Urinary biomarkers in childhood Lupus Nephritis

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Abstract

Juvenile-onset systemic lupus erythematosus (JSLE) is a rare, severe multisystem autoimmune disease affecting the kidney (Lupus Nephritis, LN) in up to 80% of children. LN is more severe in children than adults, with potential for irreversible kidney damage requiring dialysis or transplant. Renal biopsy is currently the gold standard for diagnosing and monitoring LN, however, it is invasive and associated with complications. Urine biomarkers have been shown to be better than serum biomarkers in differentiating renal disease from other organ manifestations. Over the past decade, there have been an increasing number of studies investigating specific candidate biomarkers implicated in the pathogenesis of LN or screening for urinary biomarkers using hypothesis free methods. In this review, developments in urine biomarkers for LN will be reviewed, highlighting those that are of relevance to children and have gone through validation in independent international patient cohorts, bringing them close to clinical translation.

Keywords

Urine, biomarkers, Lupus Nephritis, Childhood Lupus, JSLE

Abbreviations

AGP = α-1 acid glycoprotein,
ANA = anti-nuclear antigen
AUC = area under the curve
β2MG = Beta-2-microglobulin
BILAG = British Isles Lupus Assessment Group score
C3 = complement factor 3
C4 = complement factor 4
CKD = chronic kidney disease patients
CrCl = creatinine clearance
CXCL16 = Chemokine (C-X-C motif) ligand 16
CXCR3 = Chemokine receptor 3

Fn14 = fibroblast growth factor-inducible 14
FLCs = free light chains
FSGS = focal segmental glomerulosclerosis
GFR = glomerular filtration rate
HC's = healthy controls
IFN-γ = interferon gamma
IL = interleukin
IP-10 = Interferon-γ-inducible protein 10
ISN/RPS = International Society of Nephrology / Renal Pathology Society
JIA = juvenile idiopathic arthritis
JSLE = Juvenile-onset systemic lupus erythematosus
K-FLC= κ free light chain
KIM-1 = kidney injury molecule 1
L-FLC= lamda free light chains
LC-MS = Liquid chromatography coupled to mass spectrometry
LC/MS/MS = liquid chromatography-nanospray tandem mass spectrometry
LN = lupus nephritis
MALDI-TOF MS = Matrix-assisted laser desorption/ionization time-of- flight mass spectrometry
MCP-1 = Monocyte chemoattractant protein-1
NIH = National Institute for Health
NGAL = Neutrophil gelatinase associated lipoclain
PGDS = Prostaglandin-H2-D isomerase, alternative name LPGDS
RA = rheumatoid arthritis
RANTES = regulated on activation, normal T cell expressed and secreted
RBP-4 = retinol binding protein 4
ROC = receiver operating curve
SELDI-TOF = Surface-enhanced laser desorption/ionization time of flight technology
SLE = systemic lupus erythematosus
SLEDAI = SLE disease activity index
SLICC = Systemic Lupus Collaborating Clinics
TF = transferrin
TWEAK = Tumor necrosis factor (TNF)-like weak inducer of apoptosis
VCAM-1 = Vascular cell adhesion molecule-1
WHO = world health organization
ZA2G = Zn-alpha2-glycoprotein

1 Introduction

Juvenile-onset systemic lupus erythematosus (JSLE), also know as childhood-onset SLE, is a rare, severe multisystem autoimmune disease. Twenty percent of cases of SLE are diagnosed during childhood, with up to 80% of JSLE patients developing lupus nephritis (LN) [1], compared with 40-50% or adults [2,3]. LN may be part of the patients' initial presentation or occur later in the disease. Renal biopsy is currently the gold standard for diagnosing LN, characterizing disease activity and providing insight into the degree of scarring and irreversible damage to the kidney [4,5]. However, the invasive nature of the procedure, risk of complications such as bleeding or infection, and need for anaesthetic in children, lead to a tendency for the procedure to be avoided until deemed absolutely necessary. Clinicians therefore rely heavily upon less invasive markers including proteinuria, serum creatinine, glomerular filtration rate (GFR), urine microscopy, immunological, hematological and

inflammatory biomarkers when monitoring patients and making a decision whether to undertake a renal biopsy. At present, there is no consensus as to the optimal timing of an initial renal biopsy for diagnosing LN in children, with clinical concern that irreversible renal damage may occur prior to the onset of clinically overt disease [6].

Following a histological diagnosis of LN, on-going monitoring tends to again rely on the above blood and urine tests, however, these investigations have many limitations which require recognition. Following a LN flare, proteinuria can take a significant period of time to normalize, making it difficult to differentiate proteinuria due to irreversible damage of the glomerular capillaries or on-going LN activity [7]. Changes in serum creatinine tend to lag behind changes in glomerular filtration rate and are also influenced by the child's age, gender and height [8]. Of concern, there have been reports of 'clinically silent LN' in patients with biopsy defined LN but no proteinuria, normal urinalysis and normal renal function [9]. Repeat biopsy of patients who have shown a complete clinical renal response has found approximately one third to have on-going evidence of LN activity, and approximately 60% to display chronicity index features [10]. Normal complement and anti-dsDNA antibodies may provide reassurance that active LN is improbable, however, studies looking at the accuracy of these tests for differentiating between patients with active and in-active LN have shown conflicting results [11-13].

2 Pursuit of novel biomarkers for LN

Clearly, the performance of conventional blood and urine tests for LN diagnosis and monitoring is inadequate. LN therapy could be more effective, treatment toxicity limited, and renal outcomes improved if LN onset, severity, and treatment responsiveness could be predicted. Early treatment and rapid induction of LN remission has been shown to improve renal survival, highlighting the need for improved LN detection and monitoring [14,15]. To this end, novel, non-invasive biomarkers of disease activity and prognosis are increasingly being investigated. To translate an experimental biomarker into clinical practice, Mischak et al [16] describe a six step process which we should be mindful of when evaluating novel biomarkers studies. This process includes: (1) initial biomarker identification / verification, (2) evaluation of the results by independent experts, (3) evaluation in a suitable bio bank of existing samples or newly collected samples, (4) evaluation in a clinical trial, (5) implementation in clinical practice, and (6) proving the cost-effectiveness of the validated biomarker. The majority of LN urine biomarker studies to date have focused upon step one to three of this process.

3 The importance of assessing all novel biomarkers in children as well as adults

Juvenile and adult onset SLE differ in many ways. Juvenile onset disease is associated with more severe disease phenotype, including more renal involvement [1,17-21] and damage than in adult counterparts [17]. Compared with adult onset (18-50 years) and late onset SLE (>50 years old), juvenile onset patient have been shown to be at highest risk of renal failure and mortality [22]. Approximately 10% of children with proliferative nephritis progress to end-stage renal disease within 5 years [23], with irreversible renal damage remaining as one of the most common long-term consequences seen in JSLE [24]. It is anticipated that the differences in LN severity between juvenile and adult onset SLE populations may bear influence on the level of biomarkers reflecting the degree of renal injury within such populations. Urine biomarkers which are derived from plasma proteins may also show differences in their levels due to age dependent differences in plasma levels of the given protein. Childhood specific biomarker discovery and validation studies are therefore

important to explore these issues and in view of the fact that children tend to have fewer co-morbidities (e.g. diabetes, cardiovascular disease or hypertension) which can confound the results of urine biomarker studies. Consequently, it may be that a biomarker is useful in a paediatric but not an adult SLE population, or vice versa. This may account for some of the differences in proteins identified from paediatric and adult SLE urine proteomic studies [25-27]. In light of such observations it has been proposed that all biomarker studies should be concurrently carried out on children as well as adults with SLE [28].

4 Why urine?

In LN, urine is increasingly becoming recognized as the most useful and desirable medium for biomarker discovery due to the non-invasive nature of sample collection and its ability to reflect kidney damage, given its close proximity to native renal cells. Plasma and serum are more complex sample types which are in contact with multiple organs and therefore less likely to yield organ specific biomarkers. The smaller number of core proteins in urine than plasma / serum (approximately 2000 vs. 10,000) also make urine a better medium for harvesting biomarkers [29,30].

Under physiological conditions, a relatively small amount of protein is excreted into the urine due to the highly efficient glomerular filtration barrier which consists of fenestrated endothelium, the collagenous glomerular basement membrane, and podocytes, with their interdigitating foot processes and slit diaphragms. These three layers restrict movement of proteins into the urine on the basis of their molecular weight, size and electrical charge. In glomerular diseases such as LN, this barrier is disrupted leading to leakage of plasma / serum proteins alongside secretion of specific proteins reflective of the inflammatory state and kidney damage [31]. The pathogenesis of LN is known to involve multiple inflammatory cells, cytokines, chemokines, adhesion molecules and their receptors. Efforts to identify urinary biomarkers to date have related to specific candidate biomarkers implicated in the pathogenesis of LN or hypothesis free biomarker screens (e.g. using proteomic techniques). In the next section, developments in biomarkers for LN will be reviewed, highlighting those that have gone through validation in independent patient cohorts and are of particular relevance to children.

5 Urine biomarkers relating to the pathophysiology of LN

5.1 Inflammatory cells as urine biomarkers

The immune cells known to infiltrate the kidney in LN are known to comprise of T cells, macrophages, B cells and plasma cells [32-34]. Urinary cells have been investigated as non-invasive biomarkers of the inflammatory renal environment, with their relationship to LN outcome, therapeutic response or future flares currently under investigation. The specific origin of urinary immune cells is unknown but is speculated to relate to peri-glomerular, peri-tubular or interstitial infiltrates. In a recent study of 19 SLE patients with active LN and 55 SLE patients without LN using flow cytometry based cell quantification; urinary T cell subsets, B cells and macrophage numbers were significantly higher in active LN patients. Urinary T cell sub-sets showed excellent distinction between patients with active LN and non-LN SLE patients (CD8+ T cells, area under the curve (AUC) of 1.000 and CD4+ T cells AUC of 0.9969). CD14+ macrophages performed slightly less well (AUC = 0.9066), and CD19+ B cells were only moderately elevated, failing to reach these high standards of differentiation between patient groups (AUC = 0.7823) [35]. The same group have previously shown that high urinary CD4+ T cells numbers were observed in patients with proliferative

LN, and that over time, normalization of urinary CD4 T cell counts was associated with lower disease activity and better renal function. Persistence or an increase in urinary T cell numbers associated with higher total SLE disease activity index (SLEDAI) scores, and poorer renal outcomes in terms of serum creatinine and proteinuria [36]. These results have been confirmed by a further group who found urinary $CD4^+$ and $CD8^+$ T-cell counts to discriminate between active and inactive LN (AUC 0.92 and 0.93, respectively) [37], and to decrease significantly in response to treatment [35].

Long-lived plasma cells (CD138+) are capable of continually secreting autoantibodies. These cells live in chronically inflamed tissues like the kidneys in addition to the bone marrow/spleen. Urinary free light chains (FLCs) are synthesised by such cells with the total urinary FLC levels correlating with the number of CD138+ long-lived plasma cells within the kidney and International Society of Nephrology / Renal Pathology Society (ISN/RPS) class III/IV LN [38]. Monitoring of urinary immune cells as biomarkers is limited by the relatively large volume of urine required to get enough cells for analysis (approximately 100mls as opposed to 20-200µl in many immune-assays). The urine also needs to be fresh and analysed within six hours of voiding [35]. In the future, measurement of markers associated with these cells may provide a simpler proxy for inflammatory cell numbers, and be more suited for paediatric studies.

5.2 Cytokines, chemokines and their receptors as urine biomarkers

Cytokines, chemokines and their receptors play an important role in the pathogenesis of LN. This has led to a variety of studies testing whether they may be used as potential urinary biomarkers for LN. Multiplex bead assays have made it possible to simultaneously screen for multiple cytokines in a single sample, saving precious patient samples and time.

5.2.1 Monocyte chemoattractant protein-1

Monocyte chemoattractant protein-1 (MCP-1) is one of the most extensively investigated urine biomarkers in LN. It is a leucocyte chemotactic factor, which acts to recruit inflammatory cells to infiltrate the kidney in LN, leading to inflammation and tissue injury [39]. Increased expression of MCP-1 has been demonstrated by immunohistochemistry and in situ hybridization in endothelial cells, renal epithelial cells and infiltrating mononuclear cells present in the tubulointerstitial region of the kidney in LN [40]. There is also a correlation between glomerular expression of MCP-1 and more severe histological classes of LN (class III and IV LN), correlating with poor renal prognosis [41]. A large number of paediatric and adult cross-sectional studies have consistently demonstrated that urinary levels of MCP-1 are elevated in patients with active LN, as compared to controls (those with inactive LN and / or healthy controls) [42-49]. Although MCP-1 is associated with renal involvement in SLE, it is also elevated in other types of glomerulonephritis [50].

In a JSLE study, Watson et al [51], examined urinary MCP-1 levels in 64 JSLE patients longitudinally, demonstrating MCP-1 to be an independent predictor for active renal disease at the time of the current review, and over time, low urinary MCP-1 levels were an excellent predictor of future renal disease improvement (AUC: 0.81; p=0.013). In an adult SLE longitudinal study, Rovin et al [45] demonstrated that urinary MCP-1 levels were significantly higher during renal as opposed to non-renal flares, increasing 2-4 months before a LN flare. Patients who responded to treatment showed a decrease in urinary MCP-1 levels over several months, whereas non-responders displayed persistently elevated MCP-1 levels. MCP-1 levels were also demonstrated to be higher in patients with proliferative LN (WHO class III/IV) as opposed to membranous (class V) nephritis. A further longitudinal study of 40

adult SLE patients supports these results, with urinary MCP-1 levels falling in responders, remaining elevated in non-responders and correlating with histological severity [52]. Of note, an adult SLE study including 136 patients found urinary MCP-1 levels to be no better than anti-dsDNA antibody or C3 at differentiating renal vs. non-renal SLE flares [53]. A further study by Tian et al [54] showed that MCP-1 was unable to predict the occurrence of a LN flare in advance.

MCP-1 antagonists have been shown to ameliorate lupus nephritis in MRL-(Fas)lpr autoimmune prone mice [55], and to permit a 75% reduction in the dose of cyclophosphamide required to control diffuse proliferative LN in such mice [56]. MCP-1 antagonists have also been investigated in early phase type 2 diabetes [57] and post-traumatic neuralgia [58] trials but are not currently being investigated in LN. See Table 1 for a summary of the evidence relating to MCP-1 in children.

5.2.2 Neutrophil gelatinase associated lipocalin

Urinary neutrophil gelatinase associated lipoclain (NGAL) has also been widely investigated in LN. It is a member of the lipocalin family of carrier proteins that is expressed by many cell types including neutrophils and renal tubular epithelial cells. It is responsible for cellular iron transport, apoptosis, bacteriostasis and tissue differentiation, and is implicated in the growth and differentiation of epithelial cells [59]. NGAL is constitutively expressed at low levels in the kidneys [60], and is up-regulated in response to acute renal injury in responses to inflammation, ischemia and infection [61-65]. It has been shown to be a useful non-invasive marker of acute kidney injury following cardiopulmonary bypass surgery in children [66], and is predictive of the need for dialysis in diarrhea-associated hemolytic uremic syndrome and renal graft rejection [61,67].

The first paediatric urinary NGAL study was carried out by Brunner at al [68] in a small cohort of 35 JSLE patients. They reported higher levels of urinary NGAL in JSLE compared to juvenile idiopathic arthritis (JIA) patients. NGAL levels were strongly to moderately correlated with renal SLEDAI defined disease activity and Systemic Lupus Collaborating Clinics (SLICC) defined damage, but not with extra-renal disease activity. Subgroup analysis showed NGAL levels to be highly sensitive and specific for identifying JSLE patients with biopsy-proven nephritis. In a further study, they found the combination of NGAL together with MCP-1 and creatinine clearance to be good for identifying LN chronicity (AUC 0.83) [69]. In an independent Egyptian paediatric cohort including 35 JSLE patients, urinary NGAL was shown to correlate with the renal SLEDAI score and be predictive of class III and IV LN with 91% sensitivity and 70% specificity [70]. Pitashny et al [71] demonstrated urinary NGAL levels to be significantly higher in LN than non-LN patients in a cohort of 70 adult SLE patients. Urinary NGAL levels correlated significantly with renal but not with extra-renal SLEDAI scores. 24 hour urinary NGAL excretion has been shown to be a potential biomarker of renal damage in adult SLE patients, correlating with serum creatinine and creatinine clearance but not the SLEDAI score [72].

NGAL has also been investigated in two paediatric longitudinal studies, where it has been demonstrated to be predictive of LN flare [51,73]. Suzuki et al [73] studied 85 JSLE, 30 JIA patients, and 50 HC's. They found plasma and urinary NGAL levels to be increased in JSLE patients compared to controls, with urinary NGAL being significantly associated with worsening of renal disease. A subsequent paper reporting ongoing longitudinal follow-up (≥ 3 follow-up visits) within this cohort, described urinary NGAL levels as increasing by up to 104%, up to 3 months before worsening of LN [74]. Watson et al [51], demonstrated urinary

NGAL to be a good predictor of worsening renal disease activity (AUC 0.76; p=0.04) over time in a study involving 64 JSLE patients. Similarly, in an adult SLE study which included the exploratory Einstein Lupus Cohort and a validation cohort from University College London, urinary NGAL levels were found to rise 3-6 months before renal flare and to perform better than anti-dsDNA antibody for prediction of renal flare [75]. However, in contrast to the above, a longitudinal study involving 107 adult SLE patients was unable to identify an association between urinary NGAL and any measure of LN disease activity [76]. These conflicting results may be explained by differences in the timing of the sample and the outcome measures used. See Table 1 for a summary of the evidence relating to NGAL in children.

5.2.3 Vascular cell adhesion molecule-1

Vascular cell adhesion molecule-1 (VCAM-1) has not been investigated in paediatric urinary biomarker studies to date, and warrants further investigation in this population. It is an adhesion molecule and member of the immunoglobulin superfamily, which interacts with integrins, supporting tethering and adhesion of leukocytes to endothelial cells and migration into organs such as the kidney. Wu et al [77], demonstrated that urinary levels of VCAM-1 correlate well with urine protein levels and disease activity scores in three murine lupus nephritis models. They extended their work to compare urine samples from 38 SLE patients, 15 normal controls and six rheumatoid arthritis (RA) control patients. SLE patients displayed significantly higher urinary VCAM-1 levels than controls, with LN patients exhibiting the highest VCAM-1 levels. Urinary VCAM-1 levels also correlated with urine protein to creatinine ratios and renal SLEDAI score.

Several other small cross-sectional studies have since looked at urinary VCAM-1 in human LN. Molad et al [78], looked at 24 SLE patients compared to HC's, showing urinary VCAM-1 to be significantly increased in the SLE patients, and to correlate with SLEDAI, low C3, creatinine clearance and albuminuria. Abd-Elkareem et al [79], studied 50 SLE patients (30 active LN, 20 no LN) and found elevated urinary VCAM-1 in class III, IV and V LN but not in class I/II, or non LN SLE patients. Howe et al [80], also showed urinary VCAM-1 to be significantly increased in active versus inactive LN in 121 SLE patients (33 with active LN). More recently, Singh et al [43], found VCAM-1 to discriminate between active and inactive LN (AUC 0.92), correlating positively with the renal pathology activity index and the presence of class IV LN. In a longitudinal study of 107 SLE patients followed for up to eight clinic visits, urinary VCAM-1 levels were shown to correlate with renal SLICC score, urine protein/creatinine ratio and physicians global assessment, but not renal histology [76].

5.2.4 Tumor necrosis factor-like weak inducer of apoptosis

Tumor necrosis factor-like weak inducer of apoptosis (TWEAK) is a soluble cytokine that is mainly produced by leukocytes, but also by resident renal cells, leading to elevated TWEAK expression within the kidney and urine of adults with lupus nephritis [81,82]. Expression of Fibroblast growth factor-inducible 14 (Fn14), the TWEAK signaling receptor, is rapidly unregulated in context of tissue injury, autoimmune / inflammatory diseases, and has been shown to be present on podocytes, endothelial, mesangial and tubular cells [82]. TWEAK / Fn14 signaling mediates a variety of physiological processes, including cellular proliferation, survival, differentiation, migration, angiogenesis, induction of cell death, up-regulation of pro-inflammatory mediators and chemoattractants. The TWEAK／Fn14 pathway is felt to promote local tissue regeneration and repair after acute injury but conversely, is also involved in tissue damage and pathological tissue remodeling in diseased end organs in the context of excessive or persistent Fn14 up-regulation [83,84]. Of note, blocking TWEAK/Fn14 interaction in murine inflammatory kidney disease leads to improvement in disease [84] and

Fn14 deficiency in a lupus prone mouse strain has been shown to ameliorate the diseases clinical phenotype [85].

Substantial evidence has accumulated for the role of urinary TWEAK as a biomarker for LN in adult SLE patients. In a cross-sectional study of 83 adult SLE patients, Schwartz et al [86], found urinary TWEAK levels to be significantly higher in patients with active LN vs. non-LN SLE patients, with TWEAK levels being higher during renal vs. non-renal flares and correlating with the renal SLEDAI score. Longitudinal follow-up of a subgroup of these patients, within a larger multicenter cohort, found urinary TWEAK levels were significantly higher during a renal flare than 4-6 months prior to/following a flare. A linear mixed-effects model showed a significant association between urinary TWEAK levels and disease activity over time. Urinary TWEAK levels were again higher in LN patients that non-LN SLE patients and also controls (diabetes related kidney disease, hypertension, RA or osteoarthritis). There was no significant association between TWEAK and LN histological classes [81]. Two subsequent cross-sectional studies looking at urinary TWEAK have demonstrated significantly higher TWEAK levels in adult SLE patients with active LN compared to those with no nephritis [44,87]. Further investigation in children and young people with SLE is warranted.

5.2.5 IL-6

IL-6 has not been investigated in a paediatric study to date. Two small adult SLE studies have suggested a role for IL-6 as a urinary biomarker for LN. The first included 29 patients with active LN and showed IL-6 levels to be higher in those with WHO class IV LN on biopsy than other classes [88]. A second study of 27 SLE patient and 17 HC's also found urinary IL-6 to be higher in active LN, compared to inactive LN and HC's [89]. However, in a larger study comparing urinary IL-6 in 143 patients with SLE, 128 with a positive anti-nuclear antigen (ANA, but <3 ACR criteria) and 73 HC's, IL-6 was high in the first two groups of patients, with no difference seen between SLE patients with / without LN [90].

5.2.6 Other chemokines and their receptors

Interferon-γ-inducible protein 10 (IP-10) is a chemokine produced by monocytes, endothelial cells and fibroblasts in response to IFN-γ. IP-10 promotes migration into the kidney of T-cells expressing the IP-10 receptor (Chemokine receptor 3, CXCR3), and an increase in CXCR3+CD4+ T cells has been demonstrated in LN kidney tissue and urine, correlating with disease activity [91]. IP-10 has also been shown to promote mesangial cell proliferation in adults with proliferative glomerulonephritis [92], with tubulo-interstitial IP-10 expression decreasing in serial biopsies where a LN patient changes from having proliferative / mixed nephritis to membranous nephropathy [93]. Avihingsanon et al [94], evaluated urinary mRNA levels of IP-10 and CXCR3, demonstrating that urinary mRNA levels of IP-10 and CXCR3 were significantly higher in class IV LN compared with classes II, III and V. A significant reduction in IP-10 and CXCR3 mRNA level was seen in patients who responded to treatment, whereas levels remained elevated in treatment resistant patients. Abujam et al [53], assessed urinary IP-10 and MCP-1 levels in the urinary supernatant of 138 adult SLE patients, demonstrating a significant difference in the levels of both biomarkers between active LN, active non-renal SLE patients and HC's. In ROC analysis, MCP-1 out performed IP-10 in its ability to identify active LN (AUC 0.78 vs. 0.68 respectively). Within a paediatric study, no difference in urinary supernatant IP-10 levels could be detected between active LN, JSLE non-LN patients or HC's [42], emphasizing the need to examine urine biomarkers in both adult and paediatric studies as differences may exist.

Chemokine (C-X-C motif) ligand 16 (CXCL16), is a surface bound and soluble chemokine produced by T-cells / dendritic cells under the control of IFN-γ/TNF-α. Urinary CXCL16 has been shown to discriminate adult patients with active LN from other SLE patients with an excellent AUC of 0.934 [77]. Given the potential role for chemokines and their receptors in the pathogenesis on LN, and as biomarkers for LN, further investigation of is required, especially within JSLE populations. RANTES (regulated on activation, normal T cell expressed and secreted) is a chemokine which is involved in T cell recruitment to inflamed sites. In adult patients with diffuse proliferative LN, urinary RANTES has been shown to be significantly higher during episodes of flare [54]. No evidence relating to urinary CXCR3, CXCL16 or RANTES is available in JSLE.

	Patients	**Study design**	**Outcome**	**Ref**
MCP-1	52 in-active LN, 8 active LN, 20 HC's	Cross-sectional Renal BILAG defined LN	Significantly higher urinary MCP-1 concentrations in active LN than patients with in-active renal disease or HC's.	[42]
	12 in-active LN, 13 active LN	Cross-sectional Biopsy defined LN	Significantly higher urinary MCP-1 concentrations in active LN than patients with in-active renal disease or HC's.	[46]
	15 active LN, 15 in-active LN, 15 never had LN, 15 HC's	Cross-sectional Biopsy defined LN	Significantly higher urinary MCP-1 concentrations in active LN than patients with in-active renal disease, those who have never had LN or HC's.	[49]
	26 JSLE patients aged ≤ 18 years, 50 SLE patient >18 years old. All active LN patients within 2 months of renal biopsy.	Cross-sectional biomarker panel Biopsy defined LN	• MCP-1+AGP+CP+ protein:creatinine ratio = good for detecting LN histological activity (AUC 0.85). • MCP1+ AGP+TF+CrCl+C4 = fair for membranous LN detection (AUC 0.75). • MCP-1 +NGAL + Creatinine = good test for LN chronicity (AUC 0.83).	[69]
	47 JSLE patients at the time of renal biopsy.	Cross-sectional biomarker panel Biopsy defined LN	National Institute for Health Activity Index status predicted by MCP-1, NGAL, ceruloplasmin, adiponectin and KIM-1, Hemopexin in combination, with >92% accuracy.	[95]
NGAL	12 active LN, 23 no LN, 8 JIA controls	Cross-sectional biomarker panel SLEDAI-2K defined LN (score >0 on the renal domain)	Significantly higher urinary NGAL concentrations in active LN, than in-active/no renal disease or JIA controls. NGAL levels not correlated with extra-renal disease or damage. NGAL levels >0.6ng/mg creatinine were 90% sensitive and 100% specific for identifying JSLE patients with biopsy proven LN.	[68]
	26 active LN, 59 inactive LN, 30 JIA, 50 HC's	Cross-sectional and longitudinal (52/85 JSLE patients with ≥ 1 follow-up visit)	Significantly higher urinary NGAL levels in JSLE patients vs. controls. Moderate correlation of urinary NGAL with SLEDAI-2K defined renal diseases	[73]

	SLEDAI-2K defined LN (score >0 on the renal domain)	activity (r=0.4, p<0.008). Urinary NGAL not extra-renal SLEDAI scores. Longitudinally, urinary NGAL levels increased significantly with worsening of renal activity.	
22 active LN, 11 inactive LN	Cross-sectional Biopsy defined LN	Significantly higher urinary NGAL concentrations in active LN than in-active LN and HC's. Urinary NGAL predictive of class III and IV LN with 91% sensitivity and 70% specificity.	[70]
26 JSLE patients aged ≤ 18 years, 50 SLE patient >18 years old. All active LN patients within 2 months of renal biopsy.	Cross-sectional biomarker panel Biopsy defined LN	NGAL + MCP-1 + Creatinine = good test for LN chronicity (AUC 0.83).	[69]
47 JSLE patients at the time of renal biopsy.	Cross-sectional biomarker panel Biopsy defined LN	National Institute for Health Activity Index status predicted by MCP-1, NGAL, ceruloplasmin, adiponectin and KIM-1, Hemopexin in combination, with >92% accuracy.	[95]
111 JSLE patients with 365 follow-up episodes.	Longitudinal, at least 3 study visits. BILAG, and SLEDAI-2K defined LN	Urinary NGAL levels increased by an average of 70-104%, up to 3 months before worsening of LN. ROC analysis for ability of urinary NGAL to predict worsening LN gave an AUC of 0.78 when using SLEDAI-2K renal score as the external standard, or 0.80 when using the renal BILAG score.	[74]

Table 1: Summary of evidence for cytokines, chemokines and their receptors as urine biomarkers in children. MCP-1 = monocyte chemoattractant protein-1, LN = lupus nephritis, HC's = healthy controls. JIA = juvenile idiopathic arthritis. BILAG = British Isles Lupus Assessment Group score, AGP = α-1 acid glycoprotein, TF = transferrin, CrCl = creatinine clearance, C4 = complement factor 4, AUC = area under the curve, NGAL = neutrophil gelatinase associated lipocalin, KIM-1 = kidney injury molecule 1, SLEDAI-2k = SLE disease activity index.

6 Proteomic based urine biomarker analysis

The normal urinary proteome contains a small but discrete amount of proteins. In pathological situations the urinary proteome can provide information regarding damage to all segments of the nephron, underscoring the physiological and biological processes taking place. There are different proteomic approaches which present different advantages and disadvantages. The more traditional separation of proteins with two-dimensional gel electrophoresis followed by mass spectrometry (2D-electophoresis-MS) is labor intensive, may show poor reproducibility and requires a large amount of protein. It has a narrow dynamic range and cannot detect small (<10kDa), large (>150 kDa), hydrophobic or low

abundance proteins [96]. Liquid chromatography coupled to mass spectrometry (LC-MS) can be automated, is less time consuming, uses lower sample volumes and displays better sensitivity for smaller or hydrophobic proteins than 2DE-MS.

Surface-enhanced laser desorption/ionization time of flight (SELDI-TOF) technology uses protein-chip arrays coupled to a TOF mass spectrometer. These chips feature different surface binding properties (hydrophilic or hydrophobic materials, anionic or cationic, lectin, or antibody affinity reagents) leading to the binding of different proteins or peptides depending upon their properties. SELDI-TOF is widely used as it is high throughput and an easy to use technique that can be automated. It uses very small sample volumes, does not require pre-treatment of the sample and is sensitive for detection of low molecular weight proteins. SELDI-TOF has been widely used in LN but has two main disadvantages. Firstly, it identifies patterns of protein expression rather than specific proteins, and requires subsequent chromatographic enrichment and protein sequencing by capillary liquid chromatography-nanospray tandem mass spectrometry (LC/MS/MS), or on-chip protein sequencing using an LC/MS/MS system integrated with the SELDI-TOF mass spectrometer. The latter technique is limited to specific proteins, influencing the ability to undertake hypothesis free proteomic screening [96,97]. Secondly, intra / inter-assay coefficients of variation for protein peak abundance and intensities are approximately 20%, making the technique semi-quantitative, and requiring validation by another method [97-99]. Advancements in proteomics have provided considerable insight into the LN human urine proteome, and in this section, we will review the insights gained from paediatric and adult urine proteomic studies.

6.1 Adult proteomic studies

One of the earliest LN urinary proteomic studies by Mosley et al [100] used SELDI-TOF, identifying two proteins with masses of 3340 and 3980 which distinguished active from inactive LN, each with 92% sensitivity and specificity. This study featured a limited prospective longitudinal component including six patients, showing these proteins to predict both relapse and response to treatment earlier than traditional clinical markers. Using 2D-electrophoresis in 16 patients with LN, focal segmental glomerulosclerosis (FSGS), diabetic and membranous nephropathy, Varghese et al [25] used patterns of protein abundance to train an artificial neural network to create a prediction algorithm for identification of each disease. In an external validation set of 16 patients, the artificial neural network was found to be best for LN identification with a sensitivity of 86%, specificity of 89% and AUC of 0.84. Mass spectrometry was used to determine the identity of the proteins which included α1-acid-glycoprotein (AGP), transferrin (TF), zinc α-2-glycoprotein (ZA2G), α-1- microglobulin, α-1-antitrypsin, plasma retinol binding protein (RBP), haptoglobin, complement factor B, transthyretin, hemopexin and albumin. The same group went on to undertake a complementary study using a similar approach (2D-electrophoresis followed by mass spectrometry, artificial neural network) to differentiate ISN/RPS LN classes using urine from 20 patients immediately before renal biopsy. The sensitivity and specificity of the different combinations for the ISN/RPS classes was class II 100%, 100%; III 86%, 100%; IV 100%, 92%; and V 92%, 50%. The 50% class V LN specificity was due to inclusion of patients with mixed class III, IV, V. These patients were correctly identified as having class III or IV disease which is more clinically appropriate. The top two proteins contributing most to the tests sensitivity were found to be AGP and $\alpha\text{-}_1$ microglobulin [101].

Zhang et al [102] subsequently sought to screen the low molecular weight proteome (<20kDa) in samples taken at baseline, pre-flare, flare, and post-flare by fractionating the urine to remove proteins larger than 30 kDa, and then spotting them onto weak cation

exchanger protein chips for analysis by SELDI-TOF MS. 19 patients were included with class III (n=5), class IV (n=11), or class V (n=3) LN. 27 protein ions were found to be differentially expressed between specific flare intervals. Using on-chip peptide sequencing by integrated tandem mass spectrometry a 20 amino acid isoform of Hepcidin and an albumin fragment (N-terminal region) were found to be increased 4 months pre-flare and returned to baseline at the time of renal flare, whereas a 25 amino acid isoform of Hepcidin decreased during a renal flare and returned to baseline 4 months post-flare (marker of treatment response). They also found α-1-antitrypsin to be increased at the time of flare.

Somparn et al [26] undertook 2D electrophoresis on samples from 5 active and 5 in-active LN patients, revealing 16 protein spots whose levels differed significantly between the groups (serotransferrin, AGP, alpha-2-HS glycoprotein, haptoglobin, alpha-1-antitrypsin, albumin, ZA2G (3 isoforms), immunoglobulin kappa chain (3 forms), RBP-4, beta-2-microglobulin, transthyretin and prostaglandin-H2-D isomerase (PGDS)). They choose to validate two of the markers; ZA2G (due to the magnitude of it's change) and PGDS (because of its presence only in active LN), by ELISA in 30 active LN, 26 inactive LN, 14 non-LN glomerular diseases and 8 HC's. Urinary ZA2G levels significantly differentiated patients with active and in-active LN but were also elevated in patients with non-LN glomerular diseases. Urinary PGDS was only significantly elevated in active LN and is therefore thought to represent a LN specific biomarker.

Paediatric proteomic studies

Only two urinary proteomic studies have been carried out to date, both by Suzuki et al [27,103] in North American JSLE patients. In the first, SELDI-TOF-MS was used for proteomic profiling in 32 JSLE patients and 11 JIA patients. They identified a urinary proteomic signature consisting of eight proteins which displayed significantly higher peak intensities in patients with LN compared with controls or SLE patients without nephritis. These peaks had mass-to-charge ratios of 2.76, 22, 23, 44, 56, 79, 100, and 133. There was no significant difference in biomarker peak intensities between distinct WHO LN classes. The 22, 23, 44, 79, and 100 kDa peak were strongly correlated with renal disease activity as defined by the SLEDAI-2k score [103].

In the second study, surface-enhanced or matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used to identify TF, ceruloplasmin (CP), AGP, lipocalin-type prostaglandin-D synthetase (L-PGDS), albumin and albumin-related fragments as the proteins contained within the LN urinary protein signature. Immunonephelometry or ELISA was then used to serially assess the ability of this protein signature to monitor LN disease activity over time in 98 JSLE patients and 30 JIA controls. All proteins were significantly higher in children with SLE than JIA. Individual urinary biomarkers displayed fair to good ability to identify SLEDAI or BILAG defined active LN (AUC values between 0.68-0.81), with the AUC improving to 0.85 when all of the biomarkers were considered together. Urinary levels of TF, AGP and L-PGDS increased significantly 3 months before the clinical (SLEDAI based) diagnosis of worsening LN. However, significant increases in AGP and L-PGDS levels over time were also demonstrated in patients with stable active LN, improved LN (AGP) and inactive LN (LPGDS) [27], highlighting that further prospective studies are required to improve our understanding of the relationship of these biomarkers to disease activity over time. There is clearly overlap between some but not all biomarkers identified in adult and paediatric studies, highlighting the importance of undertaking biomarker studies in both patient groups rather than extrapolating between them.

6.2 Validation of proteomic results

The validity of previously established proteomic biomarkers must be assessed on separate groups of patients to provide independent verification of the findings and evaluation of biomarker sensitivity, specificity and predictive capabilities. This validation may either take the form of an independent proteomic study or through use of other protein binding techniques e.g. ELISA or multiplex assays, to quantify individual of combinations of biomarkers in a larger group of patients. Such techniques are quicker, usually highly sensitive and specific for the marker of interest, and readily available within many clinical or research laboratories. Table 2 lists the proteins identified in LN proteomic studies that have undergone internal or external validation using additional methods for protein quantification ± further proteomic techniques. Figure 1 also provides an overview of the evidence relating to protein urine biomarkers to date, and how they relate to the different clinical scenarios faced during the natural course of LN progression.

Protein	Original proteomic study	Validation Studies	
		Proteomic	Other methods / techniques
Biomarkers identified in ≥2 proteomic studies and validated using other methods			
AGP	**Oates [101]**	**Varghese [25]** **Suzuki [103]**	**Suzuki [27]**, 98 JSLE, ELISA's, serially assessed AGP, TF, CP, LPGDS. AUC of 0.85 for active LN identification when all biomarkers combined. **Brunner [69]**, 76 SLE patients, ELISA's. AGP+MCP1+CP+protein:creatinine ratio = good for detecting LN histological activity (AUC 0.85). AGP+MCP-1+TF+CrCl+C4 = fair for membranous LN detection (AUC 0.75). **Watson [42]**, 60 JSLE patients, ELISA. Urinary AGP significantly elevated in active LN.
TF	**Varghese [25]**	**Suzuki [103]**	**Suzuki [27]** – as described above **Brunner [69]** – as described above
ZA2G	**Varghese [25]**	**Somparn [26]**	**Somparn [26]**, ELISA, 30 active LN, 26 inactive, 14 non-LN kidney disease. ZA2G levels higher in active LN.
RBP-4	**Varghese [25]**	**Somparn [26]**	**Sesso [104],** 70 SLE patients, immunoenzymometric assay. RBP-4 significantly higher in active LN. **Marks [105]**, 21 JSLE patients, 10 active LN, 11 inactive, ELISA. RBP levels significantly higher in active LN.
PGDS or LPGDS	**Suzuki [103]**	**Somparn [26]**	**Suzuki [27]** – as described above **Somparn [26]**, ELISA, 30 active LN, 26 inactive LN, 14 non-LN kidney disease, 8 HC's, PDGS significantly elevated in active LN. **Gupta [106]** 28 SLE patients with active LN, 6 inactive SLE, 12 active non-renal disease and 19 HC's. ELISA. Significantly higher levels in active-LN. Levels fell in those responding to LN treatment and remained elevated in patients developed chronic kidney disease.
Biomarkers identified in a single proteomic study and validated using another technique			
Hemopexin	**Varghese [25]**	NA	**Brunner [95],** 47 active LN JSLE patients, ELISA. National Institute for Health Activity Index status predicted by NGAL, MCP-1, CP, adiponectin and KIM-1, Hemopexin in combination.
Hepcidin	**Zhang [102]**	NA	**Mohammed [107],** 30 active LN, 30 non-LN SLE patients and 30 HC's, ELISA. Hepcidin higher in active LN.
K-FLC	**Somparn [26]**	NA	**Hanaoka [38]**, 43 SLE patients, Nephrometric assay. Both κ/λ FLC's increased in class III/IV LN vs I/II/IV.
β2MG	**Somparn [26]**	NA	**Tsai [89],** 15 active LN, 12 with in-active LN, ELISA. Levels did not differ between groups. **Choe [108],** 64 SLE patients, ELISA, significantly higher in patients with active LN.
CP	**Suzuki [103]**	NA	**Suzuki [27] -** as described above **Brunner [69]** - as described above, **Brunner [95]** - as described above
Angiostatin	**Wu [109]**	NA	**Wu [109]**, 100 SLE, 24 CKD patients and 30 HC's. ELISA, angiostatin identified active AUC = 0.83

Table 2: Proteins identified in LN proteomic studies that have undergone validation using additional methods for protein quantification ± further proteomic techniques. Abbreviations = AGP = α-1 acid glycoprotein. TF = transferrin. CP = ceruloplasmin, MCP-1 = monocyte chemoattractant protein. CrCl = Creatinine Clearance. ZA2G = Zinc α-2-glycoprotein. HC's = healthy controls. RBP= plasma retinol binding protein. PGDS = Prostaglandin-H2-D isomerase, alternative name LPGDS = Lipocalin- type prostaglandin-D synthetase. Hepcidin = 20 & 25 amino acid isoforms. K-FLC= κ free light chain. β2MG = Beta-2-microglobulin. CKD = chronic kidney disease patients.

7 Origin / role of key proteins identified and validated in paediatric proteomic studies

It is interesting to consider the role and origin of proteins which have been identified in proteomic studies and validated extensively. AGP (also called Orosomucoid) belongs to the immunocalin family, a group of binding proteins which binds and transport small hydrophobic molecules and also has immunomodulatory functions. AGP is also one of the major acute phase proteins, which is mainly secreted by hepatocytes as part of the systemic response to inflammatory mediators (IL-1, IL-6, IL-8) or stressful stimuli such as physical trauma or bacterial infections. AGP production has also been reported in several tissues outside the liver including alveolar stimulated macrophages [110], human endothelial cells [111], cultured monocytes [112,113], resting and activated polymorphs [114,115]. L-PGDS is secretory protein of the lipocalin superfamily that acts as an enzyme responsible for the production of prostaglandin D2. It is produced in the brain by the choroid plexus or leptomeninges and is continuously secreted through cerebrospinal fluid into blood. L-PGDS is similar to albumin in chemical properties but is much smaller, allowing it to pass more readily through the glomerular capillary walls [116].

CP is involved in the metabolism of iron and carries the vast amount of circulating copper in the plasma [117]. TF is a metal binding protein with high affinity for iron, binding and moving it into cells and tissues. Iron is important to normal immune function, contributing to cell differentiation and growth. Alterations in iron homeostasis have been associated with several rheumatological and autoimmune disease [118]. Both CP and TF differ from albumin in terms of their molecular radii and isoelectric points, and have been shown to predict the onset of microalbuminuria at an earlier stage in conditions such as type 2 diabetes [119,120]. RBP-4 is mainly secreted by hepatocytes and adipose tissue, and also belongs to the lipocalin family. It is a carrier for retinol (vitamin A), delivering it to peripheral tissues, and urinary RBP-4 has been shown to be a biomarker for proximal renal tubule dysfunction [121]. Hemopexin is an acute phase reactant which is synthesized in the liver, binding and neutralizes pro-oxidant free heme, protecting the body from oxidative damage and preserving iron stores [122].

8 Metabolites

Metabolomics is a systems biology approach which investigates the overall metabolic activity of an individual, taking into account the influence of the genetic background and environment. Just as transcriptomics involves the study of gene expression, and proteomics the expression of proteins, metabolomics investigates the consequences of the activity of these genes and proteins, providing information on an individuals small molecule metabolite profile and unique chemical fingerprint [123]. To date there has been one small study looking at urinary metabolites in LN. This study included seven patients with class III/IV LN and seven with class V LN, utilising NMR spectroscopy based metabolic profiling. Significant differences were identified in citrate and taurine levels between class III/IV and V LN patients [124]. Currently there is no consensus on the best approach for normalisation of

metabolomics results from urine samples taking urinary dilution into account [125]. Further work is required extending these interesting analyses to larger patient cohorts, applying a normalisation factor and validating results using alternative techniques.

9 Conclusions - the future of urine biomarkers in LN

There have been an increasing number of promising urine biomarker studies over the last 10-15 years which have investigated a variety of biomarkers for LN. Early research into biomarker identification is becoming simpler through novel cutting edge technologies, which measure individual or combinations of biomarkers in miniscule patient samples. These data have provided insights into the pathophysiology and monitoring of LN but have not been translated into clinical practice, with the majority of studies concluding after the first or second phases of biomarker discovery. Moving such discoveries forward towards translation is complex and reliant upon studies which are carefully planned to avoid common pitfalls relating to sample size, accuracy of patient stratification over time, variation in the standard operating procedures for sample selection, collection, storage, handling, analysis and data interpretation. LN urine biomarker studies vary in terms of the outcome measures used to define active LN, with some studies relying upon composite disease activity measures (e.g. renal BILAG or SLEDAI) and others upon histological classification of LN (e.g. ISN/RPS classification, WHO criteria, National Institute for Health (NIH) activity, chronicity or tubular indices). Kidney biopsy is considered the gold standard test for LN, but unfortunately is also imperfect, relying on an invasive procedure for sampling and the subjective appearance of a few glomeruli in what can be a patchy disease.

The above methodological aspects and difficulties in LN urine biomarkers studies have impacted upon the perceived sensitivity, specificity, and predictive value of biomarkers thus far. Going forwards, it is important that the promising markers identified so far are further nurtured and internationally validated in adequately powered paediatric and adult SLE collaborative longitudinal studies, allowing us to move these discoveries towards clinical translation. Opportunities to include both paediatric and adult patients in urine biomarker studies should not be missed but considered integral to the development process, as is increasingly becoming the case in drug development. For such a study to be possible, the international community must assess and agree the measures which should be used to assess for active LN, with consideration as to whether more comprehensive validation is required of the disease activity measures for identification of active LN.

We must move from designing studies which do not only stratify patients as active / in-active LN but answer clinically important questions relating to the ability of biomarkers to predict a LN flare, identify active LN / evaluate its severity, determine response to a variety of treatments and assess for the presence of chronic renal damage over time. It has increasingly become clear that an individual biomarker will not provide optimal specificity and sensitivity in isolation, and that use of a combinatorial approach is of immense importance. Figure 2 shows a representation of the forecasted exciting future utility of urine biomarkers in LN monitoring and management. Our objective should therefore be the implementation of international collaborative efforts within paediatric and adult rheumatology communities for translating experimental findings into clinical trials, to make these ideals a reality.

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Figure Legends

Figure 1: Overview of the evidence relating to urine biomarkers to date and how they relate to distinct clinical questions in LN monitoring. Markers which are A) early predictors of flare, B) able to identify an active LN flare, C) used to evaluate flare severity, D) for identification of treatment non-responders, E) to identify chronic kidney damage. Urinary biomarkers shown in black are from paediatric studies, those in grey are from adult studies. NGAL = neutrophil gelatinase associated lipocalin. AGP = α-1 acid glycoprotein. TF = transferrin. CP = ceruloplasmin. LPGDS = Lipocalin- type prostaglandin-D synthetase. MCP1 = monocyte chemoattractant protein. VCAM-1 = vascular cell adhesion molecule 1. TWEAK = tumor necrosis like weak inducer of apoptosis. RBP= plasma retinol binding protein. Il-6 = interleukin 6. CXCL16 = Chemokine (C-X-C motif) ligand 16. RANTES = Regulated on Activation, Normal T Expressed and Secreted, ZA2G = Zinc α-2-glycoprotein. ISN/RPS = International Society of Nephrology / Renal Pathology Society classification. CrCl = Creatinine Clearance. K/L-FLC= κappa / lamda free light chains. IP-10 = Interferon gamma-induced protein 10. CXCR3 = Chemokine (C-X-C Motif) Receptor 3. NIH = national institute for health. UPUC = urine protein/creatinine ratio. KIM-1 = kidney injury molecule 1.

Figure 2: Future utility of urine biomarkers in LN monitoring and management. NGAL = neutrophil gelatinase associated lipocalin. AGP = α-1 acid glycoprotein. TF = transferrin. CP = ceruloplasmin. LPGDS = Lipocalin- type prostaglandin-D synthetase. VCAM-1 = vascular cell adhesion molecule 1. TWEAK = tumor necrosis like weak inducer of apoptosis. RBP= plasma retinol binding protein. Il-6 = interleukin 6. CXCL16 = Chemokine (C-X-C motif) ligand 16. RANTES = Regulated on Activation, Normal T Expressed and Secreted, ZA2G = Zinc α-2-glycoprotein. ISN/RPS = International Society of Nephrology / Renal Pathology Society classification. K/L-FLC= κappa / lamda free light chains. MCP1 = monocyte chemoattractant protein. IP-10 = Interferon gamma-induced protein 10. CXCR3 = Chemokine (C-X-C Motif) Receptor 3. CrCl = Creatinine Clearance. C4 = complement factor 4. NIH = national institute for health. UPUC = urine protein/creatinine ratio.KIM-1 = kidney injury molecule 1.

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