Urinary proteomic profiles distinguish between active and inactive lupus nephritis


Objectives. Key aims of the treatment of lupus nephritis (LN) are to induce and maintain remission with minimal side effects. However, assessing ongoing renal inflammatory activity is poorly served by current diagnostic tests apart from renal biopsy, but frequent biopsies cannot be justified. Our long-term aim is to identify novel biomarkers from urinary protein profiles to improve diagnosis and monitoring of activity and response to therapy in LN.

Methods. We used surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) to identify biomarkers able to discriminate between urine samples from patients with inactive (n = 49) and active (n = 26) LN. Discriminant function analysis was used to define the minimum number of proteins whose levels best distinguished between the two patient groups. Serial urines of six biopsied patients were studied prospectively, and multiple regression (MR) scores calculated.

Results. Proteins with masses of 3340 and 3980 distinguished active from inactive LN with 92% sensitivity and specificity of 92% each. The prospective study of the biopsied patients demonstrated that MR scores could predict both relapse and remission earlier than traditional clinical markers.

Conclusions. SELDI-TOF MS identified potential biomarker profiles strongly associated with activity in LN. Identification of these proteins will allow us to devise specific assays to routinely monitor disease progression, and alter immunosuppressive drug regimens accordingly. These proteins may also play a critical role in the pathogenesis of glomerulonephritis, and could therefore provide targets for therapeutic intervention.

KEY WORDS: Lupus nephritis, Biomarkers, SELDI-TOF MS.
ProteinChips with either chemically or biochemically modified surfaces to bind a subset of proteins. The bound proteins are then ionized and desorbed, and the protein ions are separated by TOF MS. Recent studies have demonstrated the usefulness of this technique in identifying protein patterns in the urine associated with renal allograft rejection [5–8], renal complications after radiocontrast medium administration [9], urolithiasis [10] and transitional cell carcinoma of the bladder [11]. The reproducibility of this method for urine samples has been examined [12], demonstrating that this is a promising platform for medium throughput protein profiling. We have therefore set out to identify protein profiles associated with lupus nephritis (LN) using SELDI-TOF MS.

Patients and methods

Patient samples

Urine was collected from 26 active and 49 inactive SLE patients during outpatient clinics. Urinary protein/creatinine ratios, haematuria, anti-dsDNA antibody levels, serum creatinine and complement C3 and C4 levels were all determined routinely. All patients were previously confirmed as having renal involvement in SLE by biopsy. At the time the sample was taken, all patients were classified by the clinician, according to standard criteria, as having active or inactive renal or systemic lupus. A diagnosis of active renal disease depended on the presence of (i) a rise in creatinine from baseline—a rise of >30 µmol/l was considered significant if the baseline was <150 µmol/l or >50 µmol/l if the baseline was >150 µmol/l and not attributable to other causes e.g. dehydration, introduction of rennin–angiotensin blockade or intercurrent illness; or (ii) a rise of total urinary protein:creatinine ratio of >50 from a baseline of <100 or >100 from a baseline of >100, or (iii) the new onset of haematuria (>10 red blood cells/high power field (hpf)] by microscopic examination. If a rise of active renal disease was suspected, a renal biopsy was performed where patients agreed. In the absence of a renal biopsy, active SLE GN was only defined if the criteria above were fulfilled and immunosuppression was escalated. Patients were defined as in remission if their protein/creatinine ratio was consistently <100 (equivalent to approximately 1 g/24 h), urine microscopy showed <10 red blood cells/hpf, and they had a stable serum creatinine, if in the normal range at baseline, or a fall in serum creatinine by >50 µmol/l if the baseline was higher than 150 µmol/l prior to change in immunosuppression. Serial samples were collected prospectively in several patients before and after renal biopsy. Active systemic lupus was defined on the basis of new onset of arthralgias, pericarditis or pleuritis, myalgias, severe alopecia, typical rash or cytopenias not attributable to drug treatments. Samples were also collected from 15 normal controls. Midstream urine was collected and stored at 4°C overnight, as early optimization experiments demonstrated that overnight storage did not affect the profiles obtained. Samples were then centrifuged at 1400g for 10 min to remove cells that could release proteins after a freeze/thaw cycle, and 1ml aliquots of the supernatant stored at −80°C. Research was carried out in accordance with the Declaration of Helsinki. All patient and control samples were obtained with informed consent and ethics approval by the Hammersmith, Queen Charlotte’s and Chelsea and Acton Hospitals Research Ethics Committee.

Protein profiling using surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS)

Urine samples were thawed and centrifuged at 1400g for 5 min. Samples were diluted in the appropriate buffer to provide an equal urinary creatinine concentration of 50 nmol/100 µl.

To establish conditions for assessing urinary proteomics to distinguish between disease groups, samples from normal control, inactive and active LN patients were analysed using normal phase (NP20), hydrophobic (H4), immobilized metal affinity capture (IMAC30), strong anion exchange (SAX2, pH4 and pH9 buffers) and weak cation exchange (CM10, pH4 and pH7) ProteinChips according to the manufacturer’s instructions (Ciphergen, Fremont, CA, USA) with α-cyano-4-hydroxycinnamic acid (CHCA) and sinapinic acid (SPA) matrices (Ciphergen and Fluka/Sigma-Aldrich, Poole, UK) and low and high laser intensities (205 and 225). IMAC30 ProteinChips were found to produce the most reproducible profiles, with numerous discrete protein ions detected. SPA matrix at 100% saturation with low laser intensity provided the best spectra in the range m/z range 2500–100000, and so these conditions were used for all further experiments.

In subsequent experiments, IMAC-30 ProteinChips were pre-treated with 100 mM CuSO4, neutralized with 0.1 M sodium acetate and washed with binding buffer (0.1 M sodium phosphate/0.5 M NaCl, pH 7). Either human serum albumin (HSA 99% pure, fatty acid free, essentially globulin free, Sigma-Aldrich) at concentrations of 1 µg–100 µg/spot (equivalent to 100 µg–10 mg/ml in urine) or LN urine samples were then applied to the ProteinChip. Samples from patients with urinary tract infections (UTIs) were excluded from the biomarker discovery phase. In some experiments, albumin was spiked into urine samples on the ProteinChip. Imidazole (0.1–10 mM, Sigma-Aldrich) was also added to urine samples to compete for binding to the ProteinChip surface. Quality control (QC) samples, which consisted of pooled urine samples from two active SLE, two inactive SLE and two control donors, were also applied to the ProteinChips. Following sample application, ProteinChips were incubated for 1h at room temperature with horizontal shaking at 1050 rpm, washed three times in binding buffer and rinsed in H2O. The spots were allowed to air dry for 10 min before adding 2 x 0.5 µl SPA matrix (Fluka). ProteinChips were then read using the Ciphergen ProteinChip Reader Iic with Ciphergen ProteinChip software, version 3.2. Data were collected in the range 0–100 000 m/z by averaging 82 laser shots using a laser intensity of 205.

Data analysis

All samples were analysed in duplicate. The normalization factor was calculated for each duplicate; all samples fell within the range 0.8–1.2. Mean intensity values were calculated for each protein ion. To examine reproducibility, QC samples were assessed and 10 peaks were compared for each ProteinChip. The coefficient of variation ranged from 17.0 to 25.5% (mean 22.4 ±0.6), which is comparable with figures published previously [12, 13].

Initially, the peak clusters in the m/z range 2500–100 000 were identified using the ProteinChip Biomarker Wizard software version 3.2 for peaks with a signal-to-noise ratio of ≥2.5. After correction for total protein, the data were normalized by log-transformation. The identified protein ions that showed significant differences between the groups (Student’s t-test) were then examined visually to ensure that the peaks were discrete. Any protein ions representing albumin (see subsequently) were disregarded. The remaining protein ions were then analysed using discriminant analysis (Systat, version 10.2, Richmond, CA, USA) to identify combinations of proteins that best discriminate between disease states. A logistic regression (LR) model was also built using the same protein ions to calculate prediction scores for each sample, allowing us to construct a receiver operating characteristic (ROC) curve based on these values. Specificity and sensitivity values for the traditional LN markers urinary protein/creatinine ratio, serum creatinine, complement factors three and four and double stranded DNA (dsDNA) were also calculated for the patient groups.
For the prospective longitudinal study, multiple regression (MR) scores were calculated using a linear equation derived from the discriminant analysis canonical discriminant functions, where \[ Y = a + (b_1 \times X_1) + (b_2 \times X_2) \] \[ (a = -2.965, b_1 = 1.18, X_1 = \log(\text{m}/\text{z} 3340 \times \text{protein}), b_2 = 0.853 \text{ and } X_2 = \log(\text{m}/\text{z} 3980 \times \text{protein}) \]. An MR score of more than 0.4 was used to classify active from inactive patients, this value being derived following an examination of the MR scores of all of the correctly classified patient samples as designated by the discriminant analysis.

**Results**

**Patient characteristics**

Patients and normal control donors were well-matched for urinary creatinine content and age. Active SLE patients had high levels of proteinuria and urinary protein/creatinine ratios, with intermediate levels in inactive SLE samples and low levels in normal controls. A large proportion of SLE patients were female due to the sex-linked prevalence of this disease (Table 1).

**Identification of urinary protein profiles associated with SLE**

Before analysing our full patient sample set, we investigated the contribution of albumin to the protein profiles. Attempts to remove albumin from the urine using immunoprecipitation or a Vivapure anti-HSA kit (Vivascience, Hanover, Germany) were unsuccessful for these samples (results not shown). The reason for this was not established, although acceptable depletion of serum albumin was achieved. To ensure that any candidate biomarkers selected were not ions representing intact albumin, we analysed HSA applied to IMAC30 ProteinChips at concentrations ranging from 1 \( \mu \text{g} \) to 10 \( \mu \text{g} \)/spot (equivalent to 100 \( \mu \text{g} \)--10 mg/ml albuminuria), and examined the spectra produced. In all, 21 protein ions were found that were derived from the albumin preparation (Fig. 1). As the MS method used cannot fragment ions the spectra produced would appear to represent both singly \((z = 1)\) and multiply \((z > 1)\) charged ions of albumin and other proteins and peptides present in the albumin preparation.

Urine samples from active and inactive LN patients were analysed using IMAC30 ProteinChips, and the Ciphergen Biomarker Wizard software was used to identify peaks representing protein ions with a signal-to-noise ratio of \( \geq 2.5 \). In the \( m/z \) range 2500–100 000, 80 protein ions were detected that were present in more than 10% of the samples. After correction for total protein, the data were normalized by log-transformation, and those ions that showed significant differences in intensity \((P < 0.01\text{, Student’s } t\text{-test})\) between the two groups were selected for further analysis. Non-discrete peaks were discounted \((n = 36)\) and peaks corresponding to albumin were also excluded from further analyses.

To examine whether the amount of albumin present in the urine could interfere with the expression of any potential biomarker, we spiked active urine samples with purified albumin and examined the effect on the six protein ions that showed the greatest significant differences between groups \((m/z 3340, 3980, 4095, 4310, 5465\text{ and } 7965)\). There was no significant difference in ion intensity on addition of up to 1 mg/ml albumin (Fig. 2). At 10 mg/ml, albumin reduced the expression of two of these protein ions \((m/z 4095 \text{ and } 4310)\) but had no effect on the remaining four. In addition, albumin binding to the ProteinChip surface

**Table 1. Patient characteristics**

<table>
<thead>
<tr>
<th></th>
<th>Active SLE ((n = 26))</th>
<th>Inactive SLE ((n = 49))</th>
<th>Normal control ((n = 15))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteinuria (g/l)</td>
<td>2.15 ± 0.39</td>
<td>0.81 ± 0.39</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>Urinary creatinine (mmol/l)</td>
<td>9.79 ± 0.98</td>
<td>10.72 ± 0.87</td>
<td>8.2 ± 2.1</td>
</tr>
<tr>
<td>Protein/creatinine ratio (mg/mmol)</td>
<td>221.02 ± 31.99</td>
<td>45.71 ± 10.90</td>
<td>4.15 ± 2.58</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>37.69 ± 2.51</td>
<td>43.72 ± 1.71</td>
<td>37.4 ± 3.2</td>
</tr>
<tr>
<td>Sex, female (%)</td>
<td>84.6</td>
<td>90</td>
<td>50</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM.

**Fig. 1.** Protein ion profile of human serum albumin. Purified human serum albumin was applied to IMAC30 ProteinChips at an excess of 100 \( \mu \text{g} \)/spot (equivalent to 10 mg/ml in urine) and analysed using SELDI-TOF MS. (A) full \( m/z \) range. (B) \( m/z \) 2500–5000. In all, 21 protein ion peaks with \( m/z \) of 2610, 2730, 2910, 3060, 3255, 3420, 3530, 3600, 3660, 3780, 4420, 8330, 9010, 13785, 15760, 22150, 33220, 44420, 59135, 66370 and 88405 were found that were derived from albumin.

**Fig. 2.** The effect of albumin on protein ion detection. Active lupus nephritis urine samples were applied to IMAC30 ProteinChips either alone (unfilled bars) or in the presence of 0.1 (diagonal hatching), 1 (black fill) or 10 mg/ml human serum albumin (stippled bars) and analysed using SELDI-TOF MS. The six protein ions that previously showed the most significant differences between samples from active and inactive patients were examined. Results are expressed as fold change in relative ion intensity of untreated urine. Asterisks denote significant difference from control \((P < 0.01\text{, Student’s } t\text{-test}\), \(n = 4\)).
was attenuated by addition of imidazole to the samples. In the presence of 1mM imidazole albumin (m/z 66370) and the protein ions with m/z of 4095 and 4310 were significantly displaced whereas the remaining protein ions remained bound to the ProteinChip at this dose, suggesting that these proteins, which have a greater affinity for the chip surface, were not related to albumin (Fig. 3).

The 32 selected protein ions were analysed using discriminant analysis. The protein ions that best discriminated between the two groups were 3340 and 3980 resulting in the correct identification of 92% (45/49) of the inactive and 92% (24/26) of the active SLE patients (Table 2, Fig. 4). The addition of a third protein ion with an m/z of 4095 only slightly improved the classification (46/49 inactive, 24/26 active), and so further analysis was performed on the 2-protein ion model. LR analysis resulted in the selection of the same proteins as discriminant analysis. An ROC curve constructed from the LR scores gave a high area under the curve (AUC) value of 0.967 (Fig. 4). Comparison with the specificity and sensitivity of traditional markers of LN calculated from this data set, demonstrated that our markers had superior discriminatory powers (Table 3).

Prospective longitudinal study

To confirm that our candidate biomarkers were responsive to the disease state, we analysed serial samples from six patients who had biopsy results indicative of active disease within 3 weeks of urine collection (Fig. 5). Patient A’s renal biopsy was classified as ISN/RPS class IV-G A/C, patient B’s as class IV-S A/C, patient C’s as class III/IV A/C, patient D’s as class IV-S A/C, patient E’s as class IV-G A/C and patient F’s as class III A. The previous data established that an MR score of >0.4 denoted active disease. At onset, the 4/6 defined as active clinically (A–D) had appropriately high MR scores of between 0.877 and 2.37, and the two clinically inactive patients (E–F) had lower scores of 0.4 and ~1.18. Both inactive patients relapsed during the course of the study. Their MR score rose to active levels 3.5 and 2.7 months before clinical diagnosis of renal relapse. All four active patients went into remission with a fall in the MR score preceding clinical diagnosis of remission. Two of the six patients (D and F) had multiple episodes of relapse and remission that were reflected by parallel fluctuations in their score. Four patients had samples collected during follow-up, which tested positive for UTIs. In all cases, the presence of an infection did not affect the predicted score.

Discussion

We have used a proteomic technique, SELDI-TOF MS, to identify urinary protein ions associated with disease state in SLE, and discovered candidate biomarkers that can distinguish between active and inactive disease. Urine analysis provides a unique method of sampling the local conditions of the whole kidney. Distinctive urinary proteins may be generated not only by increased renal synthesis, but also by a variety of factors including a rise in circulatory levels, general effects of proteinuria and haematuria, impaired tubular absorption of protein and fragmentation of larger proteins [14–16]. A number of factors need to be considered when using urine to detect biomarkers, including variations in total protein content resulting from proteinuria or hydration state, the presence of proteases and variations in handling conditions, such as differences between first-void and midstream urines. Studies by Schaub et al. [12] and Rogers et al. [13] have examined some of these issues. They demonstrated that it is necessary to use midstream urines, but showed that the addition of protease inhibitors was found to have little effect on profiles obtained, as did the time from collection at 4°C to freezing. We, therefore, incorporated these findings into our protocol. Although SELDI-TOF MS is usually performed on samples containing standardized protein levels, in the case of proteinuric samples it would not be practical to dilute these samples to the levels found in a normal control. Preliminary studies demonstrated that proportionate protein profiles patterns remain the same after dilution of the sample, providing the less intense proteins are still detectable (data not shown). We have therefore normalized according to creatinine content, which removes concerns regarding the hydration levels of samples.

A major concern when examining proteinuric samples is the albumin content, as albumin could potentially block most of the binding sites on the ProteinChips, due to its molar excess, or multiply charged albumin could be selected as a candidate biomarker. Indeed, a recent paper [17] identified a protein ion with an m/z of 66kDa as a potential biomarker for acute renal injury. In that study, although dipstick analysis of albuminuria was negative their protein profiles clearly showed a range of peaks corresponding to those that we observed with albumin alone. We demonstrated that although 21 protein ion peaks were derived from intact albumin, these were not amongst the protein ions we selected as candidate biomarkers. Recent reports have described the presence of large quantities of albumin fragments in normal urine, particularly in the 300–500 Da range, derived from renal degradation of protein [18–20]. The proportion of peptide to total protein is reduced in renal disease and the fragments are larger suggesting inhibition of albumin degradation [19, 21, 22]. We cannot rule out the possibility that our candidate biomarkers

**Fig. 3.** The effect of imidazole on protein ion detection. To compete for binding to the ProteinChip surface, active lupus nephritis urine samples were applied either alone (unfilled bars) or in the presence of 0.1 (diagonal hatching), 1 (black fill) or 10 mM imidazole (stippled bars). Protein spectra were detected using SELDI-TOF MS. Albumin (m/z 66370) and the six protein ions that previously showed the most significant differences between samples from active and inactive patients were analysed. Results are expressed as fold change in relative ion intensity of untreated urine. Asterisks denote significant difference between control and 1 mM imidazole treatment (P < 0.05, Student’s t-test. n = 4). All protein ions showed a significant difference of P < 0.005 after treatment with 10 mM imidazole.

**Table 2.** Classification of inactive and active SLE urine samples determined using protein ions with m/z of 3340 and 3980

<table>
<thead>
<tr>
<th>Sample classified</th>
<th>Inactive SLE</th>
<th>Active SLE</th>
<th>Correctly identified (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inactive (n = 49)</td>
<td>45</td>
<td>4</td>
<td>92</td>
</tr>
<tr>
<td>Active (n = 26)</td>
<td>2</td>
<td>24</td>
<td>92</td>
</tr>
</tbody>
</table>

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are derived from albumin. If so, then they could represent either specific cleavage products derived from differential expression of proteases associated with disease activity or larger fragments remaining after reduced degradation. Such fragments could therefore still act as useful biomarkers as they would be specific for activity. Recent studies [23, 24] demonstrated that albumin in the serum, rather than being detrimental to SELDI-TOF MS analysis, actually acts as a carrier protein, binding a majority of low molecular weight markers (LMM) that would otherwise be lost, resulting in amplification of the detectable levels of markers. Here, urea/3-((3-Cholamidopropyl)dimethylammonio)-1-propanesulfonate (CHAPS) denaturation of the urine samples before application of the samples to the ProteinChips had no effect on the profiles obtained (data not shown), suggesting that matrix application and ionization sufficiently removes any LMM from the albumin for detection. The lack of effect of the denaturation step also demonstrates that LMM are not solely retained on the ProteinChips due to their association with albumin, suggesting that albumin does not saturate the binding sites on the ProteinChip in our samples. Co-incubation of the samples with imidazole also demonstrated that our candidate

**FIG. 4.** Candidate biomarker discovery. Urine samples from active ($n=26$) and inactive ($n=49$) lupus nephritis patients were analysed using SELDI-TOF MS. Using discriminant analysis two protein ions with $m/z$ of 3340 and 3980 were identified as best able to differentiate between the groups. Panels (A) and (B) depict representative expression of candidate biomarkers in inactive patients and active patients respectively. Asterisk denotes peaks derived from albumin, which were excluded from analysis. (C) Plot of log$_{10}$ normalized, total protein adjusted ion expression of the candidate biomarkers for active (filled circles) and inactive (open circles) samples. RII denotes relative ion intensity. (D) ROC curve for the two biomarkers was generated using logistic regression scores, with an area under the curve of 0.967.

**TABLE 3.** Comparison of traditional markers of lupus nephritis with SELDI-TOF-MS derived biomarkers

<table>
<thead>
<tr>
<th>Test</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SELDI-TOF-MS</td>
<td>92</td>
<td>92</td>
</tr>
<tr>
<td>Urinary protein/creatinine</td>
<td>77</td>
<td>92</td>
</tr>
<tr>
<td>Haematuria</td>
<td>42</td>
<td>84</td>
</tr>
<tr>
<td>dsDNA</td>
<td>31</td>
<td>67</td>
</tr>
<tr>
<td>Serum creatinine</td>
<td>15</td>
<td>81</td>
</tr>
<tr>
<td>C4</td>
<td>13</td>
<td>89</td>
</tr>
<tr>
<td>C3</td>
<td>17</td>
<td>72</td>
</tr>
</tbody>
</table>

* are derived from albumin. *
Biomarkers are not bound to albumin, although interestingly, the two protein ions that were affected by co-incubation with excess albumin showed similar displacement patterns to albumin, suggesting a possible association. Addition of imidazole to samples in future analyses may further improve classification by reducing albumin binding to the ProteinChip and allowing more binding of less abundant proteins.

The main aim of this study was to detect proteins that are closely associated with remission and relapse, which would allow us to tailor the use of immunosuppressive drugs to each patient. Analysis resulted in the correct identification of 92% of the inactive patients (4/49 misclassified) and 92% of the active patients (2/26 misclassified), with an ROC AUC of 0.967 using protein ion peaks with 
m/z\ of 3340 and 3980. Although these two protein ions provided the best separation, there were numerous other strong candidates that could also be used. We found that there was a high degree of correlation between the ion intensity of the majority of protein ions, which limits the number of peaks entered into the discriminant analysis model. These protein ions could potentially be useful in devising future bench-based methods. This early data is very promising, particularly as our sample groups are relatively small. A pilot study performed 12 months earlier with a smaller number of patient samples revealed consistent results (83% active and 86% inactive correctly identified using M3340 and M3980; \( n = 18 \) and 22, respectively), demonstrating the reproducibility of this technique with regard to both the stability of the stored urine samples and the ProteinChip reader setup. Comparison of the relative ion intensities of protein ion peaks of patient samples present in both studies revealed no significant change in expression of our candidate biomarkers (M3340: active; study 1: 25.2 ± 7.3, study 2: 25.9 ± 4.8, \( P = 0.928 \). Inactive; study 1: 11.5 ± 2.4, study 2: 14.1 ± 2.8, \( P = 0.481 \). M3980: active; study 1: 28.2 ± 8.3, study 2: 24.0 ± 5.8, \( P = 0.684 \). Inactive; study 1: 10.1 ± 2.4, study 2: 9.5 ± 2.0, \( P = 0.873 \)), further confirming reproducibility.

For the purpose of this study, we grouped all LN patients together, regardless of their ISN/RPS biopsy classification. Further work would be needed to determine if the approach used here could be applied to individual classes of nephritis to provide even greater accuracy of disease state assessment. Post-analysis scrutiny of the ‘misclassified’ samples revealed that three of the four samples from inactive patients were previously classified by earlier biopsies as class V membranous LN. They had negative serology and complement levels, but the protein/creatinine ratios of 49, 106 and 116 were suggestive of some persistent activity. The fourth misclassified inactive patient

\[ \text{Fig. 5. Sequential multiple regression scores for biopsied patients.} \]
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has not been biopsied in the last 15 yrs, but had negative serology and complement levels and a P/C ratio of 106 associated with stable mild renal impairment. This patient is representative of one of the most difficult clinical scenarios, namely deciding whether mildly increased proteinuria in a long-term patient signifies activity that warrants escalation of treatment. The two ‘misclassified’ active patients were also examined more closely. One of these had normal anti-dsDNA and complement levels and a P/C ratio of 122, suggesting that this may indeed be an inactive patient. The remaining misclassified active patient had abnormal serology and complement and a P/C of 103, however samples collected a month before and after the one used in this study had normal P/C ratios, and the patient was classified as inactive 7 months later. This may indicate that the patient was improving at the time of analysis. These observations, rather than highlighting problems with our test, demonstrate both the weaknesses in current classification methods and the potential of our candidate biomarkers to detect early signs of relapse/remission.

To examine the responsiveness of our candidate biomarkers in predicting activity and remission, we analysed serial samples from six patients who had been biopsied within the course of our study. In all cases, the MR score predicted a change in disease state prior to clinical classification, suggesting that the MR score could detect early onset of relapse and remission, thus allowing rapid tailoring of treatment. There is a very real need to identify non-invasive biomarkers associated with active LN for early diagnosis and optimization of therapy, so that progression to end-stage renal failure may be prevented. Numerous studies have attempted to identify these, without much success [25]. However, recent preliminary studies have identified potential urinary biomarkers associated with renal activity and disease classification in SLE [26–29]. Comparison of the specificity and sensitivity of our candidate biomarkers with other non-invasive markers, and the responsiveness of the biomarkers demonstrated during our follow-up study suggests that we have developed a superior method of determining activation state in LN.

Patients with UTIs were excluded from the initial biomarker discovery experiments to prevent any bacterial proteins being selected for further analysis. Of the six patients who were entered for the prospective longitudinal study, four had UTIs on at least one occasion. In all cases the infection had no effect on their classification. This was particularly noteworthy for two inactive samples, which retained their inactive classification. This further validates our study, as false-positive classification due to infection would have resulted in potential exclusion of all infected patients during routine monitoring using our biomarkers. We cannot rule out the possibility that these biomarkers are not specific for LN, and aim to examine whether these protein peaks are present in the urine samples of patients with non-LN. However, we did not set out to identify markers that could classify patients as having LN, but aimed to identify markers of activity, which may be useful for the early diagnosis of renal relapse and monitoring of immunotherapy.

The next stage in our study will be to analyse larger patient groups and to run blinded samples to confirm the usefulness of our currently identified biomarkers. After this confirmation, we will then isolate and identify these biomarkers. Recent studies have demonstrated that SELDI-TOF MS can be used to provide reproducible results between sites [30, 31], and so theoretically a diagnostic test could be based upon the candidate biomarkers we describe here. However, our principle aim is to use SELDI-TOF as a tool to detect proteins associated with glomerular change in LN. Once these proteins have been identified, we will devise assays, such as ELISA, to monitor renal involvement in SLE.

In conclusion, we have identified two proteins in the urine of patients with SLE using SELDI-TOF MS that allow us to distinguish between patients in remission and those with active renal disease without the need for invasive procedures. Identification of these proteins will allow us to devise tests to routinely monitor patients with LN, providing us with an opportunity for early diagnosis of relapse and remission. Therapy could therefore be tailored more accurately and promptly to each patient, resulting in improved patient outcome.

### Key messages

- Two candidate biomarkers have been detected that can distinguish between active and inactive lupus nephritis.
- These biomarkers can predict early relapse and remission.

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