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Proteomic profiling of urine: implications for lupus nephritis

Najla Aljaberi[a], **Michael Bennett**[b], **Hermine I. Brunner**[a], **Prasad Devarajan**[b]

[a]Divisions of Rheumatology, Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, OH, USA;

[b]Division of Nephrology & Hypertension, Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, OH, USA

Abstract

Introduction: Lupus nephritis (LN) is a common and significant manifestation, affecting 60% of adults and 80% of children with systemic lupus erythematosus, with up to 30% of patients progressing to end stage renal disease. There remains an unmet need for non-invasive markers of disease activity, damage, and response to therapy. In addition, non-invasive biomarkers that predict therapeutic efficacy are needed to enable cost-effective clinical trials of novel agents.

Areas covered: This review examines the methodological aspects of urinary proteomics, the role of proteome profiling in identifying promising urinary biomarkers in LN, and the translation of research findings into clinically useful tools in the management of LN.

Expert opinion: Targeted and unbiased proteomics have identified several promising urinary biomarkers that predict LN activity, damage (chronicity), and response to therapy. In particular, a combination of biologically plausible urinary biomarkers termed as RAIL (Renal Activity Index for Lupus) has emerged as an excellent predictor of LN activity as well as response to therapy, being able to predict efficacy within 3 months of therapy. If validated in additional large prospective studies, the RAIL biomarkers will transform the care of patients with LN, allowing for a personalized and predictive approach and improved outcomes.

Keywords

Lupus nephritis; systemic lupus erythematosus; biomarkers; urine

1. Introduction

Systemic lupus erythematosus (SLE) is a chronic multisystem autoimmune disease with a broad range of clinical presentations. Renal involvement in SLE is a common and significant

CONTACT Prasad Devarajan prasad.devarajan@cchmc.org Division of Nephrology & Hypertension, Department of Pediatrics, University of Cincinnati Co, Cincinnati, Ohio.

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Declaration of interest

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manifestation, affecting up to 60% of adult and up to 80% of pediatric SLE patients [1,2]. Lupus nephritis (LN) has been linked to a substantial burden of morbidity, with 10–30% of patients progressing to end stage renal disease (ESRD) [2]. Although improvements in all-cause mortality in LN patients with ESRD have recently been documented [3], there remains an unmet need for close monitoring, reliable evaluation and prompt treatment of LN because achievement of remission is strongly linked to improved patient survival rates [4]. Renal biopsy is still considered the gold standard tool for evaluation of LN activity and is a valuable tool in assessing response to treatment [5–7]. Given the invasive nature of biopsies with a sizeable risk of complications particularly in children, there continues to be a quest for alternative non-invasive methods that yield reliable markers of disease activity, damage, and response to therapy. In particular, non-invasive biomarkers that predict and early identify efficacy are needed to enable cost-effective clinical trials of novel agents.

Current standard noninvasive tools used in evaluating renal disease in SLE patients consist of measures such as complement levels (C3 and C4), anti-double stranded DNA (anti-dsDNA), and the quantification of total proteinuria using various techniques. Investigations of the usefulness and validity of these tests, however, have revealed substantial shortcomings in reflecting the degree of LN activity and damage [8–10]. The convenience and non-invasiveness of specific urine bio-markers have recently fueled international scientific efforts to identify better LN biomarkers and ultimately improve the unacceptably high morbidity and mortality currently associated with LN.

Approaches utilizing unbiased proteomic techniques to profile a wide array of urinary proteins has identified many interesting and potentially useful targets in various renal and extra-renal diseases [11–13]. This review is aimed at shedding light on the technical aspects of urinary proteomics, the role of proteome profiling in identifying promising urinary biomarkers in LN, and the translation of research findings into clinically useful tools in the management of LN.

2. Proteomic profiling of urine: methodological considerations

Urine is an ideal source for a biomarker because it can be collected non-invasively and contains relatively few proteins compared to blood. In addition, there is a paucity of active proteases in the urine, which limits biomarker degradation [14,15]. Proteins found in urine are filtered from the blood or are produced by inflammatory and resident kidney cells. Hence, urine may be a valuable source of biomarkers for both systemic and primary renal diseases. Proteins from the circulation are selectively filtered through the glomerulus, based on their dimensions, charge and conformation [16–18]. Urinary proteins of less than 45 kDa are freely filtered. Cationic proteins with higher molecular weight (MW) are passed more freely than anionic proteins, which are repelled by the negatively charged glomerular basement membrane. In the healthy kidney, certain abundant proteins, such as albumin, IgG, myoglobin, and vitamin D binding protein are avidly reabsorbed through receptor-mediated endocytosis in the proximal tubule [19–21]. As a result of selective filtration and reabsorption, urine from healthy individuals contains a relatively small number of proteins. Conversely, diseases that alter glomerular filtration or tubular protein reabsorption will result in proteinuria, which greatly increases both the number and concentration of proteins in the

urine. In kidney disease, many proteins appearing in the urine are derived directly from the tubular epithelium. This is especially true of inducible tubule damage markers such as neutrophil gelatinase associated lipocalin (NGAL) [22] and kidney injury molecule-1 (KIM-1) [23]. Damaged kidney tissue releases both induced and pre-formed proteins directly into the urine, thus rendering this biofluid as a useful source of renal specific disease markers.

Despite the value of urine as a source for proteomic evaluation of kidney disease, there are several considerations that must be made in order for studies to be successful. The urine proteome is highly variable between individuals. Men and women often display variances between levels of specific proteins. Further, there are age differences, especially within the pediatric population [24,25]. Therefore, When designing urinary proteomic experiments, it is important to match subjects and controls by age and gender. Further, there is marked variation of urine protein content due to hydration status, possibly even diurnal variations, as well as intra-individual variability due to factors such as exercise, diet, and lifestyle [26]. While it is likely that these factors cannot all be controlled, it is essential to implement standardized urine collection and processing protocols.

Current consensus is that urine samples for proteomic experiments should use the mid-stream urine collection from the second morning void, rather than the first morning void or urine from a 24 hour urine collection [27,28]. This is because of possible proteolysis of urine proteins when residing in the bladder for extended periods of time. Further, there is agreement to centrifuge the urine sample at low speeds (e.g. 800–2000 g) to remove cellular debris and storage of urine samples at −80°C prior to proteomic analysis. Notably, no more than a single freeze-thaw cycle should be allowed to minimize degradation of the urine proteome.

The addition of protease inhibitors to urine samples intended for urine proteomic experiments has long been a topic of debate. More recent evidence suggests that protease inhibitors should be omitted, especially when untargeted proteomics is planned. Protease inhibitors have not been shown to help with protein identifications, and may even interfere with protein digestion procedures that are commonly needed prior to performing advance proteomic experiments [15,29,30].

The biggest hurdle to successful proteomic evaluation of urine is normalization for urine protein amounts as is needed when performing comparative analysis of the proteome. Comparing differences in the composition of the urine proteome of patients with proteinuria to that of healthy individual is challenging because there is ~ 100 times less protein in urine samples from healthy individuals. Therefore, healthy individuals are unlikely appropriate controls for urine proteomic experiments. Instead, it is generally more practical to use controls who have been diagnosed with another disease with proteinuria due to a different mechanism of pathology than suspected for the disease process under investigation (i.e. disease controls) [31,32].

3. Potential roles for urine proteome profiling in lupus nephritis

Analogous to other renal disease states, urine proteome profiling represents a promising strategy to support the diagnosis, including the findings on kidney biopsy, and to assess the treatment response of LN. Thus, ongoing investigations in subjects with LN [33–36] have focused on characterization of urinary proteins for

- distinguishing histologic classes and kidney biopsy findings
- early identification of LN flares
- correlation with LN activity and chronicity
- assessing prognosis and response to therapy
- enabling clinical trials with novel therapeutic agents

The following sections of this review will summarize current knowledge regarding the use of both targeted and unbiased urine proteomic profiling studies investigating these roles in LN.

4. Targeted urine proteomics in lupus nephritis

Targeted proteomics informed by disease pathology holds some advantages compared to untargeted approaches. This approach investigates known proteins within identified pathophysiologic pathways as biomarker candidates. Such candidates enjoy a strong biologic plausibility, especially if they are known to be expressed in the kidney and upregulated in LN. In this section, we discuss the most promising urinary biomarkers of LN identified via targeted proteomics (Table 1).

4.1. Neutrophil gelatinase-associated lipocalin (NGAL)

Neutrophil gelatinase-associated lipocalin (NGAL) is a nephroprotective protein that is induced very early after injury in renal epithelial cells in the distal and collecting nephron segments, from where it is rapidly secreted into the urine. It has been successfully utilized as a non-invasive biomarker for acute kidney injuries in a wide number of studies [37]. NGAL protein is also well known to be induced and over-expressed in kidney tubule cells in human LN. Brunner et al. [38] explored the utility of urinary NGAL as a biomarker in pediatric LN. In a cross-sectional assessment of 35 SLE patients (17 of which had biopsy-proven LN), urinary NGAL levels were significantly higher in LN patients compared to children with lupus that did not have LN, and to disease controls with juvenile arthritis. NGAL levels correlated strongly with LN activity as measured by the renal domain score of the Systemic Lupus Disease Activity Index (SLEDAI) and with LN damage as measured by the Systemic Lupus Collaborating Clinics (SLICC) damage index scores. Conversely, urine NGAL levels were not associated with extra-renal SLEDAI scores. At a cut-off value of 0.6 ng/mg urinary creatinine, urinary NGAL was 90% sensitive and 100% specific in identifying LN patients [38]. The ability of urinary NGAL to differentiate between LN and non-LN patients with a high degree of sensitivity and specificity has been recently confirmed in other cohorts with different patient ethnicities [39,40]. In a subsequent longitudinal study by Brunner et al., urinary NGAL levels were associated with worsening renal disease in SLE patients. Urinary NGAL discriminates between WHO class IV and class V LN in a subset of patients with

biopsies within 2 months of the urine samples used to measure NGAL [41]. With regards to ability to predict a flare, urinary NGAL levels showed significant increase up to 3 months prior to LN flare [42]. Conversely, Kiani et al. reported the lack of association between urinary NGAL and measures of LN in a study of 107 SLE patients [43]. However, the majority of studies that utilized this marker reported its usefulness in predicting LN activity, flares, and worsening renal disease [38–42], rendering it a desirable target in this field.

4.2. Monocyte chemoattractant protein-1 (MCP-1)

Monocyte chemoattractant protein-1 (MCP-1) is a member of the family of chemokines regulating the migration and infiltration of inflammatory cells to target tissues [44]. The association of this chemokine with the development of LN has stimulated the study of its urinary excretion and implications for LN [45,46]. Urinary MCP-1 was found to be significantly elevated with active LN as compared to inactive LN, to extra-renal lupus activity, or to healthy controls [47,48]. The levels of urinary MCP-1 decrease with high dose intravenous steroids use to levels that are comparable to MCP-1 levels of healthy control urines. Likewise low urine MCP-1 levels are present during remission of LN [47]. Over time, urinary MCP-1 levels decrease significantly with anti-inflammatory therapy and are correlated with renal SLEDAI scores [49]. Rovin et al. demonstrated an elevation of urinary MCP-1 in LN patients starting 2 and 4 months prior to LN flares, a decrease in urinary MCP-1 levels upon improvement of LN activity and continuously high MCP-1 levels in non-responders to LN therapy [50]. Collectively, these findings support the usefulness of urinary MCP-1 as a predictor for LN activity, flares, recovery, and response to treatment. Cross-sectional baseline levels of urinary MCP-1 in LN patients also demonstrated significant associations with the histological class of LN ($p = 0.036$), with the highest levels present in class IV LN [51].

4.3. Vascular cellular adhesion molecule-1 (VCAM-1)

Vascular cellular adhesion molecule-1 (VCAM-1) is an adhesion molecule that mediates the adherence of inflammatory cells to target cells in the kidney [52]. Abd-Elkareem et al. [53] demonstrated that urinary VCAM-1 levels can help discriminate between histological classes of LN with the higher levels observed with WHO class III, IV and V LN compared to class I, II and non-LN patients. Urinary VCAM-1 levels may be associated with LN histologic activity index in urine samples obtained at the time of the biopsy [54]. Urinary VCAM-1 levels were found to correlate with renal damage as measured by the renal domain score of the SLICC damage index and with the degree of proteinuria but not with renal damage as seen on kidney histology [43].

4.4. Tumor necrosis factor-like weak inducer of apoptosis (TWEAK)

Tumor necrosis factor-like weak inducer of apoptosis (TWEAK) is a cytokine of the tumor necrosis factor ligand super family that is produced mainly by leukocytes and by renal tubular epithelial cells and glomerular mesangial cells [55]. Schwartz et al. found higher levels of urinary TWEAK in LN patients compared to non-LN patients, which were also associated with LN activity as measured by the renal domain score of the SLEDAI [56]. Urinary TWEAK concentrations were superior to anti-dsDNA and complement levels in reflecting LN activity [55]. Further, urinary TWEAK was found to significantly differ

among the different International Society of Nephrology/Renal Pathology Society (ISN/RPS) classes, with higher levels in class V than in classes III/VI, when compared to class II. However, urinary TWEAK levels were unsuited to distinguish between classes III and IV [57].

4.5. Interferon-γ-inducible protein 10 (IP-10)

Interferon-γ-inducible protein 10 (IP-10), also known as CXCL10, is a chemokine produced by inflammatory cells in response to interferon-γ promoting T-cell migration, adhesion molecule expression and inhibition of angiogenesis [58]. Serum levels of IP-10 have been shown to correlate with disease activity in SLE [59]. A study by El-Gohary et al. [60] supports this finding but also suggests that IP-10 levels cannot help in discriminating LN patients from lupus patients without LN. Notably, IP10 was inferior to the urine albumin/creatinine ratio in identifying LN patients. Avihingsanon et al. reported higher levels of urinary mRNA levels of IP-10 in class IV LN patients compared to other classes. Urinary IP-10 mRNA was also significantly lower in patients who responded to treatment when followed prospectively in a subset of patients [61], supporting the potential role of IP10–10 as a marker of LN response to therapy.

Taken together, the currently available targeted proteomic studies have identified urinary NGAL and MCP-1 as promising, biologically plausible biomarkers of LN activity, flares, response to treatment, and prognosis. As described in subsequent sections, both NGAL and MCP-1 comprise critical components of the Renal Activity Index for Lupus (RAIL) biomarker panel to accurately reflect LN activity and to early predict response to therapy. It should be noted that while both urinary NGAL and MCP-1 are highly sensitive biomarkers for LN, neither is specific. For example, urinary NGAL is known to be increased in many other forms of both acute and chronic kidney injuries, and MCP-1 over-expression is characteristic of several renal inflammatory disorders.

5. Proteomic identification of markers for lupus activity

Despite availability of promising candidate biomarkers in LN identified by targeted approaches, there remains an unmet demand for urinary protein markers to improve our ability to capture LN damage and response to therapy. Unbiased discovery proteomics allows for the discovery of biomarkers that are potentially more clinically relevant than those identified using hypothesis-driven targeted proteomics. In this section, we will review important studies using discovery proteomics in the field of LN.

One of the earliest studies using this approach was carried out by Oates et al. in which urine samples from 20 patients were analyzed by 2-dimensional gel electrophoresis (2-DE) [34]. Differentially expressed protein peaks showed a good correlation with the LN chronicity index ($r = 0.87$), which was better than that for the activity index ($r = 0.77$). Six of the most sensitive biomarker spots were subsequently identified as four proteins, using matrix-assisted laser desorption-ionization tandem mass spectrometry (MALDI-TOF-TOF) as: α−1 acid glycoprotein (AGP, also known as orosomucoid), α−1 microglobulin, zinc α−2 glycoprotein (ZAG), and IgG κ light chain. The study reported high sensitivities and specificities for the different classes of the ISN/RPS classes of LN. The proximity of the

urine samples collected to the time of the renal biopsy makes the results more likely to be reflective of the identified pathology. However, these findings will require replication in a separate cohort with larger number of patients. The same group used a similar approach to explore patterns of urinary proteins that could distinguish different glomerular pathologies (focal segmental glomerulo-sclerosis, membranous nephropathy, diabetic nephropathy and LN) [62]. Among the 32 enrolled patients, 11 were LN patients. A subset of the main cohort (16 patients) was used as a separate validation set of their algorithm, which revealed a sensitivity range of 75–86% and specificity range of 67–92% for the diseases in question and an AUC of 0.84 for LN. Proteins showing the highest sensitivities were identified as: AGP, transferrin, α−1 microglobulin, ZAG, α−1 antitrypsin (A1AT), complement factor B, haptoglobin, transthyretin, plasma retinol binding protein, albumin and hemopexin [62].

Mosley et al. employed surface enhanced laser desorption-ionization time-of-flight mass spectrometry (SELDI-TOF) in a part cross-sectional, part longitudinal study of LN patients. Two proteins were found to distinguish active LN patients from inactive LN with a specificity and sensitivity of 92% for each. Six patients from the main cohort were followed prospectively with repeat renal biopsies done within 3 weeks of urine collection. The two identified proteins were able to predict early onset of relapse and recovery when compared to standard parameters (urine protein/creatinine ratio, anti-dsDNA, hematuria, complement levels and serum creatinine) [63]. The prospective part of this study showed promise in the utility of urinary biomarkers for the prediction of disease flares, particularly linked to biopsy findings. However, a larger cohort would be required to substantiate these findings.

Proteomic profiling using SELDI-TOF was also utilized in the discovery of 8 urinary biomarkers in pediatric LN patients by Suzuki et al [33]. In this study of 32 SLE patients, patients with juvenile idiopathic arthritis (JIA) patients served as disease controls. Eight proteins demonstrated a robust peak intensity pattern being significantly higher in LN patients compared to non-LN SLE patients and controls. In addition, there was a correlation between the identified proteins and renal disease activity [33]. In a follow-up validation study, those proteins were identified as: transferrin (Tf), ceruloplasmin (Cp), α1-acid-glycoprotein (AGP), lipocalin-type prostaglandin D-synthetase (L-PDGS or PGDS), albumin, and albumin-related fragments. This study included a larger sample size of SLE and JIA patients and a prospective approach with serial plasma and urine samples collected every 3 months for 18 months [36]. The identified proteins were tested via enzyme-linked immunosorbent assay (ELISA) and nephelometry. Once again, the urinary concentration of all eight proteins was significantly higher in LN patients compared to inactive LN patients, non-LN SLE patients, and JIA controls. They also performed better than standard measures of renal disease activity, including the urine protein/creatinine ratio. With regards to flare prediction, urinary levels of Tf, AGP and L-PGDS were elevated up to 3 months prior to clinical worsening. The assessment of the link between these biomarkers and renal pathology was not carried out due to limited urinary samples proximal to renal biopsy time.

In a cohort from Thailand, Somparn et al. carried out proteomic analysis with 2-DE in 10 LN patients (5 active LN, and 5 inactive LN) [64]. Sixteen protein spots were expressed differentially and were identified using electrospray ionization quadrupole time-of-flight tandem mass spectrometry (ESI-Q-TOF MS/MS). Among those proteins, ZAG and PGDS

were selected to be validated in a cohort of 78 subjects (30 active LN, 26 inactive LN, 14 non-LN glomerular disease, 8 healthy controls). Using ELISA, urinary levels of ZAG and PGDS were both significantly elevated in active LN compared to inactive LN and healthy controls. However, when it came to non-LN glomerular disease, ZAG was not helpful in differentiating between that and active LN. It also correlated with other markers of glomerular injury such as urine protein/creatinine ratio and estimated glomerular filtration rate (eGFR) thereby decreasing its specificity for LN. On the other hand, urinary levels of PGDS were significantly higher in active LN versus inactive LN, non-LN glomerular disease as well as healthy controls. PGDS correlated with urine protein/creatinine ratio but not eGFR. ROC analysis revealed an AUC for PGDS of 0.730 for the diagnosis of active LN compared to inactive LN and non-LN glomerular disease. The findings from this validation study support PGDS as a potential disease-specific biomarker for LN [64].

A few other groups have used discovery proteomics in a longitudinal fashion to explore the differential expression of urinary proteins at different parts of LN flare cycles, and to subsequently predict flares and assess treatment response. Zhang et al. [65] studied urinary protein expression patterns at baseline, pre-flare, during flare and post-flare in 19 SLE patients with 25 flare cycles. Using SELDI-TOF, this group found 19 proteins that paralleled the flare cycle. Identification of these proteins revealed two isoforms of hepcidin (hepcidin-20 and hepcidin-25), A1AT and albumin. Hepcidin-20 increased 4 months prior to a flare and normalized at the flare time. On the other hand, hepcidin-25 decreased at the renal flare and returned to baseline 4 months after the flare [65].

Aggrawal et al. [66] used 2-DE in 88 SLE patients to identify urinary biomarkers in LN. In this study, 20 subjects were used as controls (rheumatoid arthritis, diabetic nephropathy without renal failure and healthy subjects) for the validation phase, which utilized ELISA for the identified proteins and took on a prospective approach. Three proteins were found to be significantly elevated in active LN patients compared to inactive LN: α−1 anti-chymotrypsin (ACT), haptoglobin, and retinal binding protein (RBP). In the validation phase, levels of ACT and haptoglobin were higher in active LN patients compared to inactive LN and controls and showed good correlation with renal and extrarenal SLEDAI scores. Levels of RBP, however, were not as distinctive in active LN patients and showed only moderate correlation with renal and extrarenal SLEDAI score. Upon follow-up at 6 and 12 months, all three biomarkers declined [66]. There was a lack of inter-current flares in the follow-up period which hindered the assessment of flare prediction in these biomarkers.

A recent study by Go et al. [67] investigated the predictive value of certain urinary biomarkers. in the prognosis of LN patients. The initial protein quantification phase used 8 SLE patients (4 LN patients, 4 non-LN patients) to detect significant proteins related to LN. This yielded the following proteins with the highest variance between LN and non-LN patients: vitamin D binding protein (VDBP), transthyretin and RBP. Validation by ELISA in a mix of 121 SLE patients (active LN, inactive LN and non-LN) was carried out afterwards. In this phase, PGDS was added as a biomarker since it was interestingly lower in LN patients (compared to non-LN) in the initial quantification phase. This is in contrast to other studies that found significant expression of urinary PGDS in LN. Upon validation, levels of all four biomarkers were elevated in active LN patients vs inactive LN. Of note, these levels

(except for VDBP) were also higher in inactive LN compared to non-LN patients. These biomarkers also correlated to the severity of disease activity with VDBP having the best correlation. Baseline values of these biomarkers were used to estimate the prognosis of LN patients over a four-year period. In 21 LN patients, RBP was lower in those achieving remission within 12 months, while other markers were not able to predict remission. In 41 inactive LN patients, followed for a mean duration of 43 months, urinary VDBP was predictive of flare after adjustment for multiple cofounders [67].

Using a more restrictive approach, Wu et al. employed an antibody-based protein array (testing for 274 different human proteins) and found urinary angiostatin to be significantly higher in 5 SLE patients compared to 3 healthy controls. A subsequent validation study in 100 SLE patients showed that urinary angiostatin was able to discriminate between active vs inactive LN patients with good correlation to SLEDAI, renal SLEDAI and SLICC scores. It was also the highest in class IV LN and correlated to the chronicity index, but not the activity index [68].

Table 2 provides a summary of the promising urinary bio-markers identified via discovery proteomics and subsequently validated via targeted proteomics. The abovementioned studies have provided a considerable insights into the urinary proteome in LN patients. The next steps would include: (1) further validation of some of those urinary biomarkers in independent cohorts of different demographics and disease features, (2) explore urinary biomarkers related to response to treatment as well as development of fibrosis and CKD, and (3) the use of high yield urinary biomarkers alone or in combination with other biomarkers/ disease indices to create an applicable clinical algorithm. This latter concept is further discussed in the section below.

6. Pediatric renal activity index for lupus

Using traditional non-invasive measures, it has remained difficult to estimate the degree of inflammation that can be seen on a kidney biopsy with lupus nephritis. With the objective of developing a non-invasive set of urine biomarkers that could accurately reflect histologic LN activity, Brunner et al., developed the Renal Activity Index for Lupus Nephritis, or RAIL [79].

As discussed previously, a collection of urinary biomarkers have been described over the years that can be used to help diagnose LN and anticipate renal flares [38,42,50]. In addition, there is evidence that certain urinary biomarkers are associated with histological features of LN on biopsy [80]. Brunner et al. [79] measured 16 of the most promising LN urinary biomarkers in a cohort of 47 patients diagnosed with juvenile-onset SLE that required a biopsy as part of their course of clinical care. The 16 urinary biomarkers studied were NGAL, MCP-1, ceruploplasmin, adiponectin, hemopexin, KIM-1, AGP, TGF-β, hepcidin, L-PGDS, transferrin, VDBP, microalbumin, EPCR, cystatin C, and L-FABP. Traditional measures of LN, such as the renal-SLEDAI, and the Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index (SDI) were also recorded for each patient. A single expert nephropathologist interpreted biopsy samples in a blinded fashion for the study per ISN/RPS classification. The NIH-AI

and TIAI activity indices were used to group patients based on activity levels, and normalized to the NIH-CI, which quantifies chronicity and renal damage. For the analysis, they used LN-activity as the dependent variable, defined as high vs. moderate vs. low on the NIH-AI and TIAI. They first performed univariate analysis on each of the candidate urinary biomarkers and traditional measures for their ability to distinguish high vs. moderate vs. low activity. If the marker had discriminate value with a p-value of less than 0.2, they were included in the multivariate analysis for inclusion in the RAIL. A stepwise multivariate regression was used to identify the best markers to include in the RAIL. This was performed both with raw, unadjusted biomarker values, as well as concentrations standardized by urine creatinine. The ability of the final components of the RAIL to accurately reflect LN-activity status was confirmed by both principal component analysis and linear discriminant analysis, both adjusted and unadjusted for NIH-CI. Algorithms were evaluated by ROC curve analysis, then optimal cutoffs were evaluated for sensitivity, specificity, positive and negative likelihood ratios. The authors found that a combinatorial model including NGAL, MCP-1, KIM-1, adiponectin, ceruloplasmin and hemopexin was the best at predicting LN-activity status as defined by both the NIH-AI and TIAI. No traditional measures of LN-activity remained in the pool of markers with the best ability to predict LN-activity. The appropriateness of the combinatorial biomarker panel discovered in this study was also supported by principal component and linear discriminant analyses.

Using the results from the stepwise regression, the authors proposed an algorithm based on log transformed, creatinine adjusted urine concentrations of the RAIL urinary biomarkers. The RAIL algorithm is as follows: RAIL score = −4.29–0.34 * NGAL − 0.06 * ceruloplasmin + 0.89 * MCP-1 + 0.18 * adiponectin − 0.65 * hemopexin + 0.62 * KIM-1. A resulting score of ≥ 0.39 correctly identified 90% of all cases with high LN-activity as defined by the NIH-AI, which is a more clinically available score than the TIAI. The false positive rate was only 14%. As this RAIL algorithm was developed in a pediatric population, it has subsequently become known as the ‘pediatric RAIL’ or pRAIL. The importance of this type of non-invasive measure is evident in the results, demonstrating that none of the currently utilized traditional measures of LN-activity performed as well as this novel biomarker panel. In addition, not only are these markers of diagnostic value, but since their functions in the kidney are known to be involved in mechanisms that protect the kidney from inflammatory damage (Table 3), they are valuable in further delineating potential mechanisms of LN disease pathology, and identification of novel therapeutic targets. However, it should be noted that while the RAIL biomarkers are highly sensitive biomarkers for LN, they are not specific. For example, both urinary NGAL and KIM-1 are increased in many other forms of both acute and chronic kidney injuries, and MCP-1 over-expression has also been described in other renal inflammatory disorders. It is also unclear at this time whether the RAIL biomarkers can predict short term and long term renal prognosis better than traditional markers such as proteinuria and estimated glomerular filtration rate.

7. Adult renal activity index for lupus

It is often desirable to perform preliminary proteomics and biomarker studies in a pediatric population, since the results are often less influenced by comorbid conditions (e.g. hypertension, diabetes, heart disease) and lifestyle factors (including smoking, alcohol

consumption, and medication use). Children constitute a more pristine population, resulting in a non-confounded vision of changes specific to disease processes. The results must then be validated in the broader population to maximize the potential clinical utility of the findings. In terms of the RAIL biomarker panel and algorithm, Gulati et al. [81], set out to validate the RAIL panel in adults with LN. They collected urine at the time of kidney biopsy from 79 adults with LN. These patients were enrolled in the Ohio State University LN Registry. In this study, 2 expert nephropathologists were used to score the biopsies in terms of LN activity. Biomarkers were measured in the same fashion as in the original pRAIL study, and the pRAIL algorithm applied to the adult results. They also re-ran the multiple logistic regressions to determine whether alternatively weighting the 6 urinary biomarkers would yield improved accuracy in the adult cohort (A-RAIL). It was found that the p-RAIL, when applied to this adult cohort, only had a fair ability to predict LN-activity status (AUC = 0.62). However, using the logistic regression to add different weighting to the urinary biomarkers (A-RAIL) led to an excellent ability to predict LN-activity status (AUC = 0.88). The creatinine adjusted A-RAIL algorithm proposed is as follows: A-RAIL = 0.21 + 0.67 * NGAL + 0.28 * MCP-1 − 0.12 * ceruloplasmin + 0.88 * adiponectin − 0.05 * KIM-1. A cutoff of −0.97 is used to predict high activity level. Of note, all five biomarkers included in the A-RAIL are also components of the p-RAIL. It should also be noted that results did not change significantly whether the results were adjusted for creatinine levels or not. This highlights the disagreement in the field regarding whether biomarker values should indeed be adjusted for creatinine, or whether it is unnecessary [82]. This discordance is especially heightened when discussing a growing pediatric population. As creatinine is a product of muscle mass, and increases directly in proportion to age, adjusting by creatinine will differentially influence biomarker level findings based on age [24,25,83].

In addition to age differences, it is also important to consider gender differences in biomarker levels. Bennett et al. [25] measured the p-RAIL markers in the Cincinnati Genomic Control Cohort. The cohort consists of healthy pediatric patients recruited as representative of the population of the 7 counties that comprise the Greater Cincinnati, Ohio area. The study consisted of 368 children, broken into 4 age groups (3 – < 5 years; 5 – < 10 years; 10 – < 15 years; and 15 – < 18 years) as well as by gender. It was found that all RAIL urinary biomarkers except hemopexin had a weak correlation with age. NGAL, KIM-1 and MCP-1 steadily increased with age, while Adiponectin had a weak negative correlation with age. As for gender effects, NGAL, hemopexin and ceruloplasmin were found to be significantly higher in females than males. These findings indicate that biomarker adjustments for both gender and real age (as a continuous variable) are important for the urinary biomarkers in the pRAIL algorithm. The authors used a dual statistical modeling system and as a result proposed parameters for adjusting for both age and gender. Whether these age and gender calculations are necessary to increase the accuracy of the pRAIL algorithm will need to be studied further before integrating the algorithm into routine patient care.

8. Response to LN therapy

While it is of importance to be able to predict LN-activity on an ongoing basis, it is even more important to be able to determine if the treatments a given patient are receiving are

being effective in treating their disease. It is estimated that 10% of children diagnosed with LN will develop end stage renal disease (ESRD) within 10 years of diagnosis [84], and there is a 22% mortality rate for pediatric ESRD patients with LN within 5 years of requiring renal replacement therapy [85]. These negative outcomes result, in part, from a lack of timely, effective non-invasive measures of response to treatment of LN. In the clinical setting, it can take over six months to determine whether a patient is responding to treatment by traditional measures. However, it is recommended by the American College of Rheumatology and the European League Against Rheumatism to adjust therapy for lack of improvement by 3 months [5,6]. Therefore, earlier indicators of improvement are urgently needed.

To determine if any of the promising urinary biomarkers for LN-activity could also be used to gauge response to therapy, Brunner et al [86], performed a longitudinal study of candidate biomarkers following 87 patients from biopsy to 12 months post therapy induction. It was hypothesized that a selection of urinary biomarkers could be used to predict response to therapy at 6 months post biopsy. In addition to the 6 RAIL urinary biomarkers, they measured alpha −1 acid glycoprotein (AGP), cystatin-C, hepcidin, LPGDS, LFABP, osteopontin, TGF-β, transferrin and VDBP. Patients received standard treatments of cyclophosphamide or mycophenolate mofetil, with most also receiving reninangiotensin system inhibitors. Of the 87 patients, 37 responded to treatment as indicated by inactive urine sediment plus decreased proteinuria (to ≤2 g/day) plus normal or stable GFR as determined by the modified Schwartz formula [87]. Fifty patients were considered non-responders. It is important to note that responders and non-responders did not differ significantly in LN-activity, LN-chronicity or renal function at the time of biopsy. Even at the time of biopsy, it was found that 7 urinary biomarkers differed between response groups. The greatest differences were found in mean levels of TGF-β and ceruloplasmin, followed by transferrin, AGP VDBP, hepcidin and LPGDS. With the exception of hepcidin, all urinary biomarkers were higher in nonresponders than responders. The RAIL biomarkers (in addition to transferrin AGP and VDBP) all markedly differed over time with responder status. All of these markers except NGAL differed significantly as early as 3 months, whereas all biomarkers including NGAL were statistically different at 6 months post biopsy. For gross changes in biomarker levels, all markers, except osteopontin, decreased over time regardless of responder status, but this occurred more significantly in responders. At the time of biopsy, none of the biomarkers had excellent accuracy (AUC ≥ 0.9) for predicting responder status. Individually adiponectin, AGP LPGDS, transferrin and VDBP, as well as the combined RAIL showed excellent (AUC ≥ 0.9) accuracy in anticipating response to therapy at 3 months post biopsy, regardless of race, age or extrarenal disease activity. This is a study with a strictly controlled procedure for the collection and storage of urine sample, and a large longitudinal cohort. While the RAIL markers have been validated as well in adults, the ability of those markers to predict response to therapy in that population still needs to be verified. Nonetheless, being able to anticipate response to LN therapy by 3 months post biopsy could yield significant improvements in individualized care for patients with LN and potentially better outcomes.

9. Expert opinion

Lupus nephritis (LN) is a common and significant clinical problem, affecting 60% of adults and 80% of children with systemic lupus erythematosus. An unacceptably high number of patients with LN (about 30%) eventually progress to end stage renal disease. Performing a kidney biopsy has long been the ‘gold standard’ for evaluating LN activity and response to therapy. However, this is an invasive procedure that is particularly risky in children with LN. There is therefore a major unmet need for non-invasive markers of disease activity, chronicity, and response to therapy. Fortunately, recent targeted as well as unbiased proteomic profiling studies have identified several promising urinary biomarkers. In particular, a combination of biologically plausible urinary biomarkers termed as RAIL (Renal Activity Index for Lupus) has emerged as an excellent predictor of LN activity as well as response to therapy, being able to predict therapeutic efficacy within 3 months of therapy, in children as well as adults with LN. The six RAIL biomarkers include neutrophil gelatinase-associated lipocalin, monocyte chemoattractant protein-1, ceruloplasmin, adiponectin, hemopexin, and kidney injury molecule-1. The RAIL biomarkers hold promise to transform the care of patients with LN, allowing for a personalized and predictive approach and improved outcomes. We do not anticipate that the RAIL biomarkers will completely replace or supplant the kidney biopsy. However, the biomarkers can complement the initial kidney biopsy findings, and allow for a more accurate determination of severity and activity. Since serial kidney biopsies are impractical, serial measurements of the RAIL biomarkers can provide critical information regarding changes in activity, anticipation of flares, progression to chronicity, and response to therapy. RAIL biomarkers can also provide surrogate end-points for response to therapy in investigational clinical trials as well as routine clinical practice.

However, there remain some challenges before the RAIL biomarkers can be widely adopted for clinical use. First, the biomarkers still need to be validated in additional prospective studies. While current studies point to a strong association of the RAIL biomarkers with LN activity and response to therapy, their reliability will need to be further established in order for them to replace a kidney biopsy, especially given the poor prognosis of inadequately treated LN on the one hand and the significant adverse effects of appropriate therapy on the other. Second, clinical platforms for the serial automated multiplexed measurement of the RAIL biomarkers need to be developed and validated. Reliable and cost-effective clinical systems based on electrochemiluminescence, microsphere-based magnetic fluorescence, and magnetic coated bead technologies are now available, and need to be adapted for the measurement of the RAIL biomarkers. Third, standard analyte characteristics for each of the RAIL biomarkers, including optimal sample preparation and storage protocols, stability studies, and effects of interfering agents need to be determined. Fourth, and perhaps most importantly, there needs to be a culture change in the field from reliance on traditional markers and kidney biopsies to the acceptance of biomarkers. We are well in the era of personalized and predictive medicine, ushered in by some of the remarkable proteomic advances in our understanding of LN as outlined in this review. Biomarkers are indispensable for the implementation of personalized medicine, yet the reliance on kidney biopsies and other traditional markers has hampered our ability to diagnose, treat, and even

clinically study LN. In our current clinical practice, we are routinely serially measuring multiplexed RAIL biomarkers in our patients with LN, beginning at the time of initial kidney biopsy. In addition to providing information regarding the activity and response to therapy, we envision a number of refinements in the near future. We predict the emergence of a subset of biomarkers that will differentiate ongoing and/or long-term damage from repair, enabling the early identification of a recovering kidney. We also envisage the emergence of biomarkers to predict the transition from acute damage to chronic injury, allowing the early identification of chronic kidney disease. Taken together, LN biomarkers will revolutionize our ability to personalize our understanding and management of the kidney, allowing us to tailor therapies based on specific molecular and proteomic pathways.

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Article highlights

- Lupus nephritis (LN) is a common and serious manifestation, affecting 60% of adults and 80% of children with systemic lupus erythematosus; about 30% of patients progress to end stage renal disease
- Kidney biopsy is the ‘gold standard’ for evaluating LN activity, but is an invasive procedure
- There remains an unmet need for non-invasive markers of disease activity, damage, and response to therapy
- In addition, non-invasive biomarkers that predict therapeutic efficacy are needed to enable cost-effective clinical trials of novel agents
- Targeted and unbiased proteomics have identified several promising urinary biomarkers that predict LN activity, damage (chronicity), and response to therapy
- In particular, a combination of biologically plausible urinary biomarkers termed as RAIL (Renal Activity Index for Lupus) has emerged as an excellent predictor of LN activity as well as response to therapy, being able to predict efficacy within 3 months of therapy, in children as well as adults with LN
- The six RAIL biomarkers include neutrophil gelatinase-associated lipocalin, monocyte chemoattractant protein-1, ceruloplasmin, adiponectin, hemopexin, and kidney injury molecule-1
- RAIL biomarkers will transform the care of patients with LN, allowing for a personalized and predictive approach and improved outcomes

Table 1.

Urinary proteins identified in lupus nephritis using a targeted approach.

Marker	Publication (n = SLE patients)	Essential Findings
Neutrophil gelatinase-associated lipocalin (NGAL)	Brunner, 2008 (n = 35) [38] El Shahawy, 2018 (n = 70) [39] Gomez-Puerta, 2018 (n = 120) [40]	Elevated in active LN vs inactive LN and control. AUC 0.96 for active LN. Discriminates between class IV and class V LN. Elevated 3 mo prior to LN flare and remained high during flare. Levels responsive to therapy.
Monocyte chemoattractant protein-1 (MCP-1)	Noris, 1995 (n = 10) [47] Marks, 2010 (n = 25) [48] Abujam, 2013 (n = 13) [49] Rovin, 2005 (n = 41) [50] Singh, 2012 (n = 20) [51]	Elevated in active LN vs inactive LN and control. Elevated 2–4 mo prior to LN flare. Levels responsive to therapy.
Vascular cellular adhesion molecule-1 (VCAM-1)	Kiani, 2012 (n = 107) [43] Abd-Elkareem, 2010 (n = 30)[53] Singh, 2012 (n = 74) [54]	Elevated in active LN vs inactive LN and control. Higher levels in class III, IV, and V LN.
Tumor necrosis factor-like weak inducer of apoptosis (TWEAK)	Sun, 2018 (n = 65) [55] Schwartz, 2009 (n = 30) [56]	Elevated in active LN vs inactive LN and control. Higher levels in class V LN.
Interferon-γ-inducible protein-10 (IP-10)	El-Gohary, 2016 (n = 30) [60]	Elevated in active LN vs inactive LN and control. Inferior to urine albumin in identifying LN patients.

Table 2.

Urinary proteins identified in lupus nephritis via discovery proteomics and subsequent validation studies.

Marker	Discovery Proteomic Study: Publication (n = SLE patients)	Targeted Proteomic Study: Publication (n = SLE patients)	Findings
Zinc alpha-2 glycoprotein (ZAG)	Oates, 2005 (n = 20) [69] Varghese, 2007 (n = 11) [62] Somparn, 2012 (n = 10) [64]	Somparn, 2012 (n = 56) [64]	Elevated in active LN and non-LN glomerular disease vs inactive LN. Not specific for LN.
Transferrin (Tf)	Varghese, 2007 (n = 11) [62] Suzuki, 2007 (n = 32) [33]	Suzuki, 2009 (n = 98) [36]	Elevated in active LN vs inactive LN and control. AUC 0.8 for active LN. Elevated 3 mo prior to LN flare and remained high during flare.
		Brunner, 2012 (n = 76) [80]	Not correlated with rSLEDAI. Combined Tf + GFR + C4 + MCP-1 + AGP = AUC 0.75 for detecting membranous LN.
		Smith, 2017 (n = 30) [70]	Discriminated between active and non-LN. Combined AGP + CP + PGDS + TF = AUC 0.991 for active LN.
		Urrego, 2018 (n = 120) [71]	Elevated in active LN vs inactive and non-LN. Correlated with SLEDAI and proteinuria.
Alpha-1 glycoprotein (AGP)	Oates, 2005 (n = 20) [69] Suzuki, 2007 (n = 32) [33]	Suzuki, 2009 (n = 98) [36]	Elevated in active LN vs inactive LN and controls. AUC 0.76 for active LN. Elevated 3 mo prior to LN flare and remained high during flare.
		Brunner, 2012 (n = 76) [80]	Correlated with rSLEDAI. Combined MCP1 + Cp + AGP + PCr = 0.85 for activity index. AGP + CP + PGDS + TF = AUC 0.991 for active LN.
		Watson, 2012 (n = 60) [72]	Elevated in active LN vs inactive LN and healthy controls.
Prostaglandin D-synthase (PGDS)	Suzuki, 2007 (n = 32) [33] Somparn, 2012 (n = 10) [64]	Suzuki, 2009 (n = 98) [36]	Elevated in active LN vs inactive LN and controls. AUC 0.71 for active LN. Elevated 3 mo prior to LN flare and remained high during flare.
		Somparn, 2012 (n = 56) [64]	Elevated in active LN vs inactive LN, non-LN glomerular disease and healthy controls. AUC 0.73 for active LN.
		Brunner, 2012 (n = 76) [80]	Correlated with rSLEDAI. Combined AGP + CP + PGDS + TF = AUC 0.991 for active LN.
		Go, 2018 (n = 121) [67]	Elevated in active LN vs inactive LN. Higher in inactive LN vs non-LN. Correlated with urine PCr and rSLEDAI.
Alpha-1 antitrypsin (A1AT)	Varghese, 2007 (n = 11) [62] Zhang, 2008 (n = 19) [65]	NA	NA
Hepcidin	Zhang, 2008 (n = 19) [73]	Mohammed2014 (n = 60) [74]	Elevated in active LN vs non-LN. Among LN, elevated hepcidin in class IV LN vs class II/III.
		Zhang, 2012 (n = 61) [75]	Elevated in tubulointerstitial inflammation. Poor performance to classify the severity of interstitial inflammation when used alone.
Transthyretin	Varghese, 2007 (n = 11) [62] Go, 2018 (n = 8) [67]	Go, 2018 (n = 121) [67]	Elevated in active LN vs inactive LN. Higher in inactive LN vs non-LN. Correlated with urine PCr and rSLEDAI.
Retinol-binding protein (RBP)	Varghese, 2007 (n = 11) [62] Somparn, 2012 (n = 10) [64]	Sesso, 1994 (n = 70) [76] Marks, 2005 (n = 21) [77]	Elevated in active LN vs inactive and non-LN patients. Elevated in active LN vs inactive LN.
	Aggarwal, 2017 (n = 88) [66] Go, 2018 (n = 8) [67]	Aggarwal, 2017 (n = 88) [66]	Elevated in active LN vs inactive LN, RA and healthy controls. AUC 0.7 for active LN. Decreased at 6 & 12 mo upon follow-up.

Marker	Discovery Proteomic Study: Publication (n = SLE patients)	Targeted Proteomic Study: Publication (n = SLE patients)	Findings
		Go, 2018 (n = 121) [67]	Elevated in active LN vs inactive LN. Higher in inactive LN vs non-LN. Correlated with urine PCr and rSLEDAI. Baseline level lower in patients achieving remission within 12 months.
Vitamin D binding protein (VDBP)	Go, 2018 (n = 8) [67]	Go, 2018 (n = 121) [67]	Elevated in active LN vs inactive LN. Correlated with rSLEDAI. Baseline level predictive of flare upon follow-up.
Haptoglobin	Varghese, 2007 (n = 11) [62] Aggarwal, 2017 (n = 88) [66]	Aggarwal, 2017 (n = 88) [66]	Elevated in active LN vs inactive and non-LN. Correlated with SLEDAI and rSLEDAI. AUC 0.86 for active LN.
Ceruloplasmin (Cp)	Suzuki, 2007 (n = 32) [33]	Suzuki, 2009 (n = 98) [36]	Elevated in active LN vs inactive LN and control. AUC 0.68 for active LN. Not consistent with flare cycle.
		Urrego, 2018 (n = 120) [71]	Elevated in active LN vs inactive and non-LN.
Angiostatin	Wu, 2013 (n = 5) [68]	Wu, 2013 (n = 100) [68]	Elevated in active LN vs inactive LN. Correlated with SLEDAI, rSLEDAI, SLICC and chronicity index. Highest in class IV LN.
		Mok, 2018 (n = 227) [78]	Elevated in active LN vs active SLE, stable SLE and healthy controls. AUC 0.87. Correlated with SLEDAI, rSLEDAI, and PCr. No correlation with histology.

Table 3.

Characteristics of the RAIL Urinary Biomarkers.

Biomarker	Size	Renal Source	Function in Kidney
NGAL	25 kDa (monomeric)	Distal and proximal nephron	Nephroprotective, bacteriostatic, pro-proliferative, iron chelation
MCP-1	13 kDa	Epithelial, endothelial, mesangial, and fibroblasts	Regulates inflammatory infiltration into kidney tissue
KIM-1	80 kDa (ectodomain)	Proximal nephron	Nephroprotective, enhances apoptotic removal of damaged cells
Adiponectin	30 kDa	None - from adipocytes	Inhibits cell death, suppresses inflammation
Hemopexin	52 kDa	None - from liver	Heme chelation, anti-oxidant, anti-inflammatory
Ceruloplasmin	132 kDa	Glomerular epithelium	Anti-oxidant