pathways in the narrowed NIH-AI and NIH-CI analyte pools, we performed IHC staining for macrophage and neutrophil markers in the glomeruli and interstitium of kidney biopsy tissue samples. Of the 46 patients with LN and 9 biopsy controls for whom baseline kidney biopsies were available, 6 patients with LN and 1 biopsy control were



**Figure 4** Kidney biopsy IHC staining results with NIH-AI score of >8 vs ≤8 from patients with LN versus biopsy controls. Macrophage-associated markers: CD163/MPO duplex stain, MMP-9; neutrophil-associated marker: neutrophil elastase. Healthy and fibrotic glomeruli were counted together. (A) Number of CD163-positive cells/mm<sup>2</sup>. (B) Number of MPO-positive cells/mm<sup>2</sup>. (C) Number of CD163/MPO dual-positive cells/mm<sup>2</sup>. (D) Number of MMP-9-positive cells/mm<sup>2</sup>. (E) Number of neutrophil elastase-positive cells/mm<sup>2</sup>. \*p<0.01, \*\*p<0.05. CD, cluster of differentiation; IHC, immunohistochemistry; LN, lupus nephritis; MMP-9, matrix metalloproteinase-9; MPO, myeloperoxidase; NIH-AI, National Institutes of Health Activity Index.

excluded from this analysis because no glomeruli were present in the kidney biopsy samples.

Representative duplex IHC staining for macrophages with cluster of differentiation (CD) 163/myeloperoxidase (MPO) and neutrophils with neutrophil elastase of kidney biopsies from patients with LN compared with a biopsy control is shown in online supplemental figure S2. Macrophage-associated markers (CD163/MPO duplex stain, matrix metalloproteinase-9 (MMP-9)) were elevated in kidney biopsy tissue for patients classified by histopathology scoring as NIH-AI score of >8 vs NIH-AI score of ≤8 (CD163 glomeruli and interstitium, both p<0.01; MPO glomeruli, p<0.01; CD163/MPO glomeruli and interstitium, both p<0.01; MMP-9 glomeruli, p<0.01) (figure 4). Additionally, IHC staining for the neutrophil marker, neutrophil elastase, was elevated in kidney biopsy glomeruli for patients classified by histopathology scoring

as NIH-AI score of  $>8$  vs NIH-AI score of  $\leq 8$  ( $p < 0.01$ ), thus supporting increased inflammation in the kidneys of patients with LN. Fewer differences in the staining for macrophage markers were observed in the kidneys of patients with NIH-CI scores of  $>4$  vs  $\leq 4$ , while neutrophil elastase was elevated in patients with NIH-CI scores of  $>4$  vs NIH-CI scores of  $\leq 4$  (interstitium,  $p < 0.01$ ) (online supplemental figure S3).

## DISCUSSION

One method for measuring kidney disease activity is by histopathology using the NIH-AI and NIH-CI to score kidney biopsy tissue.<sup>14</sup> The aim of this study was to evaluate the use of high-throughput proteomics to identify sets of urine-based LN protein markers that correlate with kidney biopsy histopathology NIH-AI/NIH-CI scoring results for a cohort of patients with LN. The data presented here demonstrate that urine samples could be used to non-invasively track alterations in the kidney over time, as longitudinal biopsies are not routinely performed.

We identified eight urinary markers (with four each correlating to NIH-AI and NIH-CI scoring indices) and created two novel algorithms that predicted NIH-AI/NIH-CI scores with  $\geq 88\%$  specificity and  $93\%$  accuracy. Longitudinal predictions of NIH-AI and NIH-CI scores based on urinary protein expression suggested that patients with observed baseline NIH-AI scores of  $>8$  were most sensitive to NIH-AI improvement over a period of 6–12 months. In contrast, score changes were predicted to be less likely in patients with baseline NIH-CI scores of  $>4$ . Our data suggest that, although formal clinical criteria that would instigate treatment have not yet been defined, it may be possible to use urinary protein markers that correlate with pathology findings to non-invasively evaluate kidney health and provide valuable information beyond what is provided through standard clinical laboratory tests.

The NIH-AI measures kidney disease activity/inflammation, whereas the NIH-CI is a measure of kidney damage.<sup>1 14</sup> The biological plausibility of our predictive algorithms is supported by the recognised roles for the eight NIH-AI/NIH-CI-associated urine proteins. The four protein markers of our NIH-AI algorithm (ApoA-II, vWF, IL-1 $\alpha$  and IGFBP2) have all independently been shown to play key roles in inflammation and LN disease activity.<sup>10 20–28</sup> The top four identified urine NIH-CI protein markers (IL-6R $\beta$ , KIM-1, DBH and fetuin A) have been implicated in kidney damage/injury.<sup>29–39</sup> The NIH-CI canonical pathways identified (leucocyte adhesion/diapedesis,<sup>40</sup> granulocyte function<sup>41</sup> and the role of macrophages<sup>1 42</sup>) also represent key inflammatory activities that can contribute to kidney damage.<sup>1 42–44</sup> Taken together, our large, unbiased proteomics screen identified inflammation-associated and damage-associated markers that correlated with NIH-AI/NIH-CI, supporting our methodological approach.

In addition to the eight urinary markers selected for the predictive algorithms, our wider screen provided important insights into LN and SLE immunopathology. Of note, many of the 96 analytes that were differentially expressed in patients with LN (compared with HDs) were also common to the SLE and biopsy control cohorts, with only 17 analytes specific to patients with LN. These results indicate the commonalities in inflammatory markers across patients with SLE and LN, as well as non-lupus kidney disease. Given the known, widespread upregulation of inflammatory markers in patients with SLE that eventually drive organ damage,<sup>45–48</sup> our results show that kidney inflammation could be monitored using accessible urine screening prior to LN diagnosis.

Furthermore, of the 30 and 26 analytes in the narrowed analyte pools that strongly correlated with NIH-AI/NIH-CI histopathology scoring, respectively, many play key roles in SLE pathogenesis and treatment. For example, our screen identified BAFF and IFN- $\alpha$ , two immunomodulatory proteins whose activities are inhibited by monoclonal antibodies approved for the treatment of patients with SLE.<sup>43 44</sup> Furthermore, the narrowed analyte pools also included multiple macrophage-associated markers (CD163,<sup>49</sup> macrophage-stimulating factor, monocyte chemoattractant protein 1,<sup>50</sup> metalloproteinase inhibitor 1 and IL-6R $\beta$ <sup>51 52</sup>), supporting the importance of macrophage-driven inflammation in LN pathophysiology.<sup>52 53</sup>

As levels of macrophage-associated proteins correlated with kidney histopathology, and pathway analysis revealed macrophage and granulocyte pathways associated with NIH-AI/NIH-CI, we used IHC staining to evaluate neutrophil and macrophage infiltration in kidney biopsies. We found that infiltration of macrophages and neutrophils in the kidney was associated with NIH-AI scores of  $>8$  vs  $\leq 8$ . For example, the macrophage marker MMP-9 was upregulated in patients who had NIH-AI scores of  $>8$  compared with those who had NIH-AI scores of  $\leq 8$ . MMP-9 is released by macrophages in the kidney and is known to promote macrophage recruitment, supporting a role in driving the kidney inflammation captured by the NIH-AI.<sup>54</sup> As such, our IHC findings directly support the urine proteomic data on the presence of these cell types in LN kidneys.

Notably, while macrophage infiltration was still detectable in kidneys scored as NIH-CI  $>4$ , there was less distinction between macrophage markers in kidneys scored as NIH-CI  $>4$  vs  $\leq 4$  kidneys than in kidneys scored as NIH-AI  $>8$  vs  $\leq 8$ , whereas neutrophil infiltration was elevated in kidneys scored as NIH-CI  $>4$  vs those scored as NIH-CI  $\leq 4$ . Our results suggest that macrophage-driven inflammatory processes detectable earlier in the disease (measured by NIH-AI) have already induced some of the kidney damage measured by the NIH-CI. Unchecked, long-term neutrophil and macrophage activation and the subsequent release of proinflammatory cytokines contribute to the eventual chronic kidney damage in patients with LN.<sup>55</sup> Together, these findings suggest that earlier detection of

kidney inflammation could prevent long-term damage accrual.

Importantly, although we detected markers of immune cell infiltration and inflammation in the kidneys of patients scored as NIH-AI >8, our longitudinal predictions suggest that disease activity decreased over time in these patients, likely as a result of ongoing treatments. Notably, there was little change in NIH-CI over time, indicating the importance of early detection and treatment of inflammation.

Limitations of this study include that several clinical characteristics were unavailable for the SLE cohort, including SLEDAI-2K scores, urine protein concentration and antidouble-stranded DNA antibody measurements. The small cohort sizes and lack of a validation cohort were also limitations. Additionally, the LN cohort was predominantly composed of patients who were black, and it is unclear whether the same mechanisms are key in LN disease in patients of different races. Also, since patients were not standardised by treatment, observed variability could be the result of confounding factors that were not captured or measured. Further, the results were limited by the number of proteins assessed; that is, urine samples were not evaluated for all possible proteins in the human proteome. Additionally, patients with SLE can show abnormal urinalysis results from processes unrelated to LN. Unfortunately, total protein measurements were not performed on the urine samples from the control group of patients with SLE without LN. Therefore, the specificity of the urinary findings in the SLE group is not fully interpretable, and evaluation in a more thoroughly characterised SLE cohort should be conducted in future studies. Further limitations included the absence of longitudinal biopsies to confirm predicted results, and the determined coefficients of analytes in the algorithm will likely vary in other patient populations. Therefore, whereas this study assessed the utility of high-throughput proteomics measurements to generate predictive LN algorithms, further studies and external validation are needed to refine these novel models in larger independent patient populations. Although extremely logistically difficult, an ideal study would assess samples from inactive SLE, active SLE without active renal disease, LN and longitudinal samples from active SLE without active renal disease until LN is diagnosed.

The evaluation of multiple biomarkers is critical for developing a tool with robust clinical utility and for characterising the complex biological mechanisms in LN pathogenesis. Comprehensive urine screening assessments provide additional information beyond traditional clinical testing regarding kidney inflammatory activity and chronicity to improve our understanding of the overall kidney status. Urine proteomics and high-throughput multiplex approaches facilitate the discovery of key immune mediators involved in LN pathogenesis and enable monitoring of disease state. Ultimately, these strategies may lead to improved non-invasive monitoring approaches for patients with LN in an effort to inform

treatment decisions and positively impact their quality of life.

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