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14. ABSTRACT

The purpose of this project is to identify initial biomarker patterns in SLE nephritis using screening proteomic profiling. Utility of one of the biomarkers (NGAL) in predicting worsening of global and renal SLE disease activity has been validated. We have identified novel urinary biomarkers that distinguish between class IV and class V lupus nephritis, including albumin fragments and α-1 acid glycoprotein(AGP) by 2D gel electrophoresis, transferrin (Tf), ceruloplasmin (Cp), lipocalin-type prostaglandin-D synthetase (L-PGDS), and a1-acid glycoprotein (AGP) by SELDI-TOF-MS, citrate, taurine and hippurate by NMR spectroscopy-based metabolomic profiling, and apolipoprotein D, lipocalin-like prostaglandin D synthetase, hemopexin, ceruloplasmin, α-1 acid glycoprotein and orosomucoid by LCMS/MS profiling. We have validated whether these biomarkers are differentially expressed in patients with kidney biopsy-proven lupus nephritis types IV and V. Overall, these studies have identified non-invasive biomarkers that identify lupus nephritis sub-classes, and predict the clinical course of the disease. The significance of such biomarkers is that they will provide novel non-invasive tools to identify patients with lupus nephritis, to risk-stratify the subjects for therapies, and to follow the efficacy of therapies.

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Lupus nephritis, Biomarkers, Proteomics

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References……. None

Appendices… 4 published manuscripts

INTRODUCTION

In our proposal, we proposed to identify biomarker patterns in SLE nephritis by pursuing the following specific aims:

Specific Aim 1: Screening proteomic profiling: Initial high-throughput screening proteomic analysis will be done in the Devarajan Lab using 2D gel electrophoresis and Surface-Enhanced Laser Desorption/ Ionization Time-of-Flight mass spectrometry (SELDI-TOF-MS). Changes in proteomic profiles will be confirmed and enhanced using NMR- and MS-based metabonomics, by Dr. Michael Kennedy, Miami University. Changes in proteomic profiles will be compared to changes in currently available renal biomarkers (urinalysis, blood and urine chemistry), medications and other clinical outcomes (overall disease activity, renal and overall damage).

Specific Aim 2: Advanced proteomic profiling: Advanced proteomic studies on selected sample sets will be performed at Applied Biotechnology Branch, Air Force Research Lab, Wright-Patterson Air Force Base (AFRL/HEPB), where LC/MS based protein profiling will provide ultra-high resolution/mass accuracy protein identification.

Overall, these studies will identify a subset of non-invasive biomarkers that identify lupus nephritis sub-classes, and predict the clinical course of the disease. The significance of such biomarkers is that they will provide novel non-invasive tools to identify patients with lupus nephritis, to risk-stratify the subjects for therapies, and to follow the efficacy of therapies.

BODY

Research Accomplishments for Task 1: To identify initial biomarker patterns in SLE nephritis using screening proteomic profiling

1.1: Subject Recruitment

We recruited approximately 150 children with SLE, including some with and some without active renal disease. We achieved our goal of recruiting 75 patients with active renal disease, 75 patients without active lupus nephritis (LN). Seventy five children with Juvenile Idiopathic Arthritis (JIA, disease controls) and 75 normal siblings of children with JIA (healthy controls) were also recruited. All subjects had all proposed study visits. Additionally, we recruited 10 children with Focal Segmental Glomerulosclerosis (FSGS) to serve as a disease control group to better dissect mechanisms of inflammatory lupus nephritis from non inflammatory nephropathies with similar urinary findings.

1.2: Validation of NGAL as a biomarker for predicting SLE disease activity and course

Our preliminary genomic and proteomic studies in support on this application had identified neutrophil gelatinase-associated lipocalin (NGAL) as a highly promising urinary biomarker of lupus nephritis (LN) renal disease activity and renal damage. During years 1 and 2 of support, we validated the utility of NGAL in predicting impending worsening of global and renal SLE disease activity. We enrolled 111 children with SLE in a longitudinal prospective study with quarterly study visits. At each visit, global disease activity was measured using three external standards: the BILAG index, the SLEDAI score, and the physician's assessment of global disease activity. Renal and extra-renal disease activities were measured by their respective domains. Disease course over time was categorized at the most recent visit. Plasma and urinary NGAL levels were measured by ELISA at each visit. We found that significant increases in urinary and plasma NGAL levels were detected up to 3 months ***prior to*** worsening of lupus nephritis as measured by the external standards. We concluded that serial measurements of plasma and urine NGAL may be valuable in predicting impending worsening of global and renal childhood SLE disease activity. These studies have been published, and the manuscript included in the appendix ***(Hinze CH et al, Arthritis Rheum. 2009, 60:2772-2781)***.

1.3: Identification and validation of novel urinary biomarkers for active lupus nephritis

Our preliminary genomic and proteomic studies in support on this application had identified a panel of urinary biomarkers that correlated with SLE renal disease activity. During years 1 and 2 of support, we identified and validated key non-invasive biomarkers for active pediatric LN. The ISN/RPS class IV and V Lupus nephritis (LN) show different histological features and differ in prognosis. We aimed to identify non-invasive biomarkers which differentiate between class IV and V LN. Urine samples from children with class IV LN, class V LN, and FSGS (focal segmental glomerulosclerosis, disease control) were studied. All samples were collected within 60 days of a kidney biopsy. Two complementary proteomic methods were employed: 2 dimensional gel electrophoresis (2DIGE) and SELDI-TOF-MS. We found 2 proteins significantly over-expressed in class IV vs. class V by 2DIGE. MALDI-TOF-MS/MS analysis identified these proteins as human serum albumin fragments and α1-acid glycoprotein (AGP). In SELDI-TOF-MS, we used four different

types of ProteinChips and analyzed the spectra with ProteinChip Data Manager 3.07. Identification of the most robustly expressed peaks in Class IV LN revealed transferrin (Tf), ceruloplasmin (Cp), lipocalin-type prostaglandin-D synthetase (L-PGDS), and α1-acid glycoprotein (AGP). In prospective validation studies, serial plasma and urine samples were analyzed using immunonephelometry or ELISA in 98 children with SLE and 30 controls with juvenile idiopathic arthritis. All identified biomarkers (Tf, Cp, L-PGDS, AGP, and albumin) were significantly higher in patients with active versus inactive LN ($P < 0.005$), and their combined area under the receiver operating characteristic curve (AUC) for prediction of active LN was excellent at 0.85. Furthermore, increases in urinary Tf, AGP, and L-PGDS were detected up to 3 months ***prior to*** a clinical diagnosis of worsening LN activity. We concluded that urinary Tf, AGP, and L-PGDS are biomarkers of LN activity that may be valuable in predicting impending worsening of LN activity. We have published a manuscript describing these results, which is included in the appendix ***(Suzuki et al, Pediatr Res 2009; 65:530-536)***.

1.4: Association between novel urinary biomarkers and histology in Lupus Nephritis

The goal of this part of the study, completed during years 3 and 4, was to identify the relationship between LN histologic features and the novel urinary biomarkers of LN previously identified during previous years of funding by this DoD award. Urine samples from 76 patients with biopsy proven LN were collected proximate to the kidney biopsy, and assayed for key novel LN biomarkers, including lipocalin-like prostaglandin-D synthetase (L-PGDS), α1-acid-glycoprotein (AAG), transferrin (TF), ceruloplasmin (CP), neutrophil gelatinase-associated lipocalin (NGAL), and monocyte chemotactic factor 1 (MCP1). Biomarker concentrations were compared to the diagnostic histological features of LN, including mesangial expansion, capillary proliferation, crescent formation, necrosis, wire loops, fibrosis, tubular atrophy, and epimembranous deposits. The area under the receiver operating characteristic curve (AUC) was calculated to predict LN activity, chronicity, or membranous lesion. We found a diagnostic pattern of specific histological features that correlated with a urinary biomarker pattern. The combination of MCP1, AAG, and CP along with urine protein:creatinine ratio were excellent in predicting LN activity (AUC=0.85). Urinary NGAL in combination with MCP1 and creatinine clearance was an excellent predictor of LN chronicity (AUC=0.83). The combination of MCP1, AAG, and creatinine clearance was a good diagnostic test of membranous LN. In summary, these studies have shown that key urinary biomarkers of LN are associated with specific histopathological changes observed with LN activity and chronicity. Thus, our identified urinary biomarkers are well suited to non-invasively quantify LN activity, LN chronicity, and the presence of a membranous LN lesion. These studies have recently been published and included in the appendix ***(Brunner et al, Arthritis Rheum. 2012 Feb 10. doi: 10.1002/art.34426, Epub ahead of print).***

1.5: Urinary Metabonomic studies in Lupus Nephritis

Class IV and V Lupus nephritis (LN) show different histological features and differ in prognosis. We aimed to identify non-invasive metabonomic biomarkers which differentiate between class IV and V LN. Metabolic profiling was conducted using urine samples of patients with proliferative LN without membranous features (Class III/IV, n=7) and pure membranous LN (Class V, n=7). As disease controls, 10 patients with focal segmental glomerulosclerosis (FSGS) and proteinuria were also examined. Urinary profiling was performed using NMR-spectroscopy and MS-based metabonomics at Miami University, in collaboration with the laboratory of Dr. Michael Kennedy. Information about demographic and clinical data was obtained for each patient. Metabolic profiling analysis was done by visual inspection and principal component analysis. We found that urinary citrate levels were 8-

fold lower in Class V LN compared to Class III/IV, who had normal levels of urinary citrate ($P<0.05$). In contrast, Class III/IV LN patients had >10-fold lower levels of urinary taurine compared to Class V patients, who had essentially normal levels ($P<0.01$). Finally, Class V patients had normal hippurate levels compared to FSGS disease control patients, who completely lacked urinary hippurate ($P<0.01$). These studies have therefore identified novel differences in urinary metabolites between Class IV and V LN, and also between LN and FSGS patients. These studies have recently been published and included in the appendix ***(Romick-Rosendale et al, Arthritis Research & Therapy 2011, 13:R199)***

Research Accomplishments for Task 2: To identify biomarkers predictive of SLE nephritis using advanced proteomic profiling

2.1: Advanced Proteomics in Lupus Nephritis

These studies were initiated at the Applied Biotechnology Branch, Air Force Research Lab, Wright-Patterson Air Force Base (AFRL/HEPB), under the direction of Dr. Schlager. LC/MS based protein profiling of urine from SLE patients using Thermo LTQ FT-ICR was expected to provide ultra-high resolution/mass accuracy protein identification. However, these studies could not be completed by our collaborators at the Wright-Patterson Air Force Base due to unexpected and unanticipated technical difficulties.

However, our collaborators were able to complete preliminary LC-MS/MS2 studies, which have uncovered several differences between the groups. Among proteins upregulated in Class V LN were apolipoprotein D, lipocalin-like prostaglandin D synthetase, ITIH4, Caspase 10, uromodulin and CD14. Those most upregulated in Class IV LN were vitamin D binding protein, ceruloplasmin, hemopexin, A1BG and orosomucoid. A1AT has been linked to SLE flares and hemopexin is associated with glomerular disease.

Overall, these studies will identify a subset of non-invasive biomarkers that identify lupus nephritis sub-classes, and predict the clinical course of the disease. The significance of such biomarkers is that they will provide novel non-invasive tools to identify patients with lupus nephritis, to risk-stratify the subjects for therapies, and to follow the efficacy of therapies.

KEY RESEARCH ACCOMPLISHMENTS

- Completion of subject recruitment

- Validation of NGAL as a predictive urinary biomarker for impending worsening of SLE disease activity

- Identification of urinary biomarkers that distinguish between class IV and class V LN:
    - albumin fragments and α-1 acid glycoprotein (AGP) by 2D gel electrophoresis
    - transferrin (Tf), ceruloplasmin (Cp), lipocalin-type prostaglandin-D synthetase (L-PGDS), and α1-acid glycoprotein (AGP) by SELDI-TOF-MS
    - Citrate, taurine and hippurate by NMR spectroscopy-based metabolomic profiling
    - apolipoprotein D, lipocalin-like prostaglandin D synthetase, hemopexin, ceruloplasmin, α-1 acid glycoprotein and orosomucoid by LC-MS/MS profiling

- Association between novel urinary biomarkers and histology in LN
    - MCP1, AAG, and CP for predicting LN activity
    - NGAL and MCP1 for predicting LN chronicity
    - MCP1 and AAG for predicting membranous LN

REPORTABLE OUTCOMES

ABSTRACT PRESENTED:

1. Kristina Wiers, Kathleen Haines, Marissa Klein-Gitelman, Judyann Olson, Kathleen O'Neil, Kathleen Onel, Murray Passo, Nora Singer, Lori Tucker, Shannen Nelson, Prasad Devarajan, Hermine I. Brunner. New Biological Markers for Systemic Lupus Erythematosus (SLE) Renal Disease. Presented at the American College of Rheumatology Annual Meeting, 2007.
2. M Suzuki, M Bennett, L Das, K Hanes, M Klein-Gittelman, J Olson, K Onel, K O'Neil, E Silverman, L Tucker, N Singer, M Wyder, K Greis, H Brunner, P Devarajan. Urinary biomarkers for distinguishing subjects with class IV from Class V lupus nephritis. Presented at the Annual Meeting of the American Society of Nephrology, 2009, and at the American College of Rheumatology Annual Meeting, 2009.
3. Devarajan P. Validation of a novel biomarker panel for active lupus nephritis. Presented by the Principal Investigator in a platform session and a poster session by invitation at the Military Health Research Forum, hosted by the United States Army Medical Research and Materiel Command, in Kansas City, MO, on September 1, 2009.
4. Michael R. Bennett, PhD, Michiko Suzuki, MD, PhD, Shannen Nelson, Josh Pendl, Michael Kennedy, Pavel Shyianov, and Hermine Brunner, MD, Prasad Devarajan, MD. Urinary biomarkers to distinguish Class IV vs Class V lupus nephritis. Presented at the Annual Meeting of the American Society of Nephrology, 2010, and the American College of Rheumatology Annual Meeting, 2010.

MANUSCRIPTS PUBLISHED:

1. Hinze CH, Suzuki M, Klein-Gitelman M, Passo MH, Olson J, Singer NG, Haines KA, Onel K, O'Neil K, Silverman ED, Tucker L, Ying J, Devarajan P, Brunner HI. Neutrophil gelatinase-associated lipocalin is a predictor of the course of global and renal childhood-onset systemic lupus erythematosus disease activity. ***Arthritis Rheum***. 2009; 60:2772-2781.

2. Suzuki M, Wiers K, Brooks EB, Greis KD, Haines K, Klein-Gitelman MS, Olson J, Onel K, O'Neil KM, Silverman ED, Tucker L, Ying J, Devarajan P, Brunner HI. Initial validation of a novel protein biomarker panel for active pediatric lupus nephritis. ***Pediatr Res***. 2009; 65:530-536.

3. Romick-Rosendale LE, Brunner HI, Bennett MR, Mina R, Nelson S, Petri M, Kiani A, Devarajan P, Kennedy MA. Identification of urinary metabolites that distinguish membranous lupus nephritis from proliferative lupus nephritis and focal segmental glomerulosclerosis. ***Arthritis Res Ther***. 2011;13(6):R199. Epub 2011 Dec 7.

4. Brunner HI, Bennett MR, Mina R, Suzuki M, Petri M, Kiani AN, Pendl J, Witte D, Ying J, Rovin BH, Devarajan P. Non-invasive renal protein biomarkers are associated with histological features of lupus nephritis. ***Arthritis Rheum***. 2012 Feb 10. doi: 10.1002/art.34426. [Epub ahead of print]

CONCLUSION

Thus far, we have completed both Task 1 and Task 2. We have completed subject recruitment, validated one of the biomarkers (NGAL) as a predictive urinary biomarker for impending worsening of SLE disease activity, and identified a urinary biomarker signature that distinguish between class IV and class V LN. This includes albumin fragments and α-1 acid glycoprotein (AGP) by 2D gel electrophoresis, transferrin (Tf), ceruloplasmin (Cp), lipocalin-type prostaglandin-D synthetase (L-PGDS), and α1-acid glycoprotein (AGP) by SELDI-TOF-MS, Citrate, taurine and hippurate by NMR spectroscopy-based metabolomic profiling, and apolipoprotein D, lipocalin-like prostaglandin D synthetase, hemopexin, ceruloplasmin, α-1 acid glycoprotein and orosomucoid by LC-MS/MS profiling.

Overall, these studies have identified a subset of non-invasive biomarkers that identify lupus nephritis sub-classes, and predict the clinical course of the disease. The significance of such biomarkers is that they will provide novel non-invasive tools to identify patients with lupus nephritis, to risk-stratify the subjects for therapies, and to follow the efficacy of therapies. The identified association of novel biomarkers of lupus nephritis with specific histological features bears the expectation that longitudinal non-invasive measurement of lupus nephritis will become feasible, and will allow for a more effective and personalized monitoring of lupus nephritis and its therapy.

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Neutrophil Gelatinase–Associated Lipocalin Is a Predictor of the Course of Global and Renal Childhood-Onset Systemic Lupus Erythematosus Disease Activity

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Objective. **To determine whether neutrophil gelatinase–associated lipocalin (NGAL) can predict worsening of global and renal disease activity in childhood-onset systemic lupus erythematosus (SLE).**

Methods. **One hundred eleven patients with childhood-onset SLE were enrolled in a longitudinal, prospective study with quarterly study visits and had at least 3 study visits. At each visit, global disease activity was measured using 3 external standards: the numerically converted British Isles Lupus Assessment Group (BILAG) index, the SLE Disease Activity Index 2000 update score, and the physician's assessment of global disease activity. Renal and extrarenal disease activity were measured by the respective domain scores. The disease course over time was categorized at the most recent visit (persistently active, persistently inactive, improved, or worsening). Plasma and urinary NGAL levels were measured by enzyme-linked immunosorbent assay, and urinary NGAL levels were standardized to the urinary creatinine concentration. The longitudinal changes in NGAL levels were compared with the changes in SLE disease activity using mixed-effect models.**

Results. **Significant increases in standardized urinary NGAL levels of up to 104% were detected up to 3 months before worsening of lupus nephritis (as measured by all 3 external standards). Plasma NGAL levels increased significantly by as much as 26% up to 3 months before worsening of global SLE disease activity as measured by all 3 external standards. Plasma NGAL levels increased significantly by 26% as early as 3 months prior to worsening of lupus nephritis as measured by the BILAG renal score.**

Conclusion. **Serial measurement of urinary and plasma NGAL levels may be valuable in predicting impending worsening of global and renal childhood-onset SLE disease activity.**

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Drs. Hinze and Suzuki contributed equally to this work.

Dr. Klein-Gitelman has received consulting fees, speaking fees, and/or honoraria from UCB (less than $10,000) and has provided expert testimony for Robbins & Associates regarding intravenous steroid use. Dr. Devarajan has received consulting fees, speaking fees, and/or honoraria from Biosite Diagnostics and Abbott Diagnostics (less than $10,000 each). Cincinnati Children's Hospital has signed an exclusive licensing agreement with Abbott Diagnostics for the development of urinary neutrophil gelatinase–associated lipocalin as a biomarker of kidney damage and with Biosite Diagnostics for the development of plasma neutrophil gelatinase–associated lipocalin as a biomarker of kidney damage.

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Lupus nephritis is very common in childhood-onset systemic lupus erythematosus (SLE) (1–3). The onset of lupus nephritis is usually early in the disease course within 2 years after the diagnosis of SLE is made (1,4). The outcome is poor, and ~10% of childhood-onset SLE patients with lupus nephritis develop end-stage renal disease within 10 years (5).

Neutrophil gelatinase–associated lipocalin (NGAL) is a candidate biomarker for the early detection of lupus nephritis (6). NGAL is one of the most highly up-regulated proteins in experimental acute kidney injury (7,8). Urinary NGAL and plasma NGAL levels are predictive of the development of acute kidney injury after cardiothoracic surgery, with levels increasing within 2 hours after the insult (9). Additionally, NGAL is a good biomarker for chronic kidney disease, since urinary and plasma NGAL levels correlate better (inversely) with the glomerular filtration rate than do serum creatinine levels (10).

Our group has previously shown that patients with childhood-onset SLE and biopsy-proven lupus nephritis have higher urinary NGAL levels than do healthy controls or patients with childhood-onset SLE without lupus nephritis, and that urinary NGAL levels correlate with renal disease activity (11). In cross-sectional comparisons, patients with childhood-onset SLE and worsening lupus nephritis have higher urinary NGAL levels than do patients with stable or improved lupus nephritis (12). The goal of the current study was to investigate the association of longitudinal changes in plasma and urinary NGAL levels with changes in renal, extrarenal, and global disease activity in childhood-onset SLE.

PATIENTS AND METHODS

Patients. With the approval of the participating centers' institutional review boards, patients fulfilling at least 4 of 11 of the revised American College of Rheumatology classification criteria for SLE prior to age 18 years were enrolled in this prospective study (13). There were 3 categories of patients: 1) patients with newly diagnosed SLE, 2) patients with established SLE with biopsy-diagnosed lupus nephritis, and 3) patients with established SLE for at least 2 years without urinary changes suggestive of lupus nephritis. To be included in the analysis, patients (n = 111) had to have had at least 3 study visits. The study was a prospective, longitudinal trial with study visits every 3 months. Some of the patients and samples were part of previous studies of renal biomarkers (12,14,15). A list of participating centers and medical professionals who contributed to this study, in addition to the authors, is shown in Appendix A.

Laboratory assays. Urine samples were centrifuged at 4,000*g* at 4°C to remove cellular debris before storing. Plasma and urine samples were frozen within 2 hours after collection and stored at –80°C until the time of testing. Plasma and urinary NGAL levels were measured by enzyme-linked immunosorbent assay using a commercially available kit (Kit 036; AntibodyShop, Grusbakken, Denmark) as described in our previous report (12). Urine creatinine levels were measured using a quantitative colorimetric microplate assay kit (Oxford Biomedical Research, Oxford, MI). All measurements were made in duplicate. The laboratory personnel were blinded to the clinical data. Urinary NGAL excretion is presented as the amount of urinary NGAL in ng per mg of urine creatinine to correct for differences in NGAL due to urine dilution. The plasma NGAL concentration is presented in ng/ml plasma.

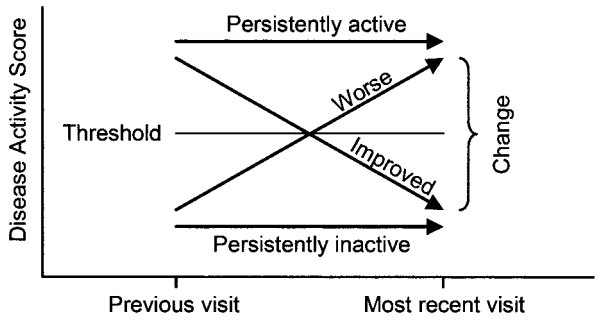
Childhood-onset SLE disease activity measures. At every study visit, global SLE disease activity was measured using 3 separate tools, as follows. The British Isles Lupus Assessment Group (BILAG) index (16) measures disease activity in 8 separate organ systems. While it was designed initially to reflect physicians' intention to treat, using 5 categories (A, B, C, D, E), for the present study we used the numerical conversion as proposed by Stoll et al (BILAG global, with a range of 0–72) (17). The second tool was the SLE Disease Activity Index 2000 update (SLEDAI-2K; global, with a range of 0–105) (18). The third tool was the physician's assessment of global disease activity (physician's global assessment), using a 10-cm visual analog scale (VAS; 0 = no disease activity and 10 = maximal disease activity). Similarly, for estimation of renal SLE disease activity, we used the following 3 separate measures: the BILAG renal domain score (range 0–9), the SLEDAI-2K renal domain score (range 0–16), and the physician's assessment of renal disease activity (physician's renal assessment; 10-cm VAS).

Extrarenal disease activity was measured using 2 tools: the BILAG global score minus the BILAG renal score (BILAG extrarenal score; range 0–63) and the SLEDAI-2K global score minus the SLEDAI-2K renal score (SLEDAI-2K extrarenal score; range 0–89). Both the BILAG and the SLEDAI-2K disease activity measures are sensitive to change in childhood-onset SLE (19).

Course of disease activity. The childhood-onset SLE disease course was categorized based on the change in disease activity at a reference time point (time 0). The respective disease activity scores were compared between 2 time points: the time of the most recent visit (time 0) and the time of the preceding visit (time –1). For example, a patient with 3 study visits could have 2 reference time points at which the disease course was determined (at the second visit and at the third visit). There were 4 categories of disease course: persistently active, persistently inactive, improved, or worsening. Details of how these categories were established are presented in Figure 1. The parameters to define the disease courses were chosen by 2 authors (HIB, PD) and were considered to represent a conservative estimate of minimal clinically important change in disease activity (20,21). The minimal clinically important change in disease activity in childhood-onset SLE is likely smaller than that in adult SLE; studies to prospectively validate these parameters in childhood-onset SLE are currently under way (22).

Statistical analysis. Levels of both plasma NGAL and urinary NGAL (standardized to the concentration of urine creatinine) were considered primary measures in this study. They were log-transformed in order to fit major assumptions of parametric statistical models in analyses. For each NGAL

A



B

	Threshold	Change
MD-Global	2	± 2
SLEDAI-2K Global/Extrarenal	4	± 4
BILAG-Global/Extrarenal	3	± 3
MD-Renal	0	± 1
SLEDAI-2K-Renal	0	± 4
BILAG-Renal	0	± 3

Figure 1. Categorization of the disease course with childhood-onset systemic lupus erythematosus (SLE). **A,** The disease course was categorized as persistently active, persistently inactive, improved, or worsening. For patients to be categorized as having persistently active (inactive) disease, the disease activity score had to remain above (below) a predefined threshold and the change could not exceed a predefined magnitude. If the change exceeded a predefined magnitude, patients were categorized as having improved (if decreased score) or worsening (if increased score) disease activity. **B,** The predefined thresholds and required changes are shown. MD Global = physician's assessment of global disease activity measured on a 10-cm visual analog scale (VAS) (a value of 0 indicates inactive SLE); SLEDAI-2K Global/Extrarenal = SLE Disease Activity Index 2000 update global score (range 0–105)/extrarenal score (range 0–89) (a value of 0 indicates inactive SLE); BILAG Global/Extrarenal = British Isles Lupus Assessment Group global score (range 0–72)/extrarenal score (range 0–63) (a value of 0 indicates inactive SLE); MD Renal = physician's assessment of renal disease activity measured on a 10-cm VAS (a value of 0 indicates inactive SLE renal disease); SLEDAI-2K Renal = SLEDAI-2K renal score (range 0–16) (a value of 0 indicates inactive SLE renal disease); BILAG Renal = BILAG renal score (range 0–9) (a value of 0 indicates inactive SLE renal disease).

measure, its change corresponding to a disease course category was assessed using a mixed-effect model, adjusting for controlling covariates, mainly the demographics (23). Because each patient had multiple (at least 3) visits, a random effect (i.e., patients) was used in the mixed-effect model to account for within-patient correlation during repeated observations. Post hoc estimates of changes in mean values were performed simultaneously among all 4 categories of disease course and adjusted for individual Type I errors using Tukey's method. Two types of changes in NGAL levels (the change between time –1 and time 0 and the change between time –2 and time –1) were analyzed in the mixed-effect models. Other numerical variables were summarized with mean ± SD values, and binary or categorical variables were summarized with frequency values (in %). Relationships between NGAL measures and between disease activity scales were assessed using Pearson's and Spearman's correlation coefficients, respectively.

In order to determine whether NGAL levels at different time points could be predictive of a worsening disease course, we applied multiple logistic regression models using the dichotomized disease course (worsening versus not worsening) as the dependent variable, and we used measurements of NGAL levels at different time points as predictors, adjusting them for the patients' demographics. The predicted logit of worsening was then transformed into the "predicted probability of worsening" for each case. A receiver operating characteristic (ROC) curve was plotted by connecting sensitivity/specificity points under all possible probabilities of worsening. The area under the curve (AUC) was used to assess the overall accuracy. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were also used to assess the discriminating and predicting performance of the NGAL measure using a specific threshold "predicted probability of worsening." Excel XP (Microsoft, Redmond, WA) and SAS 9.1 (SAS Institute, Cary, NC) programs were used for

Table 1. Characteristics of the 111 patients with childhood-onset SLE at the baseline study visit*

Age, mean ± SD years	15.9 ± 3.4
Female	89 (80.2)
Number of visits, mean ± SD	5.2 ± 1.3
Time between visits, mean ± SD months	3.4 ± 1.5
Race/ethnicity	
White	55 (49.5)
African American	36 (32.4)
Asian	14 (12.6)
Hispanic	13 (11.7)
Biopsy-proven lupus nephritis†	63 (56.8)
WHO class II	2 (1.8)
WHO class III	14 (12.6)
WHO class IV	27 (24.3)
WHO class V	16 (14.4)
WHO class III + V	1 (0.9)
WHO class IV + V	3 (2.7)
Anti-dsDNA antibody positive	56 (50.5)
Medications at baseline	
Prednisone	84 (75.7)
Cyclophosphamide	12 (10.8)
Mycophenolate mofetil	43 (38.7)
Azathioprine	12 (10.8)
Methotrexate	3 (2.7)
Hydroxychloroquine	88 (79.3)
Angiotensin-converting enzyme inhibitor	33 (29.7)

* Except where indicated otherwise, values are the number (%) of patients. WHO = World Health Organization; anti-dsDNA = anti–double-stranded DNA.

† Lupus nephritis was classified according to the revised 1995 criteria (24). Forty-nine of the 111 patients did not have systemic lupus erythematosus (SLE) renal involvement. All patients with physician-diagnosed SLE renal disease underwent a kidney biopsy.

Table 2. Disease activity and disease course during the study, as determined using the 3 external standards*

	BILAG score			SLEDAI-2K score			Physician's assessment	
	Global, 0–72	Renal, 0–9	Extrarenal, 0–63	Global, 0–105	Renal, 0–16	Extrarenal, 0–89	Global, 10-cm VAS	Renal, 10-cm VAS
Disease activity during the study, mean ± SD	4.3 ± 4.0	1.4 ± 2.5	3.2 ± 3.3	4.9 ± 4.6	1.7 ± 3.2	3.2 ± 3.0	2.1 ± 2.6	1.2 ± 1.9
Observations per disease course, no. (%)								
Persistently active	83 (22.7)	106 (29.0)	60 (16.4)	65 (17.8)	35 (9.6)	46 (12.6)	47 (12.9)	59 (16.6)
Persistently inactive	141 (38.6)	205 (56.2)	187 (51.2)	181 (49.6)	239 (65.5)	249 (68.2)	218 (60.1)	166 (46.8)
Improved	86 (23.6)	35 (9.6)	71 (19.5)	71 (19.5)	53 (14.5)	40 (11.0)	63 (17.4)	88 (24.8)
Worsening	55 (15.1)	19 (5.2)	47 (12.9)	48 (13.2)	38 (10.4)	30 (8.2)	35 (9.6)	42 (11.8)

* The 3 external standards for measuring disease activity were the British Isles Lupus Assessment Group (BILAG) index, the Systemic Lupus Erythematosus Disease Activity Index 2000 update (SLEDAI-2K), and the physician's assessment of disease activity (physician's assessment) on a visual analog scale (VAS). The disease course was categorized as persistently active, persistently inactive, improved, or worsening. For patients to be categorized as having persistently active (inactive) disease, the disease activity score had to remain above (below) a predefined threshold and the change could not exceed a predefined magnitude. If the change exceeded a predefined magnitude, patients were categorized as having improved (if decreased score) or worsening (if increased score) disease activity. Predefined thresholds and required changes are shown in Figure 1B. A total of 365 observations were made for each of the BILAG and SLEDAI-2K scores, while a total of 363 observations were made for the physician's global assessment and a total of 355 observations were made for the physician's renal assessment.

analysis. P values less than 0.05 were considered significant, and P values less than 0.1 were reported to show trends.

RESULTS

Baseline patient characteristics and treatments. Table 1 summarizes the characteristics of the 111 patients included in the study. Their mean ± SD age was 15.9 ± 3.4 years, and the majority were female (80.2%). Lupus nephritis was classified according to the original system (24), since some biopsy samples were obtained prior to the introduction of the new system in 2004 (25). Biopsy-proven lupus nephritis (often class IV and class V) was present in 56.8% of the patients. Frequently used antiinflammatory medications included prednisone (75.7%), hydroxychloroquine (79.3%), mycophenolate mofetil (38.7%), cyclophosphamide (10.8%), and azathioprine (10.8%); 29.7% of patients were treated with angiotensin-converting enzyme inhibitors at baseline.

Change in disease activity and in disease course. Table 2 summarizes the mean disease activity during the study period and the proportions of the different disease courses at the reference time point. A total of 365 observations of reference time points were available for the longitudinal analyses. The most common disease course was a "persistently inactive" course, while a "worsening" course occurred less frequently (worsening of global disease activity 9.6–15.1%, worsening of renal disease activity 5.2–11.8%, worsening of extrarenal disease activity 8.2–12.9%).

Correlation between different measurements of disease activity. Using Spearman's rank correlation coefficients corrected for tied ranks, there were strong correlations between global and extrarenal disease activity (BILAG global versus extrarenal scores: $r = 0.79$, $P < 0.0001$; SLEDAI-2K global versus extrarenal scores: $r = 0.74$, $P < 0.0001$), between global and renal disease activity (BILAG global versus renal scores: $r = 0.59$, $P < 0.0001$; SLEDAI-2K global versus renal scores: $r = 0.63$, $P < 0.0001$; physician's global assessment versus physician's renal assessment: $r = 0.51$, $P < 0.0001$), and between the different tools (BILAG global score versus SLEDAI-2K global score: $r = 0.60$, $P < 0.0001$; BILAG global score versus physician's global assessment: $r = 0.57$, $P < 0.0001$; SLEDAI-2K global score versus physician's global assessment: $r = 0.51$, $P < 0.0001$; BILAG renal score versus SLEDAI-2K renal score: $r = 0.68$, $P < 0.0001$; BILAG renal score versus physician's renal assessment: $r = 0.69$, $P < 0.0001$; SLEDAI-2K renal score versus physician's renal assessment: $r = 0.69$, $P < 0.0001$; BILAG extrarenal score versus SLEDAI-2K extrarenal score: $r = 0.47$, $P < 0.0001$). Renal and extrarenal disease activity were not correlated (BILAG renal score versus BILAG extrarenal score: $r = 0.07$, $P = 0.16$; SLEDAI-2K renal score versus SLEDAI-2K extrarenal score: $r = 0.01$, $P = 0.95$).

Distribution of plasma and urinary NGAL levels and correlation between plasma NGAL levels and urinary NGAL levels. Plasma NGAL and urinary NGAL levels were log–normally distributed in the study population (data not shown). Pearson's correlation using log-transformed plasma NGAL and urinary NGAL levels indicated that there was no correlation between plasma NGAL and urinary NGAL levels at any given

Table 3. Longitudinal levels of plasma NGAL and global, renal, and extrarenal disease course*

Tool, disease course (no. of observations)	Mean (95% CI)			*P*	
	Time −2	Time −1	Time 0	Time −2 vs. time −1	Time −1 vs. time 0
BILAG global score					
Active (83)	57.0 (49.2–66.0)	57.8 (50.7–65.8)	60.9 (54.5–68.0)	NS	NS
Inactive (141)	52.7 (46.5–59.7)	52.7 (47.3–58.6)	53.9 (49.3–59.0)	NS	NS
Improved (86)	55.4 (47.6–64.6)	55.1 (47.9–63.3)	55.8 (49.7–62.7)	NS	NS
Worsening (55)	54.7 (46.3–64.8)	65.0 (56.1–75.4)	67.8 (59.7–77.0)	0.007	NS
SLEDAI-2K global score					
Active (65)	52.4 (45.0–61.1)	55.2 (48.2–63.3)	57.5 (51.2–64.7)	NS	NS
Inactive (181)	54.0 (47.7–61.2)	51.8 (46.5–57.7)	54.0 (49.4–58.9)	NS	NS
Improved (71)	59.7 (51.1–69.7)	59.1 (51.1–68.2)	58.2 (51.4–65.8)	NS	NS
Worsening (48)	52.6 (45.2–61.2)	63.2 (55.5–72.1)	65.2 (58.0–73.4)	0.001	NS
BILAG renal score					
Active (106)	54.0 (43.6–66.9)	55.7 (45.8–67.7)	67.7 (57.8–79.2)	NS	NS
Inactive (205)	53.4 (47.3–60.3)	52.1 (47.1–57.7)	54.0 (49.7–58.6)	NS	NS
Improved (35)	61.4 (51.7–72.9)	62.7 (53.4–73.7)	57.4 (49.8–66.2)	NS	NS
Worsening (19)	51.1 (42.8–61.1)	64.5 (55.5–75.0)	59.7 (51.7–68.8)	0.0001	NS
SLEDAI-2K renal score					
Active (35)	57.3 (48.7–67.3)	57.9 (50.3–66.6)	57.9 (51.3–65.3)	NS	NS
Inactive (239)	49.4 (43.0–56.7)	50.1 (44.4–56.4)	53.5 (48.6–59.0)	NS	0.05
Improved (53)	57.8 (50.0–66.8)	58.4 (51.1–66.6)	58.8 (52.7–65.8)	NS	NS
Worsening (38)	62.6 (52.0–75.3)	68.0 (57.3–80.7)	67.7 (68.0–79.2)	NS	NS
BILAG extrarenal score					
Active (60)	61.4 (52.0–72.5)	65.3 (56.3–75.6)	67.5 (59.5–76.6)	NS	NS
Inactive (187)	52.8 (46.8–59.6)	52.3 (47.2–58.0)	53.5 (49.2–58.1)	NS	NS
Improved (71)	53.6 (46.0–62.4)	55.7 (48.5–64.0)	59.1 (52.4–66.6)	NS	NS
Worsening (47)	55.3 (46.8–65.5)	62.9 (54.0–73.2)	64.5 (56.5–73.6)	0.07	NS
SLEDAI-2K extrarenal score					
Active (46)	52.3 (43.6–62.7)	56.6 (48.2–66.5)	59.4 (51.8–68.1)	NS	NS
Inactive (249)	55.0 (48.8–61.9)	54.8 (49.6–60.5)	55.2 (50.9–59.8)	NS	NS
Improved (40)	53.4 (44.0–64.9)	55.0 (45.8–66.1)	57.7 (49.4–67.3)	NS	NS
Worsening (30)	53.1 (44.4–63.5)	60.9 (51.7–71.6)	70.1 (60.9–80.8)	0.07	NS

* Levels of plasma neutrophil gelatinase–associated lipocalin (NSAL) are shown as ng plasma NGAL/ml. Time −2 = time point 2 visits prior to the reference time point; time −1 = time point 1 visit prior to the reference time point; time 0 = reference time point at which the disease course was defined; 95% CI = 95% confidence interval; NS = not significant (see Table 2 for other definitions). See Table 2 for explanation of disease course (predefined thresholds and required changes are shown in Figure 1B).

study visit ($r < 0.01$). Pearson's correlation using log-transformed standardized urinary NGAL levels (ng/mg creatinine) and log-transformed absolute urinary NGAL levels (ng/ml urine) demonstrated a high degree of correlation ($r = 0.81$). In all remaining Results sections, urinary NGAL levels are presented standardized to the urine creatinine concentration.

Longitudinal changes in plasma NGAL levels and change in global disease activity. The relationship between the course of global disease activity (BILAG global score or SLEDAI-2K global score) and longitudinal plasma NGAL levels is shown in Table 3. Among patients who experienced worsening of global disease activity, there was a significant increase in plasma NGAL levels occurring between time –2 and time –1 (i.e., approximately 6 months to 3 months before the clinical diagnosis of a global flare was made). An identical pattern was observed when global disease activity was measured with the physician's global assessment; between time –2 and time –1, patients with worsening disease activity experienced an increase in plasma NGAL level from 57.4 ng/ml (95% confidence interval [95% CI] 47.9–68.8) to 72.7 ng/ml (95% CI 62.0–85.2) ($P < 0.001$). None of the other disease courses (persistently active, persistently inactive, or improved) was associated with a longitudinal change in plasma NGAL levels. Only 4 patients with a "worsening" disease course (as measured by the SLEDAI-2K global score) at time 0 already had a "worsening" disease course at time –1. When we excluded these patients from the analysis, similar results were obtained.

Longitudinal changes in plasma NGAL levels and change in renal disease activity. Patients with worsening renal disease activity as measured by the BILAG renal score experienced a significant increase in plasma NGAL level between time –2 and time –1. A

Table 4. Urinary NGAL levels over time and the future course of lupus nephritis*

Tool, disease course (no. of observations)	Mean (95% CI)			*P*	
	Time −2	Time −1	Time 0	Time −2 vs. time −1	Time −1 vs. time 0
BILAG renal score					
Active (106)	12.7 (9.6–16.9)	14.7 (10.1–21.4)	16.9 (11.5–24.8)	NS	NS
Inactive (205)	8.3 (6.5–10.6)	10.8 (7.9–14.7)	16.0 (12.1–21.2)	NS	0.002
Improved (35)	8.6 (5.1–14.6)	9.0 (4.4–18.6)	8.4 (4.1–17.3)	NS	NS
Worsening (19)	11.1 (6.1–20.1)	22.6 (12.7–40.4)	43.8 (25.1–76.3)	0.01	0.02
SLEDAI-2K renal score					
Active (35)	24.8 (15.3–40.0)	23.4 (11.7–46.5)	25.9 (13.4–50.3)	NS	NS
Inactive (239)	7.6 (6.0–9.6)	9.6 (7.1–13.1)	13.5 (10.2–17.9)	NS	0.007
Improved (53)	13.3 (9.0–19.7)	11.4 (5.9–21.9)	14.8 (8.3–26.3)	NS	NS
Worsening (38)	10.3 (6.9–15.5)	17.5 (11.2–27.2)	27.3 (17.3–42.8)	0.03	0.06

* Levels of urinary neutrophil gelatinase–associated lipocalin (NGAL) are shown as ng urinary NGAL/mg urinary creatinine. Time −2 = time point 2 visits prior to the reference time point; time −1 = time point 1 visit prior to the reference time point; time 0 = reference time point at which the disease course was defined; 95% CI = 95% confidence interval; NS = not significant (see Table 2 for other definitions). See Table 2 for explanation of disease course (predefined thresholds and required changes are shown in Figure 1B).

similar pattern was observed with the physician's renal assessment; between time –2 and time –1, the group of patients with worsening disease activity experienced an increase in plasma NGAL level from 53.6 ng/ml (95% CI 42.2–68.1) to 73.4 ng/ml (95% CI 59.7–90.3) ($P < 0.001$). Such increases did not reach significance when using the SLEDAI-2K renal score (see Table 3). Plasma NGAL level was not predictive of any other lupus nephritis disease course (active, inactive, or improved).

Longitudinal changes in urinary NGAL levels and lupus nephritis disease course. Between time –2 and time –1, patients with worsening SLEDAI-2K or BILAG renal scores experienced, on average, significant increases in urinary NGAL levels of 70% and 104%, respectively (Table 4). A similar increase was seen when renal disease activity was measured by the physician's renal assessment; between time –2 and time –1, the group of patients with worsening lupus nephritis experienced an increase in urinary NGAL level from 10.1 ng/mg creatinine (95% CI 6.5–15.7) to 17.2 ng/mg creatinine (95% CI 10.7–27.8) ($P = 0.04$), while no significant change of urinary NGAL level occurred during that interval in patients with any of the other disease courses. Only 4 patients with a "worsening" disease course at time 0 already had a "worsening" disease course at time –1. The exclusion of these patients from the analysis yielded similar results.

There was a significant concurrent increase in urinary NGAL level in patients who had persistently inactive disease at the reference time point. This significant increase was due to a subgroup of patients who experienced worsening at the subsequent time point according to the SLEDAI-2K renal score ($P = 0.05$), while patients who continued to have inactive disease at

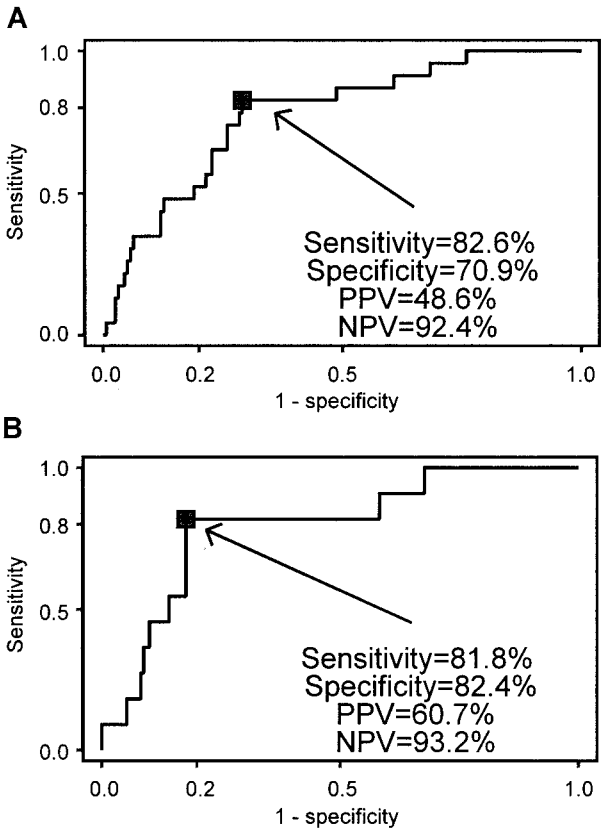


Figure 2. Receiver operating characteristic curves plotted by connecting sensitivity/specificity points under all possible probabilities of worsening of renal disease activity. Shown are the sensitivity and specificity of the predicted probability of worsening, estimated from multivariate logistic regression, using the SLEDAI-2K renal score as the external standard (area under the curve [AUC] = 0.78) **(A)** and the BILAG renal score as the external standard (AUC = 0.80) **(B)**. PPV = positive predictive value; NPV = negative predictive value (see Figure 1 for other definitions).

the subsequent time point did not experience a significant increase in urinary NGAL level (data not shown). These properties of urinary NGAL level were summarized using ROC analysis, using a dichotomized outcome (worsening versus not worsening of lupus nephritis). The sensitivity, specificity, PPVs, and NPVs were calculated for the resulting "predicted probability of worsening" (Figure 2). When using the SLEDAI-2K renal score as the external standard, the resulting AUC was 0.78, and when using the BILAG renal score as the external standard, the resulting AUC was 0.80. There was no statistically important relationship between the course of global or extrarenal disease and urinary NGAL levels over time.

DISCUSSION

The longitudinal data presented in this study demonstrate that an increase in urinary NGAL levels is predictive of worsening of childhood-onset SLE renal disease activity. Additionally, an increase in plasma NGAL levels is predictive of worsening of global and renal childhood-onset SLE disease activity. Therefore, urinary NGAL is an excellent candidate for a predictive biomarker for worsening of childhood-onset SLE renal disease activity, and plasma NGAL is an excellent candidate for a predictive biomarker for worsening of childhood-onset SLE renal disease activity and global disease activity.

SLE often follows a relapsing–remitting disease course (26). Due to the difficulty of predicting worsening of SLE disease activity, treatment is often only initiated once disease activity becomes severe and damage has occurred. Given the high morbidity and mortality in childhood-onset SLE with frequent and severe lupus nephritis (3,27), the identification of biomarkers that can predict worsening of lupus nephritis is highly desirable. The early recognition of worsening lupus nephritis, however, is difficult using routinely available laboratory tests. For example, levels of anti–double-stranded DNA (anti-dsDNA) antibodies may increase prior to the worsening of lupus nephritis (28–30), but only 50% of patients with childhood-onset SLE renal disease test positive for anti-dsDNA antibodies (2). In addition, levels of anti-dsDNA antibodies sometimes decrease concurrently with acute SLE flares, possibly due to increased tissue deposition (31), demonstrating a complex relationship between anti-dsDNA levels and SLE disease activity. Serum levels of the complement components C3 and C4 often decrease concurrently with renal flares, and thus have little predictive value (32). Results of other routine tests used to evaluate renal function, such as serum creatinine, urine protein, and examination of the urine sediment, vary not only with lupus nephritis activity but also with the presence of renal damage (33).

Additionally, there was no statistically important relationship between the future course of global, renal, or extrarenal disease and either serum C3 and C4 levels or the urine protein:creatinine ratio in our patient population. This information has already been reported for the presented patient cohort (15).

One of the difficulties of studying biomarkers for lupus nephritis has been the absence of a noninvasive criterion standard. While kidney biopsy is the gold standard for diagnosing lupus nephritis, it is impractical to perform repeated biopsies to screen for worsening of lupus nephritis. Alternative external standards must be used for the assessment of lupus nephritis and global SLE disease activity, including the BILAG and SLEDAI-2K global and renal scores. For the present study, we quantified global and renal disease activity using 3 external standards (2 for extrarenal domains) to ensure that relationships found between NGAL levels and childhood-onset SLE disease courses were not spurious. The BILAG index has been developed from the perspective of physicians' intention to treat to provide a snapshot of SLE activity by organ involvement rather than to supply a global disease activity score (34). Conversely, the SLEDAI-2K has been designed as a tool for assessing global SLE disease activity (18). There were strong correlations among the different tools for the assessment of global, renal, and extrarenal disease activity, supporting the concurrent validity of these measures in our study.

NGAL is a small, glycosylated (25-kd) protein produced in multiple normal tissues and organs, including epithelial tissues, endothelium, and bone marrow, and its production is increased in neoplastic and inflammatory conditions (35,36). Urinary NGAL levels increase markedly and immediately following acute kidney injury (37). Similarly, urinary NGAL levels are elevated with chronic kidney disease, correlating with disease severity (10,38).

Previously, we and others have shown that urinary NGAL is an excellent biomarker of concurrent lupus nephritis activity. Patients with active lupus nephritis have significantly higher urinary NGAL levels when compared cross-sectionally with patients with inactive lupus nephritis and healthy controls (11); patients with worsening lupus nephritis have higher urinary

NGAL levels when compared with patients with stable or improving lupus nephritis (12).

Our longitudinal prospective study has allowed us to examine whether NGAL could be a predictor of the future course of childhood-onset SLE. One impressive finding of our study is the marked increase in urinary NGAL levels up to 3 months prior to worsening lupus nephritis activity, irrespective of the external standard used. Our data also demonstrate a significant increase in plasma NGAL levels up to 3 months prior to worsening of global SLE disease activity and a significant increase in plasma NGAL levels prior to worsening of renal disease activity. Plasma NGAL levels also increased prior to worsening of extrarenal SLE disease activity, but changes did not reach statistical significance.

It is currently a subject of speculation why levels of urinary and plasma NGAL may increase before worsening of lupus nephritis becomes clinically detectable. One possibility is that the kinetics and specificity of the molecule may be different from those of other biomarkers. Urinary NGAL may be an immediate-early marker of general kidney injury, a notion supported by the findings reported in acute kidney injury. Another possibility is that NGAL may be produced after SLE-specific glomerular or tubular injury. While the most likely source of urinary NGAL in acute kidney injury is the distal tubules, the source of urinary NGAL in lupus nephritis is less clear. The observed increase in urinary NGAL levels may result from increased glomerular protein loss and disturbed reabsorption in the proximal nephron segment in addition to increased intrarenal production. Furthermore, based on results from experimental studies, the glomerulus may represent a source of NGAL. Mesangial cells treated in vitro with nephritogenic murine anti-dsDNA antibodies overexpress NGAL, indicating mesangial cells as a possible source (39). Additionally, a murine model of crescentic glomerulonephritis suggests that glomerular epithelial cells are a possible source of NGAL (40).

The increase in plasma NGAL levels prior to worsening of lupus nephritis, but not prior to worsening of extrarenal childhood-onset SLE, suggests a prominent role of lupus nephritis in increasing plasma NGAL levels. Similar findings are seen in other types of chronic kidney disease, with an inverse correlation between plasma NGAL levels and glomerular filtration rate (41). Several mechanisms may be postulated. Kidney injury results in dramatically increased NGAL messenger RNA expression in distant organs, especially the liver and lungs, and the overexpressed NGAL protein may constitute a distinct systemic pool (42). Additional contributions to the systemic pool may derive from NGAL released from neutrophils and macrophages. Furthermore, any decrease in glomerular filtration rate resulting from kidney injury would be expected to decrease the renal clearance of NGAL, with subsequent accumulation in the systemic circulation. The relative contribution of these mechanisms to the rise in plasma NGAL levels after acute kidney injury and in lupus nephritis remains to be determined.

Some of the problems in the clinical use of NGAL may include its nonspecific nature (i.e., the fact that urinary NGAL levels also increase after various other types of kidney injury, including ischemic and toxic injury). We anticipate that urinary NGAL may eventually be used in concert with other biomarkers to help us to better understand the nature of the underlying renal insult. Studies to identify and validate additional biomarkers for lupus nephritis are currently under way (14,15,43).

In summary, we demonstrated that urinary NGAL levels may be predictive of the development or worsening of lupus nephritis in childhood-onset SLE. In addition, an increase in plasma NGAL levels may be predictive of worsening of global and renal disease activity. As with all initial biomarker validation studies, confirmation of our findings in other cohorts is warranted. Future studies in an independent patient cohort, preferably one with childhood-onset SLE and adult SLE, are needed to verify that NGAL is a predictive biomarker. The early identification of patients at risk would be extremely helpful in order to initiate treatment early with the eventual goal of avoiding long-term morbidity and mortality due to lupus nephritis and SLE.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Brunner had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Acquisition of data. Klein-Gitelman, Passo, Olson, Singer, Haines, Onel, O'Neil, Silverman, Tucker, Brunner.

Analysis and interpretation of data. Hinze, Suzuki, Ying, Devarajan, Brunner.

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APPENDIX A: PARTICIPATING CENTERS AND MEDICAL PROFESSIONALS

Participating centers and medical professionals who contributed to this study, in addition to the authors of this article, are as follows: British Columbia Children's Hospital, Vancouver, British Columbia, Canada: Drs. David Cabral, Jaime Guzman, Kristin Houghton, Peter Malleson, Ross Petty, and Stuart Turvey (data collection); Tony Hong and Dr. America Uribe (study coordinators). Cincinnati Children's Hospital Medical Center, Cincinnati, OH: Dr. Michael Bennett (discussion); Drs. Thelma Kathman and Qing Ma (technical assistance); Dr. Susan Thompson (sample storage); Drs. Bob Colbert, Thomas Griffin, Alexei Grom, and Daniel Lovell (data collection); Shannen Nelson (study coordinating center study nurse); Jamie Meyers-Eaton (study coordinator); Shweta Srivastava (sample processing); Dr. Amber Khan, Clinical Fellow (data entry); Aimee Baker (manuscript preparation). Hackensack University Medical Center, Hackensack, NJ: Drs. Yukiko Kimura, Suzanne Li, and Jennifer Weiss (data collection); Mary Ellen Riordan (study coordination). Hospital for Sick Children, Toronto, Ontario, Canada: Lawrence Ng (study coordinator). Medical College of Wisconsin, and Children's Research Institute, Milwaukee, WI: Dr. James Nocton, Dr. Calvin Williams, and Elizabeth Roth-Wojicki, PNP (data collection); Marsha Malloy (data collection and site coordination); Joshua Kapfhamer and Noshaba Khan (study coordinators). Northwestern University Feinberg School of Medicine, Chicago, IL: Blair Dina (study coordinator). Rainbow Babies & Children's Hospital, Cleveland, OH: Dr. Elizabeth Brooks (data collection); Michelle Walette (study coordinator). La Rabida Children's Hospital, Chicago, IL: Dr. Linda Wagner-Weiner (data collection); Becky Puplava (study coordinator). University of Oklahoma Health Sciences Center, Oklahoma City: Drs. Michael Hendrickson and James N. Jarvis (data collection); Tracy Fuelling, Lisa Kempke, Linda Menifee, and Kathy Redmond (study coordinators).

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Initial Validation of a Novel Protein Biomarker Panel for Active Pediatric Lupus Nephritis

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ABSTRACT: Lupus nephritis (LN) is among the main determinants of poor prognosis in systemic lupus erythematosus (SLE). The objective of this study was to 1) isolate and identify proteins contained in the LN urinary protein signature (PS) of children with SLE; 2) assess the usefulness of the PS proteins for detecting activity of LN over time. Using surface-enhanced or matrix-assisted laser desorption/ionization time of flight mass spectrometry, the proteins contained in the LN urinary PS were identified. They were transferrin (Tf), ceruloplasmin (Cp), α1-acid-glycoprotein (AGP), lipocalin-type prostaglandin-D synthetase (L-PGDS), albumin, and albumin-related fragments. Serial plasma and urine samples were analyzed using immunonephelometry or ELISA in 98 children with SLE (78% African American) and 30 controls with juvenile idiopathic arthritis. All urinary PS proteins were significantly higher with active *vs.* inactive LN or in patients without LN (all $p < 0.005$), and their combined area under the receiver operating characteristic curve was 0.85. As early as 3 mo before a clinical diagnosis of worsening LN, significant increases of urinary Tf, AGP (both $p < 0.0001$), and L-PGDS ($p < 0.01$) occurred, indicating that these PS proteins are biomarkers of LN activity and may help anticipate the future course of LN. **(*Pediatr Res* 65: 530–536, 2009)**

Systemic lupus erythematosus (SLE) is an inflammatory autoimmune disease and lupus nephritis (LN) is one of the main determinants of poor prognosis (1). Currently, LN is gauged by measuring circulating and excreted indicators of renal dysfunction, with supporting information from kidney biopsies. The latter constitute the current standard for diagnosing LN, providing a direct assessment of the presence, severity, and activity of LN, and the degree of renal damage (2). Because of the invasive nature of kidney biopsies, clinicians base LN activity and its therapy on the results of urinary protein excretion, urinary sediment, creatinine clearance, and serum albumin. These traditional markers are not accurate in assessing whether active LN is present or not, and none of them is predictive, *i.e.*, can anticipate the course of LN.

Using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) technology, we previously identified a LN urinary protein signature (PS), consisting of eight candidate biomarkers at the mass-to-charge ratios (m/z) of 2.763, 22, 23, 44, 56, 79, 100, and 133 kDa (3).

In this study, we present the identification of the specific proteins contained in this PS of children with LN. We further assayed plasma and urine samples of patients with SLE and controls with juvenile idiopathic arthritis (JIA) to investigate the concurrent and predictive validity of the PS proteins to serve as biomarkers of LN activity.

MATERIALS AND METHODS

Patients with SLE. Children diagnosed with SLE (4) before the age of 16 y ($n = 98$) were studied every 3 mo for up to 18 mo. At each study visit, blood and random spot urine samples for research were obtained, and information on the following laboratory measures was collected: BUN (urea), serum creatinine, serum complement levels C3 and C4, presence of anti-dsDNA antibodies, urine protein:creatinine ratio (normal <0.2), and creatinine clearance approximated according to the Schwartz formula. At the participating centers, kidney biopsies are obtained in patients with SLE when abnormal urinalyses cannot be explained by mechanisms other than SLE. Thus, all

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Abbreviations: AGP, α1-acid-glycoprotein; **BILAG,** British Isles Lupus Activity Group; **Cp,** ceruloplasmin; **JIA,** juvenile idiopathic arthritis; **L-PGDS,** lipocalin-type prostaglandin-D synthetase; **LN,** lupus nephritis; MALDI-TOF MS, matrix-assisted laser desorption/ionization–time of flight mass spectrometry; **PS,** protein signature; **NGAL,** neutrophil gelatinase-associated lipocalin; **PMFs,** peptide mass fingerprints; **AUC_{ROC},** area under the receiver operating characteristic; SELDI-TOF MS, surface-enhanced laser desorption/ionization-time of flight mass spectrometry; **SLE,** systemic lupus erythematosus; **SDI,** Systemic Lupus International Collaborating Clinics/ACR Damage Index; **Tf,** transferrin; **SLEDAI,** 2k-version of the Systemic Lupus Erythematosus Disease Activity Index

children without kidney biopsies were considered to have SLE without LN. The study was approved by the Institutional Review Board (IRB) of the Cincinnati Children's Hospital Medical Center, and the IRBs of all other participating centers, with informed consent obtained before any study-related procedures.

SLE disease measures. At each study visit, two widely accepted measures of disease activity were completed: the 2k-version of the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) (5), and the British Isles Lupus Activity Group (BILAG) Index (6), an index that has been developed specially to assess organ-specific disease activity. SLEDAI or BILAG scores of 0 indicate inactive disease, and higher scores represent higher disease activity. Renal disease activity corresponds to SLEDAI or BILAG renal scores of >0 or >1, respectively. At study entry, the Systemic Lupus International Collaborating Clinics/ACR Damage Index (SDI; 0 = no damage) was completed (7).

Controls with juvenile idiopathic arthritis. Like SLE, JIA is an inflammatory autoimmune disease. Although it rarely involves the kidney primarily, theoretically nephrotoxic medications are the mainstay of JIA therapy. Thirty children with JIA (26 White, 4 African American; F:M = 27:3) served as controls, none of them had current or preceding laboratory abnormalities suggestive of a chronic renal disease. Only cross-sectional data of 20 patients with active and 10 with inactive JIA (as rated by their pediatric rheumatologist) were available for analysis.

Peptide mapping and protein identification. Details on the approach to develop the LN PS with its eight candidate biomarkers have been published (3). Briefly, these biomarkers were detected on at least two different ProteinChips, and displayed a >100-fold increase in peak intensity between groups. Subsequently, three urine samples from each WHO class of patients with LN and controls were lyophilized and redissolved with Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) for SDS-PAGE, using 8% or 12% Tris-Glycine gels with molecular weight standard markers (Invitrogen, Carlsbad, CA). Bands that showed the same molecular weights as the candidate biomarker proteins (SELDI-TOF MS) were excised, then digested with trypsin, and recovered for mass spectrometry (8,9). One-third of the individual band was treated with elution solution (50% formic acid, 25% acetonitrile, 15% isopropranolol, 10% water) to extract the proteins contained in each band. These proteins were analyzed on a normal binding ProteinChip to confirm the aimed mass spectrum. Peptides recovered from the in-gel digest were identified either *via* peptide mass fingerprints (PMFs) on the SELDI-TOF platform, or MALDI-TOF/TOF MS *via* MS/MS fragmentation with sequencing individual peptides. The use of both methods was necessary as albumin or albumin fractions often were present, and none of the various albumin removal approaches [albumin depletion kit (QIAGEN, Qproteome albumin/IgG depletion kit, Valencia, CA), immunoprecipitation (Dynabeads Protein G, Invitrogen, Carlsbad, CA), urea treatment, and anion exchange spin column (ProteinChip Q spin column, Bio-Rad laboratories)] succeeded in removing the albumin fraction effectively.

For protein identification by SELDI-TOF MS, samples were dried on a surface chip target plate followed by matrix application. In this format, the SELDI system may be comparable with a conventional MALDI-TOF instrument and can be used to collect PMFs spectra directly. Alternatively, samples were applied onto the ProteinChip SEND-ID array. The peptide mapping data were standardized using the All-in-One-Peptide Software (Bio-Rad Laboratories).

For MALDI-TOF/TOF MS, the excised peptides were desalted and concentrated on C18-micro-ZipTips as recommended by the vendor (Millipore, Billerica, MA) and then spotted on the target plate in 2.5 mg/mL CHCA containing 10 mM monobasic ammonium phosphate dissolved in 50% acetonitrile. The monobasic ammonium phosphate suppresses ionization of matrix clusters and enhances low mass range detection of peptides (10). PMFs and MS/MS-fragmentation data were collected for each sample. Both MALDI-TOF and TOF/TOF approaches were used, since the extreme abundance of albumin fragments interfered with the PMF identification for many of the bands. The acquired peptide data from SELDI-TOF MS were searched *via* Mascot (Matrix Science, Boston, MA) database search engine and the International Protein Index (IPI) human protein database. For the MALDI-TOF MS/MS spectra, data were processed using an integrated GPSExplorer interface from Applied Biosystems coupled to a local Mascot Server (Matrix Science) with database searches against the entire NCBInr database. In either case, standard Mascot statistical criteria were used to indicate positive protein identification.

Quantitative testing of the identified proteins. We measured plasma and urinary transferrin (Tf), plasma ceruloplasmin (Cp), plasma α-1-acid-glycoprotein (AGP, also: orosomucoid), as well as plasma and urine lipocalin-type prostaglandin-D synthetase (L-PGDS) by immunonephelometry (Dade Behring BNII Prospect, Marburg, Germany). Urinary Cp was quantified by ELISA (Human Ceruloplasmin ELISA Quantitation Kit; Genway Biotech, Inc., San Diego, CA); and urinary AGP by ELISA (Human Orosomucoid ELISA Quantitation Kit; Genway Biotech).

Statistical analysis. We inspected the central tendency, dispersion, and skewness of PS-protein levels and found them to all fit well into normal distributions after log transformation. Hence, log-transformed PS-protein levels were used in the formal statistical analyses, and results related to the PS proteins are presented using geometric means after their log-transformed means were converted back to original values by taking exponentials.

Using data from the first study visit only, PS-protein concentrations in three groups of patients with SLE (children with active LN; those with inactive LN and those without LN) and between two control groups with active JIA or inactive JIA, respectively, were tested for statistically important

Table 1. *Demographics and disease features of children with SLE at baseline*

Parameter	*n*	Mean (SE)	Number of patients with score = 0
Gender (female:male)			
81:17	98		
Race			
American Indian	2		
Asian	2		
African American	76		
Pacific Islander	1		
White	17		
Ethnicity			
Hispanic	12		
Non-Hispanic	86		
Age (y)	98	15.4 (0.49)	
Disease duration (y)	98	4.3 (1.11)	
Current medications			
Prednisone (mg/d)	67	17.2 (2.0)	
Azathioprine, mycophenolate mofetil, methotrexate	52		
Cyclophosphamide*	23		
Angiotensin blocking agents	29		
No lupus nephritis	36		
Lupus nephritis†			
WHO Class 2	5		
WHO class 3	17		
WHO class 4	22		
WHO class 5	18		
Disease activity			
SLEDAI‡			
Renal		2.0 (0.35)	68
Extrarenal	98	3.5 (0.31)	25
BILAG§			
Renal		2.0 (0.34)	61
Extrarenal		3.4 (0.31)	13
Disease damage			
SDI‖			
Renal	98	0.07 (0.03)	91
Extrarenal		0.45 (0.11)	72

* Six patients were treated with cyclophosphamide at enrollment.

† Classified as per Churg J, Bernstein J, Glassock RJ. Renal Disease: Classification and Atlas of Glomerular Diseases. 2nd Ed. Igaku-Shoin, New York, 1995.

‡ SLEDAI: Systemic Lupus Erythematosus Disease Activity Index, version 2k; 0 = inactive disease. Renal disease activity equals the sum of the items addressing renal disease. Extrarenal disease activity considers scores of all but the renal domain items.

§ BILAG: British Isles Lupus Activity Group index. Renal disease activity corresponds to the renal domain score of the BILAG. Extrarenal disease activity considers all other BILAG domain scores. Alphabetical BILAG score were converted into numericals as follows: A = 9; B = 3; C = 1; D or E = 0; 0 = inactive disease.

‖ SDI: Systemic Lupus International Collaborating Clinics/ACR Damage Index; 0 = no damage.

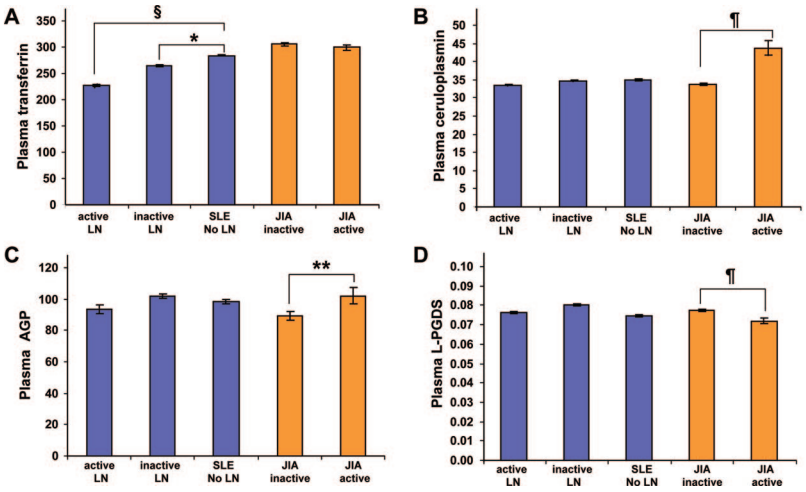

Figure 1. Plasma concentrations of the PS-proteins. Values are means and SE in mg/dL. Significant differences are based on Tukey post-hoc testing. The histograms show the level of the PS-proteins, Tf (*A*), Cp (*B*), AGP (*C*), and L-PGDS (*D*). SLE patients with active lupus nephritis (LN), inactive LN or without LN are compared with groups were defined by the SLEDAI. Twenty children with active and 10 with inactive JIA served as controls. Significant differences between groups are indicated as follows: $*p < 0.02$; $**p < 0.01$; $¶p < 0.001$; $§p < 0.0002$.

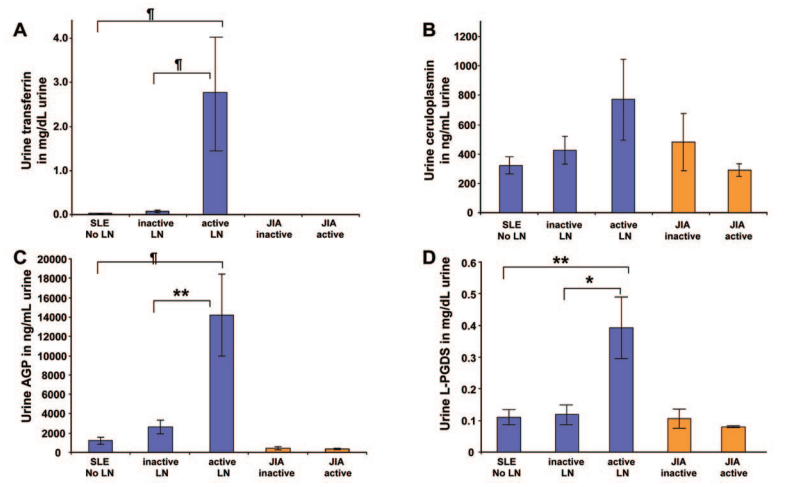

Figure 2. Urinary concentration of the PS-proteins. Values are means and SE. Significant differences are based on Tukey post-hoc testing. The histograms show urinary concentrations of Tf (*A*), Cp (*B*), AGP (*C*), and L-PGDS (*D*) for the groups defined as Figure 1. Uncorrected PS-protein levels (per mL or dL of urine) are depicted. Significant differences between groups are indicated as follows: $*p < 0.004$; $**p < 0.002$; $¶p < 0.00001$.

differences under a multivariate fixed effect model (or ANOVA model) framework, after adjusting for patients' characteristics such as age, gender, and race. Active LN was defined as a renal SLEDAI score >0 or a renal BILAG score >1 (11), respectively. For analysis of longitudinal data with repeated observations on each patient, a random effect (*i.e.*, the patient) was added to the previous fixed effect models to account for within-patient correlation. Receiver operating characteristic (ROC) curves were constructed, and the area under each ROC curve (AUC_{ROC}: range 0–1) was calculated (12) to assess performance of the PS proteins in discriminating between the presence *vs.* absence of LN activity. An AUC_{ROC} of 1.0 represents a perfect biomarker whereas a value of 0.5 is no better than expected by chance. Statistical computations were conducted using SAS version 9.1 (SAS, Cary, NC) software. p values <0.05 were considered statistically significant.

RESULTS

Characteristics of patients with SLE. Characteristics of the children with SLE are summarized in Table 1. At study entry, there were 26 patients with active LN (renal SLEDAI > 0), 36 with inactive LN, and 36 who never had LN. For patients with SLE, a total of 347 visits (249 follow-up visits) were available for analysis with all children having had a minimum of two study visits.

Identification LN protein signature proteins. The LN urinary PS consisted of eight proteins with MS peaks-to-charge ratios (m/z) of 2.763, 22, 23, 44, 56, 79, 100, and 133 kDa (3). We identified the 23 kDa band as L-PGDS; the 56 kDa as AGP or orosomucoid; the 79 kDa as Tf; and the 133 kDa as Cp, respectively. The remaining four bands of the LN urinary PS represented albumin or albumin fragments, which were not further, examine for their relationship to the features of LN because we were unable to extract any specific proteins that might have been contained in these bands by our methods.

In the following, the plasma concentrations of Tf, Cp, AGP, and L-PGDS are reported in mg/dL; urinary concentrations of the PS-proteins are reported as 1) absolute concentrations in the urine: Tf and L-PGDS in mg/dL, Cp and AGP in ng/mL urine, respectively; 2) corrected for urinary creatinine (in mg/mL); and 3) corrected for nonselective proteinuria as estimated by the protein:creatinine ratio.

Differences between JIA and SLE. At the first study visit, the mean ± SE of the urinary concentrations (per mL urine) of Tf, Cp, AGP (all $p < 0.0001$), and L-PGDS ($p < 0.0025$) were markedly higher in children with SLE than those with JIA. Plasma levels of all PS proteins were comparable between children with SLE *vs.* JIA, with the exception of plasma Tf, where levels were higher with JIA than SLE (JIA: 304 ± 9.6 *vs.* SLE: 250 ± 5.8; $p < 0.002$).

PS proteins in patients with SLE. PS proteins were unrelated to SLE patients' weight, gender, race, ethnicity (Hispanic/Non-Hispanic), the use of angiotensin blocking medications, or disease duration.

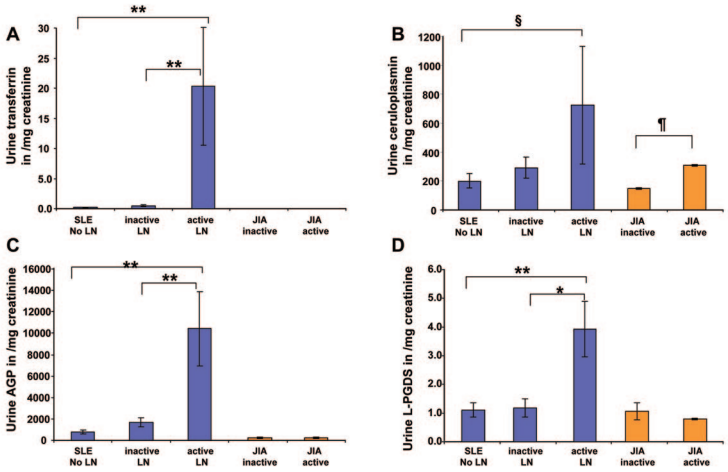

Figure 3. Urinary concentration of the PS-proteins. Values are means and SE. Significant differences are based on Tukey post-hoc testing. The histograms show urinary concentrations of Tf (*A*), Cp (*B*), AGP (*C*), and L-PGDS (*D*) for the groups defined as Figure 1. PS-protein excretion standardized by urine creatinine (mg/mL urine) is shown. Significant differences between groups are indicated as follows: $*p < 0.0005$; $**p < 0.0001$; $§p < 0.05$; $¶p < 0.001$.

Comparison of PS-protein plasma concentrations in the three groups of patients with SLE (no LN, active LN, inactive LN) and the two groups of controls (active JIA, inactive JIA) is shown in Figure 1, supporting only statistically significant differences of Tf plasma levels among the groups of patients with SLE, whereas the plasma levels of the other PS proteins appear not to be relevant biomarkers of LN.

Figures 2-4 depict the comparison of urinary concentrations of the PS proteins considering absolute levels (per dL or mL of urine; Fig. 2), levels standardized by urinary creatinine (Fig. 3) or nonselective proteinuria (Fig. 4), respectively. Patients with SLE with active LN had much higher levels of all PS proteins per mL or dL of urine (Fig. 2) or standardized by urinary creatinine (Fig. 3), with statistically significant differences indicated in the figures. Corrected for nonselective proteinuria, only urinary Tf and CP levels continued to be higher with active LN, suggesting that their excretion increases to a higher degree than nonselective proteinuria. Conversely, although urinary AGP and L-PGDS were significantly higher with active compared with inactive LN (Table 3), increases were less pronounced than those of nonselective proteinuria. Significant differences between SLE groups persisted only for Tf and L-PGDS once PS proteins were corrected for nonselective proteinuria, as is indicated in Figure 4. Use of the BILAG instead of the SLEDAI to classify SLE groups according to LN activity yielded comparable results as shown for the SLEDAI in Figures 1 to 4.

PS proteins differentiate better than traditional measures with the features of LN. Table 2 provides a cross-sectional comparison of PS proteins and traditional laboratory measures for their ability to identify active LN or renal damage. Besides the protein:creatinine ratio, the levels of none of the other traditional laboratory markers, including serum creatinine and BUN (data not shown), demonstrated important differences among patients with active *vs.* inactive LN. Among seven patients with SLE with renal damage, both the levels of plasma Tf and all urinary PS proteins were significantly higher than in patients with SLE without renal damage. However, six patients with renal damage had concomitantly active LN.

The AUC_{ROC} was calculated to assess the concurrent validity of the PS proteins and the traditional renal biomarkers to diagnose the presence of active LN as measured by the SLEDAI and the BILAG, respectively (Table 3). Individual urinary PS proteins in performed all in the fair to good range according to current ROC interpretation standards (12), they were all better diagnostic markers of active LN than traditional renal biomarkers (all $AUC_{ROC} < 0.63$) with the exception was the urine protein:creatinine ratio with an AUC_{ROC} at 0.91 (SLEDAI) and 0.85 (BILAG), respectively.

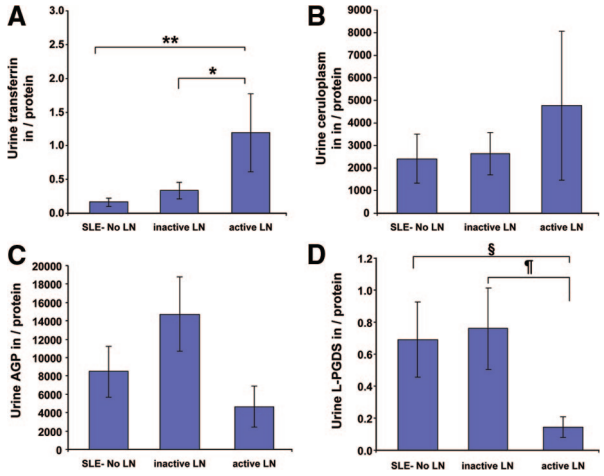

Figure 4. Urinary concentration of the PS-proteins. Values are means and SE. Significant differences are based on Tukey post-hoc testing. The histograms show urinary concentrations of Tf (*A*), Cp (*B*), AGP (*C*), and L-PGDS (*D*) for the groups defined as Figure 1. PS-protein excretion standardized by nonselective proteinuria is depicted with significant differences between groups indicated as follows: $*p < 0.05$; $**p < 0.005$; $§p < 0.02$; $¶\, p < 0.009$.

PS-proteins may predict the future course of LN. Figure 5 depicts the absolute levels of urinary PS proteins over time in relation to changes in LN activity as measured by the SLEDAI. Urinary levels of Tf, AGP, and L-PGDS significantly increased (SLEDAI: all $p < 0.009$) at least 3 mo before the clinical diagnosis of worsening LN activity (month 0) and continued to be elevated at the time of the clinically diagnosed LN flare. Cp levels did not show a consistent pattern with the course of LN. None of the traditional biomarkers, including the protein: creatinine ratio was predictive of the course of LN. Similar results were observed when the BILAG instead of the SLEDAI was used to determine the course of LN.

Table 2. *Concurrent validity in SLE—biomarker concentrations with active and inactive lupus nephritis**

Disease activity	Protein signature proteins†				Traditional biomarkers‡			
	Urinary Tf	Urinary Cp/1000	Urinary AGP/1000	Urinary L-PGDS	GFR	C3	C4	Protein creatinine ratio
SLEDAI renal								
Absent	0.01 (0.01–0.03)	0.49 (0.36–0.66)	1.55 (1.15–2.08)	0.09 (0.07–0.12)	78 (67–133)	64.3 (56.1–73.7)	9.5 (8.1–11.0)	0.05 (0.04–0.08)
Present	0.09 (0.04–0.22)	0.98 (0.63–1.50)	2.53 (1.74–3.68)	0.16 (0.12–0.22)	64 (50–128)	57.9 (47.3–70.9)	11.3 (8.9–14.3)	0.32 (0.19–0.52)
p	<0.0001	0.004	0.005	<0.0001	NS§	NS	NS	<0.0001
SLEDAI extrarenal								
Absent	0.02 (0.01–0.04)	0.45 (0.29–0.72)	1.45 (0.98–2.15)	0.09 (0.07–0.13)	67 (52–86)	72.5 (58.8–89.3)	13.3 (10.4–16.9)	0.09 (0.05–0.17)
Present	0.03 (0.01–0.06)	0.66 (0.48–0.90)	1.91 (1.40–2.61)	0.11 (0.09–0.15)	76 (66–89)	59.4 (52.0–67.9)	9.01 (7.8–10.4)	0.09 (0.06–0.14)
p	NS	NS	NS	NS	NS	NS	0.007	NS
BILAG renal								
Absent	0.01 (0.00–0.02)	0.44 (0.32–0.61)	1.33 (0.98–1.81)	0.09 (0.07–0.12)	80 (68–95)	66.5 (57.5–77.0)	9.7 (8.2–11.5)	0.04 (0.03–0.06)
Present	0.11 (0.05–0.26)	0.92 (0.62–1.37)	2.67 (1.88–3.79)	0.14 (0.11–0.19)	64 (52–79)	55.3 (46.2–66.3)	10.2 (8.3–12.5)	0.23 (0.16–0.35)
p	<0.0001	0.003	<0.0001	0.004	NS	NS	NS	<0.0001
BILAG extrarenal								
Absent	0.01 (0.00–0.03)	0.54 (0.31–0.95)	1.54 (0.97–2.44)	0.08 (0.06–0.12)	63 (46–86)	72.7 (56.6–93.4)	10.8 (7.9–14.6)	0.11 (0.05–0.23)
Present	0.03 (0.01–0.06)	0.60 (0.44–0.81)	1.80 (1.32–2.46)	0.12 (0.09–0.15)	76 (66–88)	59.9 (52.6–68.3)	9.73 (8.4–11.3)	0.09 (0.06–0.13)
p	0.032	NS	NS	NS	NS	NS	NS	NS
SDI renal								
Absent	0.02 (0.01–0.04)	0.59 (0.41–0.85)	1.53 (1.07–2.17)	0.10 (0.07–0.13)	82 (69–98)	62.4 (53.7–72.4)	9.10 (7.5–11.0)	0.09 (0.06–0.15)
Present	2.48 (0.15–42)	2.02 (0.55–7.44)	9.65 (2.23–41.65)	0.36 (0.13–0.99)	51 (28–91)	42.2 (25.0–71.4)	10.6 (5.5–20.6)	0.83 (0.17–3.98)
p	0.001	0.080	0.018	0.016	NS	NS	NS	0.011
SDI extrarenal								
Absent	0.02 (0.01–0.04)	0.65 (0.43–0.98)	1.47 (0.99–2.20)	0.09 (0.07–0.13)	84 (69–102)	61.9 (52.2–73.2)	8.92 (7.2–11.0)	0.10 (0.06–0.18)
Present	0.08 (0.02–0.37)	0.65 (0.32–1.31)	2.61 (1.28–5.31)	0.17 (0.10–0.29)	68 (49–94)	57.2 (43.2–75.7)	10.0 (7.1–14.3)	0.14 (0.06–0.36)
p	NS	NS	NS	NS	NS	NS	NS	NS

* Values are geometric means (95% confidence intervals).
† Urinary concentrations of Tf in mg/dL, Cp in ng/mL, AGP in ng/mL, and L-PGDS in mg/dL.
‡ C3 and C4 all in mg/dL; GFR in mL/min/1.73m^2; protein:creatinine ratio (no units).
§ NS, not significant.
For additional legend, see Table 1.

Table 3. *Area under the ROC curve of protein biomarkers for LN activity and damage**

Measure of LN	SLEDAI-2k	BILAG	SDI
Protein signature proteins			
Plasma Tf	0.54	0.57	0.69
Urinary Tf	0.80	0.81	0.84
Urinary Cp	0.68	0.80	0.73
Urinary AGP	0.76	0.81	0.87
Urinary L-PGDS	0.71	0.73	0.79
All LN protein biomarkers	0.84	0.85	0.88
Traditional renal biomarkers			
Creatinine clearance	0.45	0.50	0.39
Protein–creatinine ratio	0.91	0.85	0.76
Complement C3	0.58	0.63	0.75
Complement C4	0.60	0.49	0.64

* Urine concentration of proteins is shown per ml urine.
For additional legend, see Tables 1 and 2.

DISCUSSION

There is a need for high-quality accurate biomarkers to judge LN activity and renal damage with SLE. In this study, we chose a proteomic approach for the discovery of novel LN biomarkers and identified a set of PS proteins (*i.e.*, Tf, Cp, AGP, and L-PGDS). In quantitative analysis, particularly urinary rather than plasma levels of the PS proteins increased significantly with the presence of active LN. The increased urinary excretion of the PS proteins could not simply be explained by concomitant increases of nonselective proteinuria. Different from all traditional laboratory measures of LN, we have initial evidence that Tf, AGP, and L-PGDS constitute predictive biomarkers of worsening LN activity.

We found high and increasing urinary levels of Tf associated with active LN and impending worsening of LN flares. Tf is coregulated by interferon-α, involved in iron delivery, and the innate immune system. Plasma Tf levels were correlated to global SLE disease activity in the past (13). Thus, our study confirms these earlier findings in SLE, and new evidence is provided that urinary Tf excretion may represent a predictive biomarker for LN.

Cp plays a critical physiologic role in controlling the rate of iron efflux from cells with mobilizable iron (14). Like Tf, plasma Cp has been recommended as a marker of global SLE disease activity (13,15). Conversely, our results support that urinary Cp concentrations only differ with LN activity rather than extrarenal disease activity. Possibly, because urinary Cp levels vary widely in SLE, we were unable to detect a meaningful relationship to the course of LN.

AGP is a predictive biomarker for diabetic renal disease (16), and we provide initial evidence that this is also the case for LN. More importantly, urine concentrations of AGP (similar to Tf and L-PGDS) seem useful to anticipate LN flares, *i.e.*, these markers may allow clinicians to preemptively adjust therapy before the appearance of overt worsening of LN. Previous studies proposed plasma AGP to be a biomarker of SLE global disease activity (17,18). Our results support this

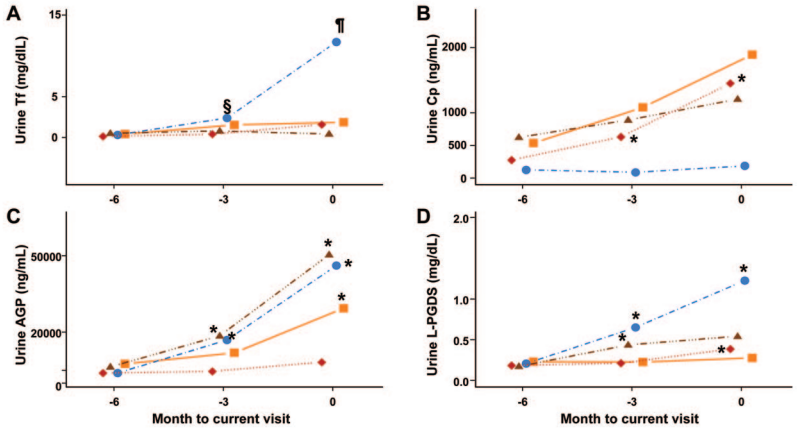

Figure 5. Changes of the PS-proteins in relationship to future changes in LN activity. Values are geometric means of uncorrected urinary levels of Tf (*A*), Cp (*B*), AGP (*C*), and L-PGDS (*D*) at months −6, −3 and 0, respectively. Month 0 is the time point when the clinical diagnosis of the course of LN is made and months −3 corresponds to the time point of 3 mo before the clinical diagnosis of the LN flare. "Improved LN" describes the course of LN with decreasing renal SLEDAI scores; "worse LN" describes the course of LN associated with an increase of the renal SLEDAI scores; "stable active LN" describes patients with stable renal SLEDAI scores >0; and "inactive LN" describes the course of continuously inactive LN (renal SLEDAI = 0). Significant differences in the levels between two consecutive visits are indicated in the plots as follows. §$p < 0.009$; ¶$p < 0.0001$; *$p < 0.001$. The above defined LN courses are depicted as follows: Improved LN, *squares*; Worsened LN, *circles*; Stable active LN, *triangles*; Inactive LN, *diamonds*.

(data not shown) but we also provide evidence that urinary AGP constitutes a biomarker of LN rather than extrarenal disease activity.

Lipocalins play a role in many biologic processes, among them immune responses and prostaglandin synthesis. L-PGDS, a lipocalin, is involved in nitric oxide regulation and the induction of apoptosis in the kidney. L-PGDS has not been previously found to be a LN biomarker. Urine and plasma L-PGDS are considered sensitive indicators of chemotherapy-induced renal damage and diabetes-associated hypertension (19,20). We found urinary L-PGDS unrelated to the creatinine clearance in both JIA and SLE; L-PGDS also did not significantly change with cyclophosphamide exposure in patients with SLE. Reason for these discrepancies might be that our patients had all normal or only minimally decreased creatinine clearance, and that there was at least a 3-wk time lag from a previous i.v. cyclophosphamide dose.

We confirm the result of a recent study that Tf and AGP are part of the LN PS (21) but were unable to detect hepcidin, a protein recently identified *via* SELDI-TOF MS (22). This might have been due to differences in the experimental approach, including the study of children instead of adults with SLE. Biomarker discovery in children may have a distinct advantage as children generally lack comorbidities, increasing the likelihood of detecting specific biomarkers.

Further research is needed to compare the usefulness of the PS proteins to that of other recently discovered LN biomarkers, including neutrophil gelatinase-associated lipocalin (11) or various urinary chemokines.

At present, there is no agreement how best to interpret protein biomarkers derived by protein profiling. Hence, it remains unclear whether absolute uncorrected concentrations in the urine, levels standardized by urine creatinine excretion, or even protein-adjusted levels are most suited for analysis. Like others (22), however, our data support that correction of absolute biomarker levels for urinary creatinine does not importantly change the principle properties of the PS proteins for detecting LN activity. Even when we corrected the PS proteins for the nonselective proteinuria, statistically significant differences between patients with SLE without LN, inactive LN, and active LN often persisted. The results of the latter analyses will need to be confirmed under consideration of the limitations of protein:creatinine ratios, possibly correcting for microalbuminuria rather than nonselective proteinuria (23).

At present, there is no universally accepted gold standard for the measurement of LN activity. For this study, we chose to use the two widely accepted SLE disease activity indices (SLEDAI and BILAG). The relevance of our findings is strengthened by the fact that the PS proteins performed similarly well to capture and anticipate the course of LN, irrespective of the index used. Compared with the BILAG, the SLEDAI considers only proteinuria and the urinary sediment in the calculation of the LN activity score. Thus, a close association between the protein:creatinine ratio and LN activity and an AUC_{ROC} were expected when using the SLEDAI. Given the sensitivity of moderately elevated protein:creatinine ratio to angiotensin blocking medications and its unproven ability for predicting LN flares, we consider Tf, CP, AGP, and L-PGDS to be promising LN biomarkers, as their levels do not seem to change with the use of angiotensin inhibiting medications and even help to discriminate patients who are at risk of a future LN flare.

A limitation of our study may be that we could not consider effectively the findings of renal biopsy specimens for their relationship to the PS proteins. We did not have a sufficient numbers of urine samples collected at the time of kidney biopsy to present sound results on the relationship of the PS proteins and the complex features of LN histology. Similarly, the relationship of the PS proteins to the presence or development of renal damage will need further study, as the majority of children with renal damage also had active LN concurrently.

In summary, Tf, Cp, AGP, and L-PGDS are promising LN biomarkers. Their initial validation suggests superior measure-

ment properties compared with most traditional LN biomarkers and that Tf, AGP, and L-PGDS are candidates of a novel set of predictive LN biomarkers. Additional validation studies are mandatory to evaluate the usefulness of such a LN renal panel to predict the course of LN, the severity of kidney pathology, and the future development of renal damage with SLE.

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NON-INVASIVE RENAL PROTEIN BIOMARKERS ARE ASSOCIATED WITH HISTOLOGICAL FEATURES OF LUPUS NEPHRITIS

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Conflict of Interest Statement

Dr. Devarajan is a co-inventor on NGAL patents for the diagnosis of acute kidney injury. Dr. Devarajan and Brunner are co-inventors on patents covering biomarker panels for the diagnosis of lupus nephritis.

ABSTRACT

Objective: To investigate the relationship of urinary biomarkers (UBM) and established measures of renal function (EMRF) to the histological findings with lupus nephritis (LN); and to test whether certain combinations of the above mentioned laboratory measures are diagnostic of specific histological features of LN.

Methods: Urine samples of 76 patients were collected within 2 months of a kidney biopsy and assayed for the UBM: lipocalin-like prostaglandin-D synthetase (L-PGDS), α1-acid-glycoprotein (AAG), transferrin (TF), ceruloplasmin (CP), neutrophil-gelatinase associated lipocalin (NGAL), and monocyte chemotactic factor 1 (MCP1). Using non-parametric analyses, UBM and EMRF levels were compared to histological features seen with LN: mesangial expansion, capillary proliferation, crescent formation, necrosis, wire loops, fibrosis, tubular atrophy, and epimembranous deposits. The area under the receiver operating characteristic (AUC) curve was calculated to predict LN activity, chronicity or membranous LN.

Results: There was a differential increase of the UBM that formed a pattern reflective of specific histological features seen with active LN. The combination of MCP1, AAG, CP plus protein:creatinine ratio were excellent in predicting LN activity (AUC=0.85). NGAL together with creatinine clearance plus MCP1 was an excellent (AUC=0.83) and MCP1, AAG, creatinine clearance plus C4 (AUC=0.75) a good diagnostic test of LN chronicity and membranous LN, respectively.

Conclusions: Select UBM are associated with specific tissue changes observed with LN activity and chronicity. Especially in combination with select EMRF, UBM are well-suited to non-invasively quantify LN activity, LN chronicity, and the presence of membranous LN.

INTRODUCTION

Systemic Lupus Erythematosus (SLE) is a multi-system inflammatory autoimmune disease, and renal involvement is one of the main determinants of poor prognosis (1). The histological features seen on kidney biopsy constitute the current criterion standard for the diagnosis of lupus nephritis (LN) and are used to guide LN treatment. Kidney biopsies provide a direct assessment of the presence and severity of acute changes due to active LN and give insight into the chronicity of LN (2). Obtaining kidney biopsies is necessary because traditional measures of LN such as blood pressure, proteinuria, urine sediment, complement components C3 and C4, and glomerular filtration rates (GFR) are considered too inaccurate to reliably discriminate between the acute inflammatory changes that are amenable to immunosuppressive therapy and the chronic degenerative changes that will not improve despite control of SLE activity.

Using proteomic techniques, we identified previously novel urinary biomarkers (UBM) of LN. These include transferrin (TF), ceruloplasmin (CP), α-1-acid-glycoprotein (AAG; also known as orosomucoid), lipocalin-type prostaglandin-D synthetase (L-PGDS), monocyte chemotactive factor 1 (MCP1; also known as chemokine ligand 2), and neutrophil gelatinase associated lipocalin (NGAL) (3-5). We have shown that these UBM correlate with and are responsive to clinical measures of LN activity, and that some UBM are even suited to predict future LN flares (6-8). The relationship of these UBM to specific histological features of LN, however, has not been examined and was the focus of this study.

The objectives were to (1) study the relationship of the UBM and traditional laboratory measures of LN to histological findings seen on kidney biopsy in both children and adults with LN; and (2) test whether certain combinations of the above mentioned laboratory measures are diagnostic for specific histological features of LN.

MATERIALS & METHODS

Patients

Children and adults diagnosed with SLE (9) who required a kidney biopsy as part of standard of care therapy were included in this study, if a random spot urine sample was available that was collected within 60 days of the kidney biopsy. On the day of the urine sample collection, information about patient demographics, medications, and disease activity was collected. Key laboratory measures were obtained, including complement C3 and C4 levels, anti-dsDNA antibodies (present/absent), amount of proteinuria as estimated by the protein to creatinine ratio (P/C ratio) in a random or 24-hour urine sample, serum creatinine, and glomerular filtration rate (GFR) as estimated by age-appropriate calculation of the creatinine clearance (10, 11).

The renal domain score of the Systemic Lupus Disease Activity Index (SLEDAI-R; range 0 – 16; 0 = inactive LN) served as the clinical measure of LN activity (12). The Systemic Lupus International Collaborating Clinics/ American College of Rheumatology Damage Index items addressing kidney damage (SDI-R; range 0 – 3; 0 = no LN damage) were recorded as a clinical measure of kidney damage in patients with LN (13).

Kidney Histology

The histological characteristics of each kidney biopsy, as per report from the local pathologists, were reviewed in a blinded fashion by one expert nephropathologist (DW), as per the International Society of Nephrology/ Renal Pathology (ISN/RPS) Classification (14).

The following histological features reflective of active inflammation with LN were recorded: mesangial proliferation, endocapillary karyorrhexis (also: fibrinoid necrosis); cellular crescents; capillary proliferation, subendothelial deposits identifiable by light microscopy (also: wire-loops). We also noted features representing LN chronicity or degenerative damage. These included glomerular sclerosis (segmental or global), fibrosis including fibrous adhesions and fibrous crescents, as well as tubular atrophy.

Almost all studies in LN employ a previously developed scoring system to quantify the amount of overall LN activity and overall LN chronicity as is present in the kidney biopsy specimen (15). The features of activity and chronicity listed above were categorized as follows: 0 (no lesions), 1 (lesions in up to 25% of glomeruli), 2 (lesions in 25–50% of glomeruli) or 3 (lesions in >50% of glomeruli). Using these numeric values, a *Biopsy Activity Index (BAI) score* (range 0 - 24) and a *Biopsy Chronicity Index (BCI) score* (range 0 - 12) can be calculated, where higher scores represent higher LN activity or chronicity, respectively.

Epimembranous deposits, although not included in the BAI or the BCI scores, were also recorded. Depending on the findings of active inflammation**,** and chronic changes

observed on kidney biopsy, LN is classified in six categories. Pronounced predominance of epimembranous deposits is compatible with Class 5 of LN.

The ISN/RPS Classification, the BAI and the BCI have all been validated for use in adults and children with LN (16, 17). Risk factors for poor LN outcome include BAI scores of 7 or higher and BCI scores of 4 or higher (16, 18-25).

Urinary Biomarker Assays

Urine samples were frozen at -80 degree celsius prior to batch processing. We measured urinary concentrations of TF and L-PGDS by immunonephelometry (Dade Behring BNII Prospect, Marburg, Germany). Urinary CP was quantified by ELISA (Human Ceruloplasmin ELISA Quantitation Kit; Assaypro, St.Charles, MO, USA). Intra and inter-assay coefficients of variation of these assays (%CV) were 3.4% and 2.5% for TF, 2.3% and 6.5% for L-PGDS, and 4.1% and 7.1% for CP, respectively. Likewise, urinary AAG (5.0% and 8.5%) was measured using an ELISA kit (Human Orosomucoid ELISA Quantitation Kit; Genway Biotech, Inc., San Diego, CA, USA). MCP1 levels were also measured by ELISA (R&D Systems, Minneapolis, MN, USA). The respective intra-assay and inter-assay CV was 5.0% and 5.1%. We used all these commercial ELISA kits as per manufacturers' instructions, while NGAL was measured as previously reported by our group (6, 7). Intra and inter-assay CV of the NGAL assay were 5.0% and 5.1%, respectively.

Concentrations of the UBM (in ng/ml for AAG, NGAL, CP and MCP1 and in mg/dl for TF and L-PDGS) were standardized for urinary creatinine levels (in mg/mL).

Laboratory personnel measuring the UBM were blinded to the clinical and histological information.

Statistical analysis

We inspected the central tendency, dispersion, and skewness of the UBM and traditional markers of LN (C3, C4, GFR, P/C ratio) and found them not to fit well into normal distributions. Therefore, medians and interquartile ranges (IQR) were calculated as measures of central tendency for continuous variables, while categorical variables were summarized by frequency (in percentages). We used Spearman correlation coefficients to examine the strength of the association between numerical variables and Wilcoxon rank sum test to assess for statistically significant differences between types of histological features and UGM and traditional renal markers, respectively.

Because of the skewness, we log-transformed the concentrations of the UBM and traditional measures of LN prior to considering them in univariate and multivariate logistic regression modeling to determine relevant predictors of *key LN features* that are associated with poor prognosis (BAI score $\geq$ 7, BCI score $\geq$ 4) or that may require differential therapy (i.e. ISN/RPS Class 5 LN) (26).

We also calculated the relative change of the median and IQR of the laboratory measures with the presence vs. absence of a histological feature or a particular LN outcome (ISN/RPS Class 5 LN; BAI score $\geq$ 7; BCI score $\geq$ 4). Hence, values of 100% signify that the UBM (or traditional measure of LN) is present in the same amount with the presence vs. absence of a histological features or a particular LN outcome. Values >100% represent scenarios where the laboratory measure increases, and values <

100% where they decrease with the presence of the histological feature or a particular LN outcomes compared to its absence.

Included in the multivariate logistic models were all candidate biomarkers, and traditional biomarkers measures of LN with p-value of <0.15 on univariate analysis.

As published by our group in the past (5), the diagnostic accuracy of each biomarker and biomarker combination was assessed by the receiver operating characteristic (ROC) curve analysis, and the corresponding area under the curve (AUC, range 0 – 1) was calculated. The accuracy of the biomarker and biomarker combinations in predicting LN histology features was considered outstanding, excellent, good, fair, and poor if the AUC was in the range of 0.9 –1.0,0.81–0.90, 0.71–0.80, 0.61–0.70, and 0.50–0.60, respectively.

The sensitivity and specificity to predict LN outcomes (presence of ISN/RPS Class 5 LN; BAI score $\geq$ 7; BCI score $\geq$ 4) were determined for particular cut-off values of each biomarker combination, generally that for sensitivities around 75%.

Furthermore, we tested whether biomarker concentrations and specific kidney biopsy features systematically changed with patient age and explored whether the lag time between urine collection and kidney biopsy conduct was important for the association between UBM and histological features seen with LN.

Statistical analyses were done using SAS version 9.2 software (SAS, Cary, NC, USA). P-values < 0.025 were considered statistically significant. The study was approved by the Institutional Review Boards and Ethics Review Committees of the participating centers.

RESULTS

Patient Characteristics & Features of Kidney Biopsy

A total of 76 patients with a median age of 23 years (range: 9 - 51 years) was included in the study, and 26 patients were 18 years or younger (***Table 1***). At the time of urine collection, almost all patients were treated with glucocorticosteroids, many with immunosuppressive medications, and the median SLEDAI-R score was 8 (range: 0 - 16). Elevated levels of anti-dsDNA antibodies were present in 75% (49/65) of the patients with available information. Only three patients had renal damage as per the SDI-R.

The median time interval between kidney biopsy and urine sample collection was 3.5 days, and for 50% (38/76) of the patients the urine sample was collected prior to or on the day of the kidney biopsy. The histological diagnoses included proliferative LN (Class 3 or 4) in 50% (41/ 76), and ISN/RPS Class 5 LN in 38% (29/76) of the patients. Epimembranous deposits (ISN/RPS Class 5 or together with Class 3 or 4 LN) were observed in 43% (33/76) of the biopsies.

As expected, histological features were seen often concomitantly in the same kidney biopsy specimen. Generally, features reflective of active inflammation were clustered as were those representing LN chronicity. For example, in kidney biopsies with capillary proliferation, there was often (85%) moderate mesangial proliferation. Among patients with tubular atrophy, 95% also had fibrotic changes of the renal tissue (***Table 2***).

Associations of Clinical and Laboratory Measures with Histological Features

The age of the patients was significantly associated with serum creatinine (r = 0.27; p< 0.017) and the BCI score (r= - 0.45; p< 0.0001) but not with any of the UBM levels or other traditional measures of LN.

The concentrations of all the UBM were at least weakly correlated ($r \geq |0.2|$) with each other. An exception was TF which was strongly correlated with CP (r = 0.74; p < 0.0001) and AAG (r = 0.61; p = 0.005). Among traditional measures of LN, the only strong correlation (r = 0.79) was, as expected, between the GFR and serum creatinine levels. Notably, levels of C3, C4 and the P/C-ratio were unrelated (r<|0.2|).

This suggests that concentrations of the UBM do not simply increase in the urine due to increased proteinuria and supports the notion that the UBM provide additional information about LN over and above the traditional measures of LN.

Association of SLEDAI Renal Domain Score with LN histology

There were statistically significantly correlations of the SLEDAI-R scores with concentrations of NGAL (r= - 0.39; p< 0.0007), MCP1 (r= 0.23; p< 0.07), CP (r= 0.23; p< 0.05), AAG (r= 0.35; p< 0.003) and L-PGDS (r= 0.28; p< 0.016), respectively. Serum creatinine (r= 0.35; p< 0.002), the P/C-ratio (r= 0.40; p< 0.0004), and C3 levels (r= - 0.34; p <0.0043) were also correlated with SLEDAI-R scores. Conversely, urine concentrations of TF, C4 and the GFR were unrelated to the SLEDAI-R scores (r < |0.2|). BAI scores were weakly correlated with the SLEDAI-R (r= 0.29; p < 0.01)

Relative changes of the biomarkers with histological features of LN activity

The relative excretion of the UBM and traditional measures of LN with histological features of LN is shown in ***Figure 1.*** There was a relative increased excretion of some UBM with mesangial proliferation (Panel A), capillary proliferation (Panel B), cellular crescents (Panel C), and fibrinoid necrosis (Panel D). There was a trend towards more pronounced relative increases of the UBM with the presence of wire-loops but none of these changes reached statistical significance (Figure 1, Panels E). With the exception of the P/C ratio, there were no statistically significant differences in the levels of traditional measure of LN with any of the histological measures of LN activity. The levels of MCP1, CP, TF, AAG, and the P/C ratio all discriminated between low versus high BAI scores (<7 vs. ≥7) (Figure 1, Panels F). Of note, urinary L-PGDS and NGAL were not differentially associated with any of the histological features under consideration. The same was true of the levels of complement C3 and C4.

Relative changes of the laboratory measures with histological features of LN chronicity and epimembranous deposits

The levels of the UBM differed especially with features of active LN but not with features of LN chronicity (Figure 2, Panel A – C). Only the GFR and the serum creatinine levels importantly differed with LN chronicity features and distinguished between high vs. low BCI scores (≥ 4 vs. < 4).

None of the UBM or traditional measures of LN differed significantly with the presence vs. absence of epimembranous deposits. However, there was a trend towards the UBM showing larger relative differences in urinary excretion with the presence vs.

absence of epimembranous deposits. Only the GFR differed with Class 5 as compared to Class 2 - 4 LN significantly [median (IQR) in ml/min/1.73m^2: 125 (54) vs. 85 (54)].

Univariate and multivariate logistic modeling to predict LN outcomes

In univariate logistic regression, we assessed the diagnostic accuracy (AUC) of each of the biomarkers for *key LN features*, i.e. BAI scores $\geq$ 7, BCI scores $\geq$ 4 and the presence of ISN/RPS Class 5 LN. Good to excellent accuracy to diagnose patients with high BAI-scores were present only for AAG, TF (both AUC = 0.76), CP (AUC = 0.79), MCP1 (AUC = 0.82), and the P/C ratio (AUC= 0.76). Excellent predictors of high BCI scores were the GFR and serum creatinine (both AUC = 0.82). Univariate analysis did not reveal a single UBM or traditional measure of LN that was at least a good diagnostic measure (AUC $\geq$ 0.7) of Class 5 LN. Lastly, the SLEDAI-R scores were a poor proxy measure of BAI scores (AUC = 0.5).

The results of multivariate modeling to predict key LN outcomes (high BAI score, high BCI score, Class 5 LN) were summarized in ***Table*** 3. A combination of four different biomarkers each was excellent for diagnosing high BAI scores (AUC = 0.85) as the combination of NGAL, GFR and MCP1 for diagnosing high BCI scores (AUC = 0.83). Combinations of five biomarkers yielded a good diagnostic test for ISN/RPS Class 5 LN (AUC = 0.75).

Exclusion of Urine Samples Collected after the Kidney Biopsy

Although we did not have access to recent changes of medications just prior or after the study visit, we hypothesized that LN therapy was intensified after the results of

the kidney biopsy had become available. When only considering patients from whom urine samples were collected no later than the day of the kidney biopsy (n= 38), urinary NGAL concentrations were significantly lower with the presence of cellular crescents [median (IQR) in present vs. absent: 8.74 (44.9) vs. 48.5 (64.3); p< 0.0033], and L-PGDS concentrations were significantly lower with the presence of tubular atrophy [median (interquartile range) in present vs. absent: 695 (591) vs. 928 (1021); p< 0.0234]. There were no significant relative changes between LN features and any of the other UBM or traditional measures of LN when only considering this subset of 38 patients. The patterns of relative biomarker excretion in relation to LN histology, including those observed in the subanalysis are summarized in ***Figure 2, Panel F.***

DISCUSSION

We examined recently discovered UBM and traditional measures of LN and found especially individual UBM related to specific histological findings representing LN activity. The combination of MCP1, CP, AAG and the P/C was excellent in estimating histological LN activity. NGAL together with GFR and MCP1 were excellent diagnostic tests of LN chronicity. Combinations of biomarkers provided good markers for the presence of Class 5 LN.

Our previous research supports that the UBM correlate with and are responsive to change in clinical measures of LN activity (3, 7). These observations are confirmed by the findings of this study where the UBM were associated with histological features of LN activity. Surprisingly, urinary NGAL and L-PGDS were not differentially associated

with features of LN activity in our entire cohort of patients with urine samples collected within 2 months of a kidney biopsy.

We previously reported L-PGDS to be a biomarker of LN activity as measured, among others, by the SLEDAI-R (3). The significance of this protein for inflammatory processes with LN has been confirmed in animal studies (27). Our study found L-PGDS to be weakly associated with clinical measures of LN activity (SLEDAI-R), other UBM and traditional measures of LN but not with a specific histological feature of LN. This might be due to the observation that elevated levels of L-PGDS are reflective of increased permeability of injured glomerular capillary walls (28), a feature not directly visible in histological standard stains of kidney biopsies. Alternatively, as is suggested by the findings of our subanalysis where L-PGDS excretion was 46% higher in patients with tubular atrophy, it may represent an early biomarker or one that rapidly declines with immunosuppressive therapy.

NGAL is an early and predictive urinary biomarker which is rapidly induced by active inflammation with LN, and promptly declines with therapy (5). Thus, when we excluded patients whose urine sample was collected after the kidney biopsy, i.e. already on intensive treatment for LN, and instead considered only the remaining patients (n=38), we found NGAL to be much lower in patients with cellular crescents, an important histological feature of active proliferative LN.

The biologic function of NGAL is still under investigation (29). In the acute setting, the biological role of NGAL appears to be a protective anti-apoptotic mechanism that limits tubule cell damage and enhances proliferation (30). Hence, the low NGAL levels found in patients with cellular crescents may represent a failure to protect from

structural changes typically associated with active LN. Besides its role in LN activity, NGAL is also associated with LN damage (6). The importance of NGAL as a biomarker of LN chronicity is supported by its role as a predictor of high BCI scores in this study. Urinary NGAL is also elevated in adults with chronic kidney disease, in whom NGAL is inversely correlated with GFR and positively correlated with tubular atrophy (31). The increased production of NGAL in this chronic context likely constitutes a pathophysiological pathway that leads to progressive renal failure (32).

MCP1 has long been known to be a predictive biomarker of LN flares and LN severity (4). Lupus-prone MRL-lpr/lpr and MCP1 knockout mice exhibit significantly lower proteinuria and prolonged survival (33), indicating a role for MCP1 in LN pathogenesis, in addition to its demonstrated capacity as a urinary biomarker for LN (4). The findings of our study are in line with these previous reports because MCP1 was differentially excreted with features of LN activity and high BAI scores. Multivariate models that predict the presence of membranous LN (ISN/RPS Class 5) included MCP1 as an important predictor, supporting previous observations in idiopathic membranous nephropathy of high urinary MCP1 and high expression of MCP1, especially in the tubular epithelial cells (34).

AAG was markedly increased in the urine of patients with mesangial proliferation and crescents. This finding is line with AAG being a known marker of LN activity whose urinary levels are also elevated with other inflammatory kidney diseases. AAG is produced in epithelial cells and is thought to play an important role in regulating the dynamic properties of the glomerular capillary wall by reducing the permeability towards macromolecules such as albumin (35).

We found TF to be associated with mesangial and capillary proliferation and cellular crescent formation, an observation that is congruent with previous reports from IgA nephropathy (36). Physiologically, recycled and absorbed iron is delivered to the main iron-transporting protein in blood, transferrin. Some TF normally enters the glomerular filtrate, but it is retrieved by specific receptor-mediated uptake in the kidney tubular system (37). Thus, tubular injury will lead to increased urinary TF concentrations.

Similarly, urinary concentrations of CP were higher, especially with mesangial or capillary proliferation, crescent formation and fibrinoid necrosis. We reported CP, an oxidative stress-related protein, to be a biomarker of LN activity in the past (3). CP has been associated with tissue remodeling in the kidney after renal tubular injury as can be observed with LN (38).

Individually, neither the UBM nor the traditional measures of LN are suited to determine whether there are epimembranous deposits. Likely because the various histological features of LN often are seen together in the same histological specimen, the GFR was only found to importantly differ with the presence vs. absence of LN Class 5 but not of epimembranous deposits. We speculate that this is also the reason for the differences in trends of the other LN measures in patients with LN Class 5 compared to those patients whose kidney biopsies showed some epimembranous deposits but who did not have LN Class 5.

Although combinations of the biomarkers included in this study yielded excellent diagnostic tests for LN activity and chronicity, the presented analyses also suggest that

additional markers are needed to provide the highly accurate (AUC > 0.9) diagnostic tests that are urgently needed by clinicians to help guide LN therapy.

Our study must be seen in the light of certain limitations. Given the diverse medication regimens used, the multiplicity of distinct kidney biopsy features and their considerable overlap in a given patient, our study findings will need to be confirmed in a larger cohort. Nonetheless, the association of novel as well as traditional biomarkers of LN with specific histological features bears the expectation that accurate longitudinal non-invasive measurement of LN activity and chronicity is feasible. If confirmed this will allow for a more effective and personalized monitoring of LN and its therapy. The availability of standardized clinical platforms for the reliable measurement of the urinary biomarkers will enable the testing of this hypothesis in the near future (39).

LEGEND

Figure 1:

For the 76 patients considered in the study, relative changes of the median levels with the presence vs. absence of specific histological features associated with LN activity are shown (whiskers are percentage change of the IQR) of the biomarkers: NGAL, MCP1, CP, AAG, TF, L-PGDS; serum creatinine, GFR; complement C3 and C4, P/C ratio; and clinical disease activity as measured by the SLEDAI-R score. *Y-axis:* Values of 100% signify that there is no difference of the biomarker with the presence vs. absence of the histological feature under consideration, while values of < 100% (> 100%) imply that the levels of the biomarker decrease (increase) with the presence of the histological features.

Panel A: MCP1, CP, AAG and TF significantly increase with mesangial proliferation;

Panel B: MCP1, CP and TF significantly increase with capillary proliferation;

Panel C: AAG and TF and the P/C ratio increase with cellular crescents;

Panel D: CP and the SLEDAI-R significantly increase with fibrinoid necrosis;

Panel E: Especially AAG and TF increase with wire-loops but changes do not reach statistical significance at $p < 0.025$.

Panel F: MCP1, CP, AAP, TF and the P/C ratio significantly increase with high Biopsy Activity Index Scores (BAI).

Figure 2:

In **Panels A – C,** relative changes of the median levels (whiskers are percentage change of the inter-quartile ranges) of the biomarkers with the presence versus absence of histological feature or scores associated with LN chronicity are shown. Details on the biomarkers and the interpretation of the values of the y-axis are provided in the legend of figure 1.

Panel A: None of the biomarkers changes significantly with tubular atrophy;

Panel B: Only the serum creatinine increases significantly with fibrosis;

Panel C: Only the serum creatinine and the GFR increase significantly with high Biopsy Chronicity Index Scores (BCI);

Panel D: None of the biomarkers changes significantly with of epimembranous deposits;

Panel E: Only the GFR increases significantly with ISN/RPS Class 5 LN

Figure 3:

A summary of significant changes with the presence vs. absence of histological features as are shown in Figure 1 and in Panel A – C of Figure 2. BLUE dots represent changes seen in urine samples that were collected within 2 months of the kidney biopsy (n=76), while GREEN dots represent additional significant differences if only urine samples collected prior to the kidney biopsy are considered (n= 38). The novel urine biomarkers are differentially excreted with histological changes of LN activity but not with membranous changes or LN chronicity. The GFR, serum creatinine and the P/C ratio do not allow for the differentiation between active, chronic or membranous changes of LN.

LITERATURE

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Table 1: Patient Demographics, medications and renal status at the time of the urine collection

			Number of patients with available information	*n of N (%)*	*Median*	*Interquartile range*
Disease-onset	Childhood-onset LN		76	28 (37%)		
	Adult-onset LN			48 (63%)		
Females				64 (84%)		
Race	Black		76	35 (46%)		
	White			33 (43%)		
	Other+			8 (11) †		
Medications	Oral prednisone		76	73 (96%)		
	Pulse methylprednisolone			33 (43%)		
	Mycophenolate mofetil			23 (30%)		
	Azathioprine			3 (4%)		
	Cyclophosphamide			13 (17%)		
	Methotrexate			4 (5%)		
	Angiotensin blocking agent			41 (54%)		
LN Status	GFR < 60 ml/min/m^2		76	14 (18%)		
	Protein:creatinine ratio > 0.5		76	68 (89%)		
	Renal SDI score > 0		22	3 (14%)		0 - 2
	Renal SLEDAI score		76		8	0 - 16
	Presence of double-stranded-dsDNA		65	49 (75%)		
Timing of urine collection	Time interval to biopsy*		76		+3.5 days*	-60 to +60
	>30 days before biopsy		76	5 (7%)		
	>30 days after biopsy			17 (22%)		
Histological Features present	ISN/RPS class[&]	Class 2	76	6 (8%)		
		Class 3		12 (16%)		
		Class 4		29 (38%)		
		Class 5		29 (38%)		
		Mesangial expansion	76	73		
		Capillary proliferation		38		
		Cellular crescents		22		
		Fibrinoid necrosis		22		
		Wire -loops		21		
		Fibrosis		53		
		Tubular atrophy		58		
		Epimembranous deposits		33		
		BAI score[‡]	76		3	0 - 15
		BCI score[Δ]			2	0 - 9

* Positive value indicates that the urine was collected after the kidney biopsy

† American Indian 1; Asian 3; Mixed racial 5; [&]There were no patients with Class 1 or Class 6 LN

‡ Biopsy Activity Index; range 0 – 24; 0 = inactive LN

Biopsy Chronicity Index; range 0 – 12; 0 = LN without chronic changes

Table 2: Relationship between Histological Features on Kidney Biopsy†

Histological Features		*N*	*Capillary proliferation*	*Cellular crescents*	*Fibrinoid necrosis*	*Wire-loops*	*Fibrosis*	*Tubular atrophy*	*Epimembranous deposits*	*BAI Score ≥ 7*	*BCI Score ≥ 4*
Mesangial proliferation	Moderate	27	85%	44%	52%	62%	78%	74%	37%	41%	14%
	No / Mild	49	31%	20%	16%	8%	65%	78%	47%	2%	14%
	p-value		<0.0001	-	0.0016	<0.0001	-	-	-	<0.0001	-
Capillary proliferation	Yes	38		53%	39%	49%	79%	76%	34%	32%	13%
	No	38		5%	18%	5%	61%	76%	53%	-	16%
	p-value			<0.0001	-	<0.0001	-	-	-	<0.0001	-
Cellular crescents	Yes	22			68%	55%	77%	73%	32%	41%	9%
	No	54			13%	15%	67%	78%	48%	6%	17%
	p-value				<0.0001	<0.0001	-	-	-	0.0004	-
Fibrinoid necrosis	Yes	22				57%	68%	64%	23%	41%	9%
	No	54				15%	71%	82%	52%	6%	17%
	p-value					0.0005	_	-	0.02	-	-
Wire-loops	Yes	20					80%	65%	20%	60%	15%
	No	55					67%	80%	53%	0	15%
	p-value						-	-	0.02	0.0001	-
Fibrosis	Yes	53						95%	42%	17%	21%
	No	23						39%	48%	13%	-
	p-value							0.0001	-	-	-
Tubular atrophy	Yes	58							47%	12%	19%
	No	18							33%	28%	-
	p-value								-	-	-
Epimembra-nous deposits	Yes	33								9%	12%
	No	43								21%	16%
	p-value									-	-
Biopsy Activity Score	≥7	12									17%
	<7	64									14%
	p-value										-

† Only p-values < 0.025 are stated

Table 3: Prediction of Key Biopsy Features with Lupus Nephritis

Model Outcome Variable	Predictor Variables	Area under the ROC Curve‡ (95% confidence interval)	Sensitivity*	Specificity
Biopsy Activity Score $\geq$ 7	MCP1, CP, AAG, P/C ratio	0.85 (0.69- 1.0)	72%	66%
Biopsy Chronicity Score $\geq$ 4	NGAL, GFR, MCP1	0.83 (0.67 – 0.93)	73%	67%
Membranous Lupus Nephritis (Class 5)	MCP1, GFR, AAG, TF, C4	0.75 (0.62- 0.86)	75%	48%

‡. The area under the ROC (receiver operating characteristic curve) ranges between 0 - 1
* Clinically relevant point on ROC with sensitivity of at least 70%

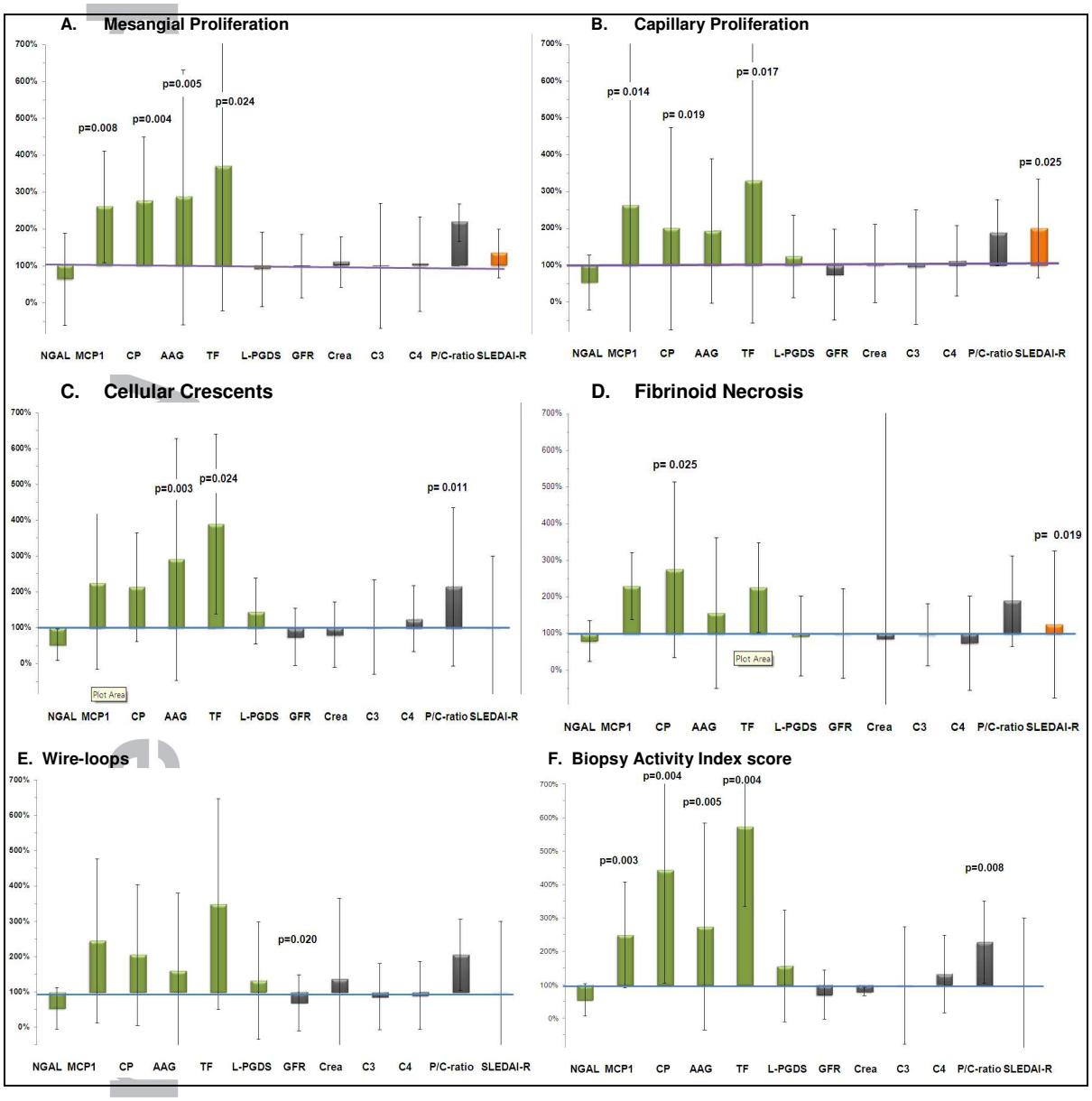

Figure 1 – Lupus nephritis activity: For the 76 patients considered in the study, relative changes of the median levels with the presence vs. absence of specific histological features associated with LN activity are shown (whiskers are percentage change of the IQR) of the biomarkers: NGAL, MCP1, CP, AAG, TF, L-PGDS; serum creatinine, GFR; complement C3 and C4, P/C ratio; and clinical disease activity as measured by the SLEDAI-R score. *Y-axis:* Values of 100% signify that there is no difference of the biomarker with the presence vs. absence of the histological feature under consideration, while values of < 100% (> 100%) imply that the levels of the biomarker decrease (increase) with the presence of the histological features. **Panel A:** MCP1, CP, AAG and TF significantly increase with mesangial proliferation; **Panel B:** MCP1, CP and TF significantly increase with capillary proliferation; **Panel C:** AAG and TF and the P/C ratio increase with cellular crescents; **Panel D:** CP and the SLEDAI-R significantly increase with fibrinoid necrosis; **Panel E:** Especially AAG and TF increase with wire-loops but changes do not reach statistical significance at $p < 0.025$. **Panel F:** MCP1, CP, AAP, TF and the P/C ratio significantly increase with high Biopsy Activity Index Scores (BAI).

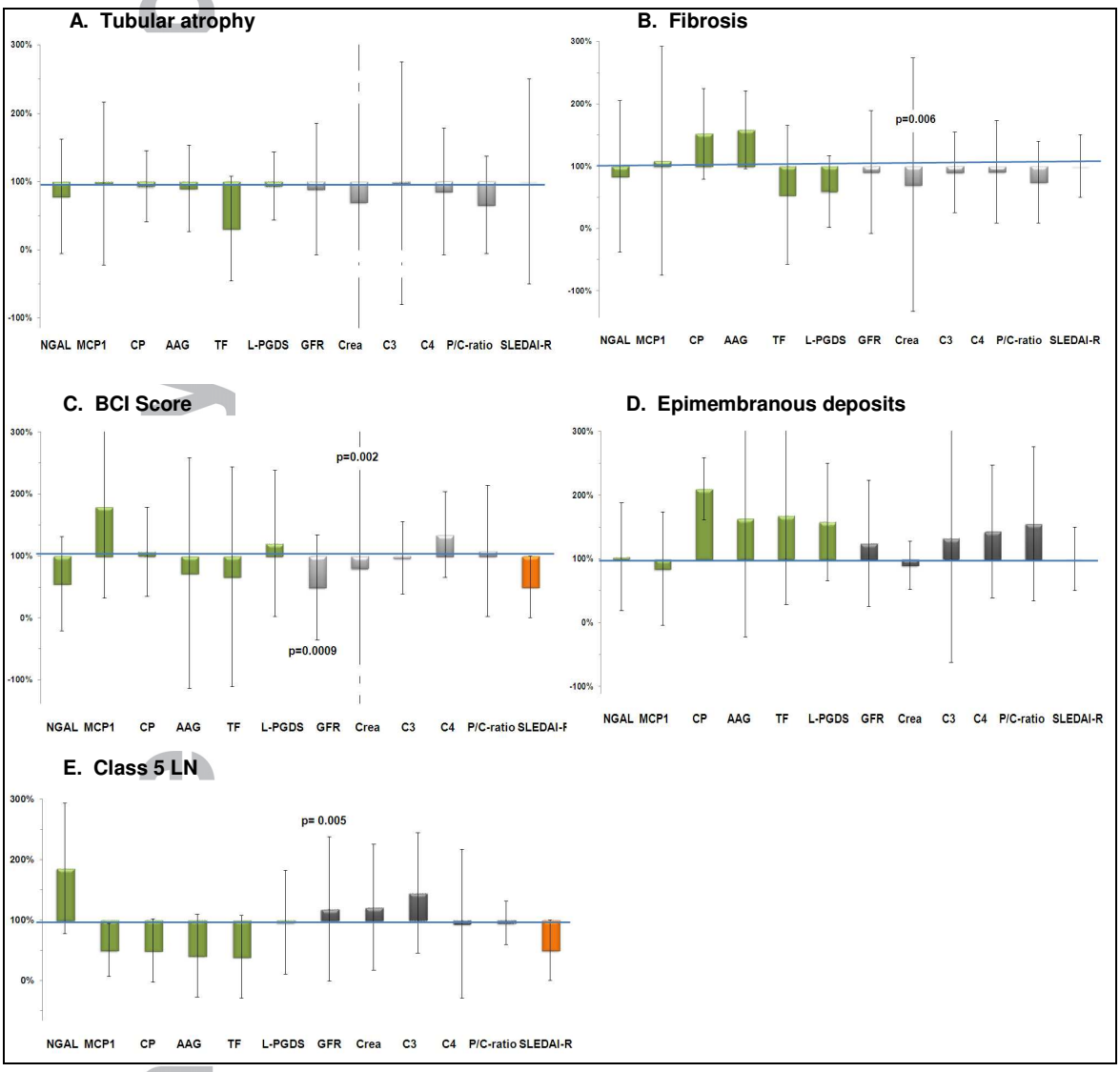

Figure 2 – Lupus nephritis chronicity: In **Panels A – C,** relative changes of the median levels (whiskers are percentage change of the inter-quartile ranges) of the biomarkers with the presence versus absence of histological feature or scores associated with LN chronicity are shown. Details on the biomarkers and the interpretation of the values of the y-axis are provided in the legend of figure 1. **Panel A:** None of the biomarkers changes significantly with tubular atrophy; **Panel B:** Only the serum creatinine increases significantly with fibrosis; **Panel C:** Only the serum creatinine and the GFR increase significantly with high Biopsy Chronicity Index Scores (BCI); **Panel D:** None of the biomarkers changes significantly with of epimembranous deposits; **Panel E:** Only the GFR increases significantly with ISN/RPS Class 5 LN

Biomarkers‡ / Histological features	NGAL	MCP1	CP	AAG	TF	L-PGDS	C3	C4	P/C-ratio	GFR	Serum creatinine
Mesangial proliferation		●	●	●	●						
Capillary proliferation		●	●		●						
Cellular crescents	●			●	●						
Fibrinoid necrosis			●								
Wire-loops										●	
BAI Score		●	●	●	●				●		
Fibrosis											●
Tubular atrophy						●					
BCI Score										●	●
Epimembranous deposits											
Class 5 lupus nephritis										●	

Legend – Patterns of LN Biomarker Changes: A summary of significant changes with the presence vs. absence of histological features as are shown in Figure 1 and in Panel A – C of Figure 2. BLUE dots represent changes seen in urine samples that were collected within 2 months of the kidney biopsy (n=76), while GREEN dots represent additional significant differences if only urine samples collected prior to the kidney biopsy are considered (n= 38). The novel urine biomarkers are differentially excreted with histological changes of LN activity but not with membranous changes or LN chronicity. The GFR, serum creatinine and the P/C ratio do not allow for the differentiation between active, chronic or membranous changes of LN. *Abbreviations:* ‡ Lipocalin-like prostaglandin-D synthetase (L-PGDS), α1-acid-glycoprotein (AAG), transferrin (TF), ceruloplasmin (CP), neutrophil-gelatinase associated lipocalin (NGAL), and monocyte chemotactic factor 1 (MCP1). Neutrophil gelatinase associated lipocalin (NGAL); protein:creatinine ratio (P/C ratio); glomerular filtration rate (GFR).

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Identification of urinary metabolites that distinguish membranous lupus nephritis from proliferative lupus nephritis and focal segmental glomerulosclerosis

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Abstract

Introduction: Systemic lupus erythematosus (SLE or lupus) is a chronic autoimmune disease, and kidney involvement with SLE, a.k.a. lupus nephritis (LN), is a frequent and severe complication of SLE that increases patient morbidity and mortality. About 50% of patients with SLE encounter renal abnormalities which, if left untreated, can lead to end-stage renal disease. Kidney biopsy is considered the criterion standard for diagnosis and staging of LN using the International Society of Nephrology/Renal Pathology Society (ISN/RPS) classification, which was developed to help predict renal outcomes and assist with medical decision-making. However, kidney biopsy-based classification of LN is highly invasive and impractical for real-time monitoring of LN status. Here, nuclear magnetic resonance (NMR) spectroscopy-based metabolic profiling was used to identify urinary metabolites that discriminated between proliferative and pure membranous LN as defined by the ISN/RPS classification, and between LN and primary focal segmental glomerulosclerosis (FSGS).

Methods: Metabolic profiling was conducted using urine samples of patients with proliferative LN without membranous features (Class III/IV; $n = 7$) or pure membranous LN (Class V; $n = 7$). Patients with primary FSGS and proteinuria ($n = 10$) served as disease controls. For each patient, demographic information and clinical data was obtained and a random urine sample collected to measure NMR spectra. Data and sample collection for patients with LN occurred around the time of kidney biopsy. Metabolic profiling analysis was done by visual inspection and principal component analysis.

Results: Urinary citrate levels were 8-fold lower in Class V LN compared to Class III/IV patients, who had normal levels of urinary citrate ($P < 0.05$). Class III/IV LN patients had > 10-fold lower levels of urinary taurine compared to Class V patients, who had mostly normal levels ($P < 0.01$). Class V LN patients had normal urinary hippurate levels compared to FSGS patients, who completely lacked urinary hippurate ($P < 0.001$).

Conclusions: This pilot study indicated differences in urinary metabolites between proliferative LN and pure membranous LN patients, and between LN and FSGS patients. If confirmed in larger studies, these urine metabolites may serve as biomarkers to help discriminate between different classes of LN, and between LN and FSGS.

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Introduction

Systemic lupus erythematosus (SLE or lupus) is a chronic autoimmune disease [1]. Kidney involvement with SLE, a.k.a. lupus nephritis (LN), is a frequent and severe complication of SLE that increases patient morbidity and mortality [2]. About 50% of patients with SLE encounter renal abnormalities which, if left untreated, can lead to end-stage renal disease [3,4].

Kidney biopsy is considered the criterion standard for diagnosis and staging of LN using the International Society of Nephrology/Renal Pathology Society (ISN/RPS) Classification [5]. This classification was developed to help predict renal outcomes and assist with medical decision-making. Treatment of patients diagnosed with ISN/RPS class III or class IV LN requires combination therapy with corticosteroids plus immunosuppressive medications [6], whereas therapeutic choices for class V LN are still under considerable debate [7]. The ISN/RPS class of a patient with LN is not static. Over time, histological features of LN may improve in response to therapy or degenerative changes can accrue. The lack of sensitive and specific non-invasive biomarkers that assist with distinguishing between various LN classes makes it virtually impossible to dynamically monitor changes in LN classes in real time. This impairs the timely initiation of therapy and impairs monitoring of treatment response.

It is particularly difficult to discriminate proliferative LN (class III/IV) from pure membranous LN (class V) clinically, as both are associated with pronounced proteinuria, and changes in blood pressure and renal function. Pronounced proteinuria is also the hallmark of primary focal segmental glomerulosclerosis (FSGS). One of the histological characteristics of FSGS is podocyte injury, which results in different degrees of proteinuria and potentially, hypoalbuminemia, that is, these clinical and histological features can also occur with active LN [8].

In the past, we and others have used proteomics to discover candidate protein biomarkers for LN [9,10]. Alternative biomarker discovery approaches include metabolomics, that is, the systematic study of small-molecule metabolite profiles or unique chemical fingerprints that are the result of specific cellular processes, and metabonomics, which can be defined as the quantitative measurement of metabolite changes in such metabolic profiles [11-13]. Nuclear magnetic resonance (NMR) finger printing is currently the method of choice for metabonomics because it provides uniform detection of equal sensitivity for all proton-containing small molecules and can provide valuable information on metabolites directly from biofluids with little sample preparation [14-16]. Metabonomics is achieved by maximum data capture through NMR spectroscopy followed by pattern recognition statistics [17].

The objective of this study was to identify urinary metabolites that discriminated between proliferative LN (class III/IV), pure membranous LN (class V), and FSGS, using NMR spectroscopy-based metabonomics. Metabolic profiles of urine samples were investigated using high-field (850 MHz) solution-state NMR spectroscopy. Two urinary metabolites, citrate and taurine, were found to accurately distinguish between class III/IV and class V LN patients. Urinary citrate levels were eight-fold lower than normal in class V compared with class III/IV LN patients, who had normal levels of urinary citrate. Also, class III/IV LN patients had more than 10-fold lower than normal levels of urinary taurine compared with class V patients, who had mostly normal levels of urinary taurine. Finally, urinary hippurate levels accurately distinguished between class V patients, who had normal levels of urinary taurine, in comparison with FSGS patients, who completely lacked urinary hippurate.

Materials and methods

Patients and samples

All research was conducted in compliance with the Helsinki Declaration. Informed consent was obtained from all enrolled patients. The study was approved by the institutional review boards of both the Johns Hopkins Hospital and the Cincinnati Children's Hospital Medical Center. Children and adults diagnosed with SLE [1] who required a kidney biopsy as part of standard of care therapy were eligible for inclusion in this study if a random spot urine sample was available that was collected within 60 days of the kidney biopsy. On the day of the urine sample collection, information about patient demographics, medications, and disease activity was collected. Key laboratory measures were obtained, including complement C3 and C4 levels, anti-dsDNA antibodies (present/absent), amount of proteinuria as estimated by the protein to creatinine ratio (P/C ratio) in a random or 24-hour urine sample, serum creatinine, and glomerular filtration rate (GFR) as estimated by age-appropriate calculation of the creatinine clearance [18,19]. For SLE patients to be included in the study, they had to have undergone kidney biopsy, found to have either class III or IV LN without membranous features (class III/IV) or pure membranous class V LN as per the ISN/RPS classification [5], had an available stored urine sample collected within 60 days of a kidney biopsy, and signed the informed consent form.

The histological characteristics of each kidney biopsy, as per report from the local pathologists, were reviewed in a blinded fashion by one expert nephropathologist, as per the ISN/RPS classification [5]. The following histological features reflective of active inflammation with LN

were recorded: mesangial proliferation, endocapillary karyorrhexis (also: fibrinoid necrosis); cellular crescents; capillary proliferation, subendothelial deposits identifiable by light microscopy (also: wire-loops). We also noted features representing LN chronicity or degenerative damage. These included glomerular sclerosis (segmental or global), fibrosis including fibrous adhesions and fibrous crescents, as well as tubular atrophy. The results of these classifications are summarized in supplementary Table S1 [see Additional data file 1].

Almost all LN studies employ a previously developed scoring system to quantify the amount of overall LN activity and overall LN chronicity present in kidney biopsy specimens [20]. The features of activity and chronicity listed above were categorized as follows: 0 (no lesions), 1 (lesions in up to 25% of glomeruli), 2 (lesions in 25 to 50% of glomeruli) or 3 (lesions in > 50% of glomeruli). Using these numeric values, a Biopsy Activity Index (AI) score (range 0 to 24) and a Biopsy Chronicity Index (CI) score (range 0 to 12) can be calculated, where higher scores represent higher LN activity or chronicity, respectively. The ISN/RPS classification, the AI and the CI have all been validated for use in adults and children with LN [21,22]. Risk factors for poor LN outcome include AI scores of seven or higher and CI scores of four or higher [21,23-30]. The AI and CI scores of the patients are also listed in supplementary Table S1 [see Additional data file 1].

Epimembranous deposits, although not included in the AI or the CI scores, were also recorded. Depending on the findings of active inflammation, and chronic changes observed on kidney biopsy, LN is classified in six categories. Pronounced predominance of epimembranous deposits is compatible with class V of LN.

For patients with LN, key laboratory measures were recorded, including complement C3 levels, anti-dsDNA antibodies (present/absent), amount of proteinuria as estimated by the P/C ratio in a random or 24-hour urine sample, serum creatinine, and GFR as estimated by age-appropriate calculation of the creatinine clearance [18,19]. The renal domain score of the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI-R; range 0 to 16; 0 = inactive LN) served as the clinical measure of LN activity [31]. The Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index items addressing kidney damage (SDI-R; range 0 to 3; 0 = no LN damage) were recorded as a clinical measure of kidney damage with LN [32]. The results of these laboratory measurements are summarized in supplementary Table S2 [see Additional data file 1].

Ten patients with biopsy-proven primary FSGS and proteinuria served as a disease control group. For controls with FSGS, data and urine samples were collected during visits to the pediatric nephrology clinics.

The demographics and kidney status of all patients included in the study are summarized in Table 1.

Metabolic profiling

Preparation of urine samples for NMR analysis

Urine samples were stored at -80°C after collection and thawed on ice prior to preparation for NMR analysis. A 1 ml aliquot of each sample was centrifuged for 10 minutes at 2655 × g, and then 350 μl of clear urine was pipetted into a 1.5 ml microcentrifuge tube. A volume of 350 ml of buffer (300 mM KH_2PO_4, 2 mM NaN_3, 0.2% trimethylsilyl propionate (TSP) in 20% D_2O, pH 7.4) was added to each urine sample. A volume of 600 μl of the urine/buffer mixture was then pipetted into a 5 mm NMR tube (Norell ST500-7, Norell, Inc., Landisville, NJ USA).

NMR data collection and processing

All NMR experiments were carried out on a Bruker Avance™ III spectrometer (Bruker Biospin, Rheinstetten, Germany) operating at 850.10 MHz ^{1}H frequency and equipped with a room temperature 5 mm triple resonance probe with inverse detection and controlled by TopSpin 2.1.4 (Bruker Biospin, Rheinstetten, Germany). All experiments were conducted at 298 K. All data were collected using a spectral width of 20.0 ppm. Three ^{1}H NMR experiments, optimized by Bruker (Bruker BioSpin, Billerica, MA, USA) for use with metabonomic studies, were run on all samples: a standard 1D presaturation (zgpr), a 1D first increment of a nuclear Overhauser effect spectroscopy (NOESY; noesygppr1d) experiment, and a CPMG (cpmgpr1d) experiment. All experiments included presaturation of the water peak. The transmitter offset frequency (O1) was set to 4002.80 Hz to obtain optimal water suppression. The 90° pulse width was determined for every sample using the automatic pulse calculation feature in TopSpin. All pulse widths were between 13 and 16 μs. Water suppression was achieved by irradiation of the water peak during a recycle delay of 4.0s with a pulse power level of 55.92 dB.

One-dimensional zgpr ^{1}H NMR spectra were acquired using two transients and two dummy scans, 65 K points per spectrum giving an acquisition time of 1.92 s, -0.01 Hz of exponential line broadening, and a recycle delay of 4 seconds. Once the zgpr spectrum was determined to be of acceptable quality, based on the line width (< 1 Hz) and line shape (resolved C13 satellites) of the TSP internal standard, the other two experiments were run. The first increment of the 1D NOESY experiment was collected using eight transients with four dummy scans, 65 K points per spectrum giving an acquisition time of 1.92 seconds, a mixing time of 10 ms, and apodized using a Gaussian line broadening parameter of 0.01, and a 4 seconds recycle delay. The CPMG experiment was

Table 1 Patient demographics, medications and renal status at the time of the urine collection.

		Focal segmental glomerulosclerosis		Proliferative LN (class III or IV)		Pure membranous LN (class V)	
		n of 10 (%)	Median (Range)	n of 7 (%)	Median (Range)	n of 7 (%)	Median (Range)
Females		3 (33%)		4/7 (57%)		5/7 (71%)	
Race	Black	2/10 (20%)		3/7 (42%)		3/7 (42%)	
	White	5/10 (50%)		2/7 (29%)		2/7 (29%)	
	Other	3/10 (30%)*		2/7 (29%)†		3/7 (43%) **	
Medications	Oral prednisone	4/10 (40%)		7/7 (100%)		4/7 (57%)	
	Mycophenolate mofetil	5/10 (50%)		3/7 (43%)		5/7 (71%)	
	Cyclophosphamide	-		2/7 (29%)		1/7 (14%)	
	Angiotensin blocking agent	9/10 (90%)		2/7 (29%)		6/7 (86%)	
Kidney Status	GFR < 60 ml/min/m^2	4/10 (40%)		1/7 (14%)		1/7 (14%)	
	Protein: creatinine ratio > 0.5	5/5 (100%)		7/7 (100%)		7/7 (100%)	
	Renal SDI score > 0¥	-		0/3		1/4 (25%)	
	Renal SLEDAI score‡	-			4 (0-16)		8 (4-12)
	Presence of anti double-stranded-dsDNA	-		7/7 (100%)		4/7 (67%)	

‡ Systemic Lupus Erythematosus Disease activity Index (SLEDAI) - renal component score; ¥ Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index - renal component score; *American Indian 1, Asian 1, Mixed racial 1; † Asian 1, Mixed racial 1; ** Asian 1, Unknown

collected in order to eliminate any broad peaks present in the spectrum. The CPMG experiment used 64 transients with four dummy scans, 65 K points per spectrum giving an acquisition time of 1.87 seconds, a T_2 filter loop of 128 with an echo time of 1 ms, apodized using -0.01 Hz of exponential line broadening, and a 4 seconds recycle delay.

All NMR spectra were phased, baseline corrected, and corrected for chemical shift registration relative to TSP in TopSpin 2.1.1 (Bruker Biospin, Billerica, MA, USA).

Box and whisker plot analysis

Box and whisker plots were generated in excel using a template provided by Vertex42 LLC

Principal component analysis

The data were subjected to multivariate statistical analysis using AMIX software version 3.9.7 (Bruker Biospin, Billerica, MA, USA). All NMR spectra were normalized to total intensity prior to principal component analysis (PCA). NMR spectra were binned into 0.03 ppm-wide buckets, using simple rectangular bucketing, over the region of δ10.0 to 0.2 ppm. The region of δ 4.75 to 5.0 was excluded from the analysis to avoid effects of imperfect water suppression. Buckets with variances less than 5% were also excluded from PCA.

Unsupervised PCA was performed without consideration of group information (class III/IV; class V, FSGS). The algorithm employed to calculate the principal components (PC) is discussed by Rousseau *et al*. [33]. As is commonly done with metabolomic data, visualization of the data was accomplished by inspection of the PC scores plots and loadings plots.

Statistical significance analysis of NMR data

Statistical significance analysis was performed for the comparison of class III/IV and class V LN, as well as the comparison of class V LN and FSGS, using AMIX 3.9.7 (Bruker Biospin, Rheinstetten, Germany), as outlined by Goodpaster *et al*. [34]. A critical value of alpha of 0.05 was selected to ensure no greater than a 5% false positive rate. In order to correct for multiple simultaneous testing, a Bonferroni correction was applied to the critical value to ensure a constant family-wise false positive rate [34]. The Bonferroni corrected critical value was calculated by dividing alpha by the number of buckets used in the PCA, resulting in a stringent *P* value threshold for determination of statistically significant changes in resonance intensities between groups being compared. The number of buckets used for statistical significance analysis was determined by the number remaining after omitting buckets that contained less than 5% variance and after omitting buckets in the excluded regions. A change in bucket intensity between groups was determined to be statistically significant if its *P* value was less than the Bonferroni corrected critical value.

Mahalanobis distance and F-value calculations

Mahalanobis distance calculations and F-value calculations were performed in MatLab as described by Goodpaster *et al*. [35]. Critical F-values were calculated using a critical F-value calculator [36].

Identification of metabolites

Experimental NMR spectra obtained from study samples were compared with spectra of known metabolites using the ChenomX NMR Suite (ChenomX Inc., Edmonton, Alberta, Canada). The ChenomX database was used to filter for resonance frequencies at chemical shifts corresponding to those identified as outliers by visual comparison as well as in the loadings plot. Spectra present in the ChenomX database were examined to verify if the pattern of peaks matched those observed in the experimental data.

Results and discussion

Patients

A total of seven patients with proliferative LN without membranous features (class III/IV), seven patients with pure membranous LN (class V), and 10 disease controls with primary FSGS were included in the study.

NMR metabonomics data analysis

Due to slight variations in the pH of the urine samples, the NMR peaks of some metabolites experienced pH-dependent chemical shifts, making it difficult to identify variances for these peaks by PCA using standard rectangular bucketing. Therefore, all spectra were also visually inspected to validate NMR resonances that were potentially changing between groups, and then these peaks were locally aligned to enable reliable *P* score calculations.

Visual inspection of the NMR spectra of class III/IV and class V LN patients led to the identification of one metabolite, citrate, that had an eight-fold higher urinary concentration (Table 2) (*P* score = 0.0477) in class III/IV LN patients (1.11 ± 0.97 mM) compared with class V patients (0.14 ± 0.15 mM). The concentration of citrate in the class III/IV group fell into the normal range in human urine [37] whereas the citrate levels in the class V group were at the lower limit of published values [37]. A receiver operator characteristic (ROC) analysis indicated the citrate had 100% specificity at 86% sensitivity and an overall 88% accuracy [see Additional data file 1] for distinguishing between class III/IV and class V LN patients. Urinary citrate levels were also compared with the SLEDAI-R, CI, and P/C ratio (Figure 1). The comparisons shown in Figure 1 further indicated that there was a strong correlation between disease class and citrate levels; however, no correlation was observed between class and SLEDAI, CI, or P/C ratio between class III/IV and class V LN patients. Complete summaries of measured patient indices, including others not presented in Figure 1, are presented in Tables S1 and S2 of the Supplementary Material.

Visual comparison of the NMR spectra of urine samples of patients with class III/IV versus class V LN also

Table 2 Concentrations and fold-changes of urinary metabolites measured in Class III/IV, Class V LN and FSGS patients.

Citrate	Class IV (mM)	Class V (mM)	Fold change
	0.59	0.15	
	2.16	0.32	
	2.68	0	
	0.85	0.36	
	1.15	0.055	
	0	0	
	0.37	0.083	
ave (std)	1.11 (0.97)	0.14 (0.15)	8.04
Taurine	Class IV (mM)	Class V (mM)	Fold change
	0	1.84	
	0	7.51	
	0	2.32	
	0	0.32	
	0	2.61	
	0	0.85	
	0	0.55	
ave (std)	0	2.29 (2.47)	> 10
Hippurate	Class V	FSGS (mM)	Fold change
	1.79	0	
	0.70	0	
	0.55	0	
	0.47	0	
	1.49	0	
	1.66	0	
	1.89	0	
		0	
		0	
		0	
ave (std)	1.22 (0.62)	0	> 10

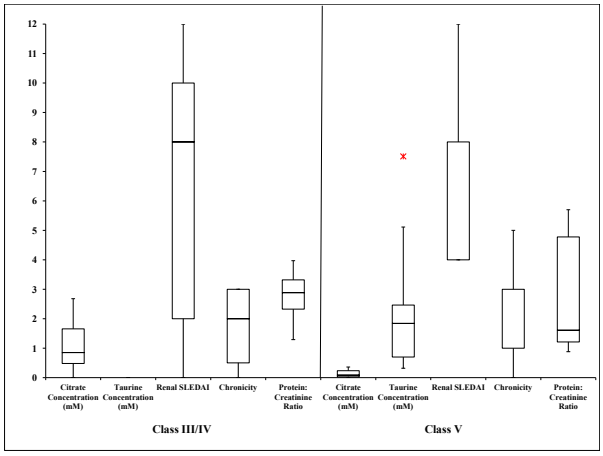

Figure 1 Box and whisker plots. The plots shown are comparing citrate concentration (mM), taurine concentration (mM), SLE Disease Activity Index (SLEDAI), chronicity index (CI), and urine creatinine (mg/mL) for class III/IV versus class V LN patients.

revealed higher concentrations (> 10 fold) of taurine in class V LN (2.29 ± 2.47 mM) compared with class III/IV LN patients, which lacked taurine altogether (Table 2) (*P* score = 0.00141). Taurine is normally present urine at levels in the range from 50 to 750 μM [38]. Therefore, the complete absence of taurine in class III/IV patients indicated a renal pathology that was distinct from class V patients who appeared to have mostly normal levels of taurine, with the exception of one patient who had more than five times the normal amount of taurine in the urine (Table 2). Discrimination of the two groups (class III/IV versus class V) based on urinary taurine levels was confirmed by PCA by restricting the spectral analysis just to the region of the ^{1}H NMR spectra that contained the taurine-specific triplet at δ 3.425 ppm (Figure 2). Inspection of the PC scores plot of these samples showed a significant separation of the class III/IV from the class V populations, primarily based on higher levels of taurine in the urine of patients with class V LN (Figure 2a). The magnitude of the cluster separation was quantified by calculating the Mahalanobis distance between the cluster centroids and the statistical significance of the cluster separation was evaluated by calculating the F-value and comparing it with the critical F-value (Figure 2a). The Mahalanobis distance of 2.282 between class III/IV and class V LN group centroids and the corresponding F-value of 8.353 (critical F-value 3.982) indicated a statistically significant separation of the two groups. The PC loadings plot corresponding to the PC scores plot shown in Figure 2a is shown in Figure 2b. The difference in the mean intensities of the bucket at δ 3.425 ppm, which corresponded to taurine, was found to be statistically significant with a *P* value of 0.00151.

ROC curve analysis indicated that taurine was a perfect predictor for discrimination between class III/IV and class V LN patients with 100% specificity, 100% sensitivity and 100% accuracy [see Additional data file 1]. Urinary taurine levels were also compared to SLEDAI, CI, and P/C ratio (Figure 1). As indicated above, the data failed to reveal any correlation between LN class and SLEDAI, CI, or urinary P/C ratios; however, a strong correlation between class III/IV and class V LN and was observed with a complete absence of urinary taurine in class III/IV patients compared with mostly normal levels of taurine in class V patients.

Unsupervised PCA was also carried out on normalized ^{1}H NMR spectra obtained from urine samples from Class V LN and FSGS patients. Inspection of the PC scores plot showed separation of the class V from the FSGS group based on the levels of hippurate, which were in the normal range found in human urine [37] in the class V LN group (1.22 ± 0.62 mM), compared with the FSGS group, which completely lacked hippurate (Figure 3a; Table 2). The Mahalanobis distance between the cluster centroids of the class V and FSGS groups was 1.781, with a corresponding F-value of 6.096 (critical F-value 3.739) indicating a statistically significant separation of these groups. NMR spectra of patients with class V LN or FSGS were visually compared to validate the differences in hippurate concentrations indicated by PCA. Figure 4 shows a region of

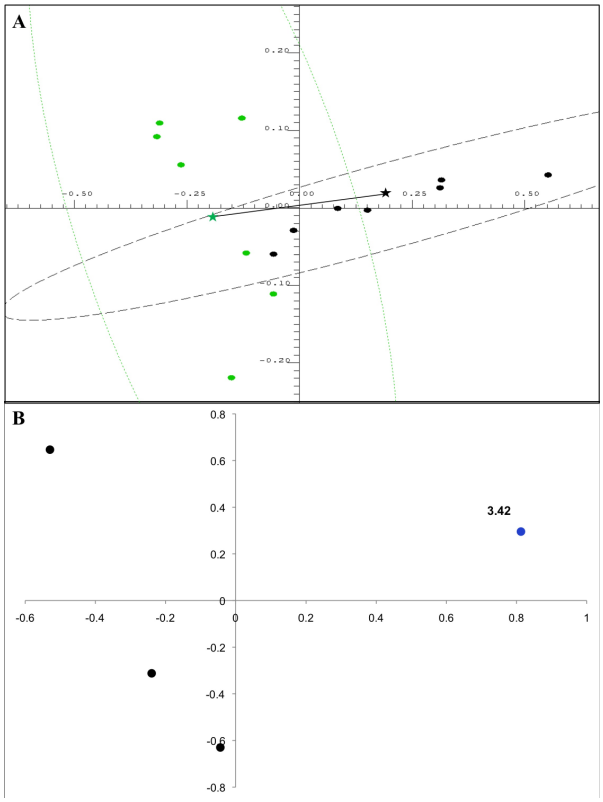

Figure 2 Principal component analysis of urine samples from patients with class III/IV LN and class V LN. **(a)** Two-dimensional principal component analysis scores plot of urine samples from patients with class III/IV LN (green) and class V LN (black) for peaks in the region from δ 3.40 to 4.50 ppm calculated using the first two principal components. Each point in the scores plot represents the NMR spectrum of an individual patient projected onto the two-dimensional space defined by the first two principal components. The dashed lines encircling the points define the 95% confidence intervals for each group. The color-matched stars indicate the centroid of each group and the line connecting the stars represents the Mahalanobis distance between the group centroids. **(b)** The loadings plot corresponding to the scores plot shown in Figure 1a. The labeled bucket (point) corresponds to the triplet belonging to taurine in the ^{1}H NMR spectra. The coordinates of each point indicate the PC loadings for that bucket, and represent how strongly that bucket is weighted in the eigenvector defining either the first or second principal component. The loadings plot points are heat map color-coded according to bucket *P* value: Black (> 1.25×10^{-2}), Blue (1.25×10^{-2}-10^{-5}). The Bonferroni corrected α-value was 0.0125.

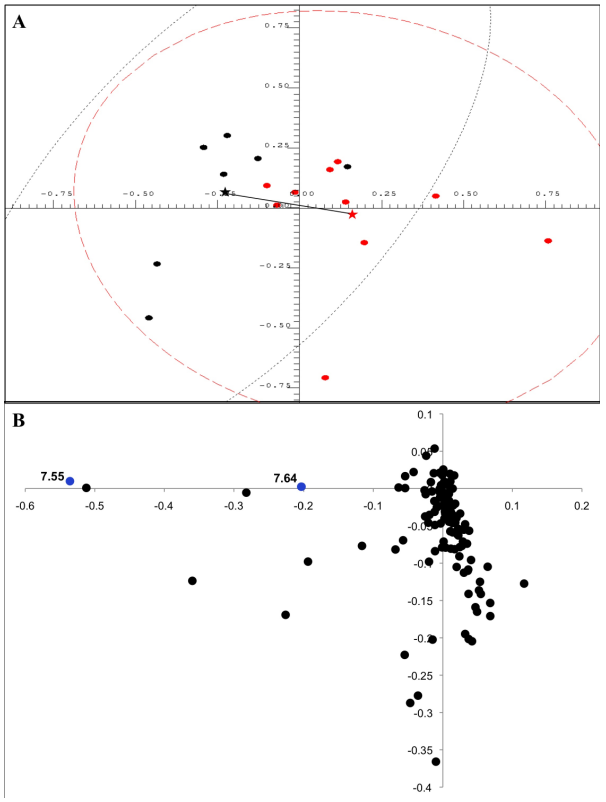

Figure 3 Principal component analysis of urine samples from class V LN patients and focal segmental glomerulosclerosis patients. **(a)** Two-dimensional principal component analysis scores plot of urine samples from patients with class V LN patients (black) and focal segmental glomerulosclerosis patients (red) using the first two principal components. The dashed lines encircling the points define the 95% confidence intervals for each group. The color-matched stars indicate the centroid of each group and the line connecting the stars represents the Mahalanobis distance between the group centroids. **(b)** The loadings plot corresponding to the scores plot in Figure 2a. The buckets shown are in the region from δ 0.02 to 10.0 ppm. The loadings plot is heat map color-coded according to bucket *P* values: Black (> 1.730×10^{-4}), Blue 1.730×10^{-4}-10^{-5}). The Bonferroni corrected α-value was 1.730×10^{-4}.

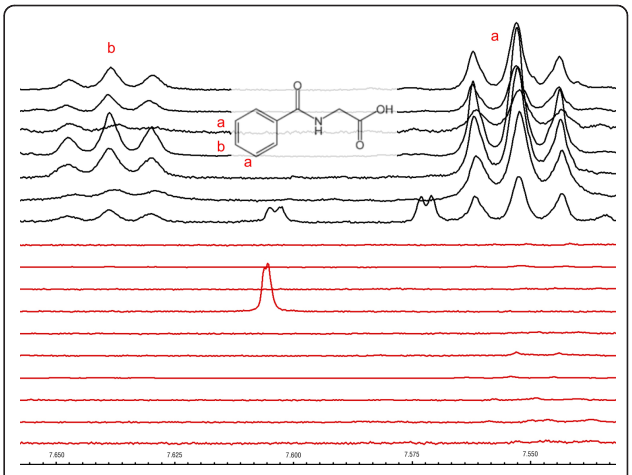

Figure 4 NMR urine spectra in the region from 7.650 to 7.550 ppm of class V LN patients (black) and focal segmental glomerulosclerosis patients (red). The triplets at δ7.64 and δ7.55 belong to the metabolite hippurate, as indicated in the inset.

the NMR spectrum that contains two triplets unique to hippurate located at δ 7.64 and δ 7.55 ppm confirming that hippurate was present in the class V group but completely absent in the FSGS group. The differences in the mean bucket intensities at δ 7.55 ppm and δ 7.64 between the two groups (> 10-fold change) were found to be significant: δ 7.55 *P* value = 0.000171, and δ 7.64 *P* value = 0.000379, respectively. ROC curve analysis indicated that hippurate was a perfect discriminator for distinguishing between class V LN and FSGS patients with 100% specificity, 100% sensitivity, and 100% accuracy [see Additional data file 1]. Interestingly, the complete lack of hippurate in the FSGS group indicated a unique pathology in FSGS compared with LN that leads to depletion of the normal urinary levels of hippurate.

Kidney biopsies are currently required to distinguish between different classes of LN, and between LN and other glomerular disorders, based on characteristic histological features. Obtaining kidney biopsies is invasive, and repetitive performance to guide day-to-day medical decisions is not practical. Although diagnostic, kidney biopsies are not suited to pinpoint altered metabolic processes or biological pathways involved in LN, which if detected, could lead to the identification of novel therapeutic targets. As the kidneys filter and reabsorb metabolites to maintain a metabolic equilibrium, existence of renal pathologies can impair the filtration of small metabolites through the glomerulus and their subsequent reabsorption in the renal tubules leading to changes in metabolic profiles [39]. Using NMR-spectroscopy, we found the metabolites taurine, citrate, and hippurate differentially excreted in the urine of patients with proliferative LN, membranous LN, and FSGS.

Comparison of the metabolic profiles of class III/IV LN versus class V LN patients indicated that class III/IV patients had normal urinary citrate levels but low urinary taurine levels whereas class V LN patients exhibited low urinary citrate levels but elevated urinary taurine levels. Based on previous reports, citrate and taurine are both measures of tubular cell function [40]. A possible explanation for the reduced excretion of urinary citrate in the class V LN group could be the presence of metabolic acidosis, which is known to cause decreased urinary excretion of citrate in humans [41]. It is believed that patients experiencing low urinary citrate output may have renal tubular cells that are more acidotic than in the healthy normal populations [42]. The proximal

tubules are responsible for the regulation of re-absorption and excretion of citrate [43,44]. The body's response to metabolic acidosis includes an increase of sodium/citrate co-transporter activity in the kidney, which causes increased citrate transport across the apical membrane into the tubule lumen [44]. Up-regulation of the co-transporter activity results in the increased re-absorption of citrate and reduced excretion of the metabolite into the urine. The cytosolic citrate metabolism also plays a key role in regulating the amount of citrate excreted into the urine. During metabolic acidosis, alterations in the enzyme ATP citrate lyase also results in decreased urinary citrate excretion [44].

Class III/IV LN patients had a striking absence of urinary taurine. Although the pathology leading to a complete absence of urinary taurine is not obvious, the body's store of taurine is known to be regulated by the kidneys and taurine is known to act as an anti-oxidant in a variety of *in vitro* and *in vivo* systems, and is used to treat renal dysfunction [45]. Therefore, it is possible that under the conditions of the most severe LN in class III/IV patients the kidneys utilize all available taurine in an attempt to manage or repair the kidney pathology.

Although the majority of patients with class V LN have normal levels of taurine, one patient had more than five times the normal amount of taurine. A possible explanation for the elevated level of taurine in the urine of this exceptional class V LN patient may be a consequence of inadequate re-uptake of taurine into the cells [46]. Taurine is excreted through both bile and urine, but its total body pool is primarily controlled by the kidneys via the renal tubules [47]. Previous studies suggest that tubular dysfunction is a risk factor of taurine deficiency [48]. Patients in renal failure often have low muscle and plasma concentrations of taurine. Although it has been suggested in the past that this was due to reduced taurine synthesis [48], our results suggest that low taurine levels are actually the result of increased urinary taurine excretion.

Increased urinary taurine may also be a result of changes in cysteine metabolism. Hypercysteinemia is associated with alterations of the sulfur metabolism and/or sulfate transport [49]. Taurine is known to play a critical role in these processes [49], and patients with class V LN may be unable to adequately cope with oxidative stress and the elimination of free radicals. Interestingly, based on animal studies, the acquisition of age-related renal fibrosis can be decreased by taurine supplementation [50], and taurine also has anti-hypertensive effects [51].

Histological scoring and quantification of proteinuria are key methods used to survey disease activity and severity in patients with LN [52,53]. Pirani *et al*. created a scoring system that is semi-quantitative [54], which was later adapted by Austin *et al*. [20] This system was developed to calculate the activity of LN (SLEDAI) by assessing six histological factors focusing on the severity of active lesions in the glomeruli, and the chronicity of the disease by evaluating four histological parameters focusing on the reversibility of LN [53]. Wallace *et al*. provides a table (Table 55-5 in the original article) that outlines the scoring strategies for both the AI and CI [55]. The degree of proteinuria is determined by measuring the P/C ratio in a 24-hour urine collection. This ratio has previously proven to be a reliable predictor of proteinuria in a study of LN patients [56]. The changes in citrate and taurine levels in the patients included in this study were plotted against the SLEDAI, CI, and P/C ratios in order to determine whether a correlation existed between any of these conventionally measured indices and biopsy-determined LN class (Figure 1). The data showed that no clear correlation existed between these conventional indices or P/C ratios and the LN class; however, our data indicated strong correlations between citrate and taurine levels and LN class. The lack of correlation between renal activity and chronicity with disease class is not surprising given that other studies have shown that when applying these indices to all World Health Organization classes of LN, rather than just diffuse proliferative LN, these indices lack an association with long-term prognosis [57,58]. The inability to relate P/C ratio to disease class is also expected because differing degrees of proteinuria are present throughout the patient population. Visual comparison of the above indices as they related to LN class further demonstrated a strong need for continued development of reliable biomarkers that allow for LN class differentiation, as was seen in the changes in the metabolites identified in this study.

Our pilot study also identified one urinary metabolite, hippurate, whose levels differentiated class V LN and FSGS patients. Specifically, class V LN patients had normal levels of urinary hippurate whereas FSGS patients completely lacked hippurate. Although the pathological link associated with a complete lack of urinary hippurate is not evident, the complete lack of urinary hippurate in FSGS patients is striking. A possible pathological cause could be related to having a distinct gut microbial biota, which has been linked to depleted excretion of hippurate in patients with Crohn's disease [59].

Conclusions

Currently, it is difficult to distinguish between class III/IV and class V LN, and FSGS using conventionally measured indices. Diagnoses require invasive and time-consuming procedures involving biopsies and histological analyses, which make monitoring of real-time changes in the disease pathology impossible using current technologies. Ideally, one would like to develop a non-

invasive and rapid biomarker-based methodology to distinguish between class III/IV and class V LN, and FSGS. Here we report putative urinary biomarkers for this purpose obtained from a pilot study. Using NMR spectroscopy, we found that the metabolites taurine, citrate, and hippurate were differentially excreted in the urine of patients with proliferative LN, membranous LN, and FSGS. Not only do these metabolites represent potential biomarkers for distinguishing classes of LN and FSGS, but consideration of the metabolic pathways involving these metabolites should lead to a better understanding of the pathology of the respective disease states. Although the small size of this pilot study limits its statistical power, it is in the realm of similar pilot studies for other diseases [60]. Nonetheless, our study has generated several hypotheses regarding the etiology of LN and FSGS and further validation of our findings is planned using an independent cohort of patients.

Additional material

Additional file 1: There are two additional tables included in this file, including Table S1, which provides a summary of classifications from histological analyses of kidney biopsy samples, and Table S2, which is a summary of laboratory test scores for LN patients. The file also contains Figure S1, which includes the receiver operator characteristic curves for citrate, hippurate and taurine.

Abbreviations

AI: activity index; CI: chronicity index; FSGS: focal segmental glomerulosclerosis; GFR: glomerular filtration rate; ISN/RPS: International Society of Nephrology/Renal Pathology Society; LN: lupus nephritis; NMR: nuclear magnetic resonance; NOESY: nuclear Overhauser effect spectroscopy; P/C ratio: protein to creatinine ratio; PC: principal component; PCA: principal component analysis; ROC: receiver operator curve; SLE: systemic lupus erythematosus; SLEDAI-R: Systemic Lupus Erythematosus Disease Activity Index-Range; SDI-R: Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index-Range; TSP: trimethylsilyl propionate.

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Authors' contributions

HIB was involved in the study design, data collection, and sample management as well as the interpretation of the data and the development of the manuscript. MRB, RM, SN, MP, and AK were all involved in the data collection, and sample collection and management and the development of the manuscript. PD was responsible for the study design, the interpretation of the data, and the development of the manuscript. LRR was responsible for NMR sample preparation, NMR data collection and analysis, investigation of biological interpretation of data and development of the manuscript. MAK was involved in NMR data analysis, interpretation of data and development of the manuscript. All authors read and approved the final manuscript.

Competing interests

Drs. Prasad Devarajan and Brunner are holding a patent on the use of some of the renal biomarkers evaluated for this study.

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