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14. ABSTRACT
The purpose of this project is to identify initial biomarker patterns in SLE nephritis using screening proteomic profiling, and advanced proteomic profiling. Subject recruitment has been completed (150 children with SLE, out of which 75 have lupus nephritis). Utility of one of the biomarkers (NGAL) in predicting worsening of global and renal SLE disease activity has been validated. We found 2 proteins significantly over-expressed in Class IV vs Class V lupus nephritis by 2D gel electrophoresis: albumin fragments (25kDa) and α-1-B glycoprotein (60kDa). We found over 30 proteins differentially expressed in Class IV vs Class V lupus nephritis by SELDI-TOF-MS. Additional proteomic profiling studies using NMR- and MS-based metabonomics as well as LC/MS based protein profiling using Thermo LTQ FT-ICR have been initiated. 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.

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INTRODUCTION AND SCOPE OF WORK

We propose 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 using Thermo LTQ FT-ICR will provide ultra-high resolution/mass accuracy protein identification, using the LTQ FT-ICR hybrid instrument (Thermo Electron North America LLC). The data will be analyzed by using Bioworks 3.2 software for protein identification along with statistical calculations for protein/peptide probabilities.

BODY

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

1.1: Subject Recruitment
All subjects with systemic lupus erythematosus (SLE) targeted for this study have now been recruited. We have 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) have been recruited. All subjects had at least six study visits to date, and the majority have completed all 7 study visits. Additionally, we have 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
During the first year of this study, we identified a panel of urinary biomarkers that correlated with SLE renal disease activity. Of the biomarkers, neutrophil gelatinase-associated lipocalin (NGAL) appeared to be the most promising. During this (second) year, we validated the utility of NGAL in predicting impending worsening of global and renal SLE disease activity. A total of 111 patients with SLE were enrolled in a longitudinal, prospective study with quarterly study visits and had at least three study visits. At each visit, global disease activity was measured using three external standards: numerically converted BILAG index, SLEDAI-2K and physician assessment score. Renal and extra-renal disease activity was 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 ELISA, and urinary NGAL was standardized to urinary creatinine. The longitudinal changes in NGAL levels were compared to the changes in SLE disease activity using mixed effects models. Significant increases in standardized urinary NGAL levels of up to 104% were detected up to three months before worsening of lupus nephritis (as measured by all three external standards). Plasma NGAL levels increased significantly by as much as 20% up to three months before worsening of global SLE disease activity as measured by all three external standards. Plasma NGAL levels increased significantly by 26% as early as three months prior to worsening of lupus nephritis as measured by the renal BILAG domain score. We then assessed the diagnostic accuracy of NGAL as a predictive biomarker of the course of SLE nephritis. As shown below in the Receiver operating characteristic curves in Figure 1, urine NGAL predicted probability of worsening kidney function as assessed either by the SLEDAI-2K renal score (panel A) or the BILAG renal score (panel B), with an area under the curve in the 0.80 range. Corresponding sensitivities and specificities at a n optimal cut-off are also shown in Figure 1. We concluded that serial measurement of urinary and plasma NGAL levels are valuable in predicting impending worsening of global and renal SLE disease activity. A manuscript describing these results has now been published in ***Arthritis and Rheumatism.*** The manuscript is attached in the Appendix.

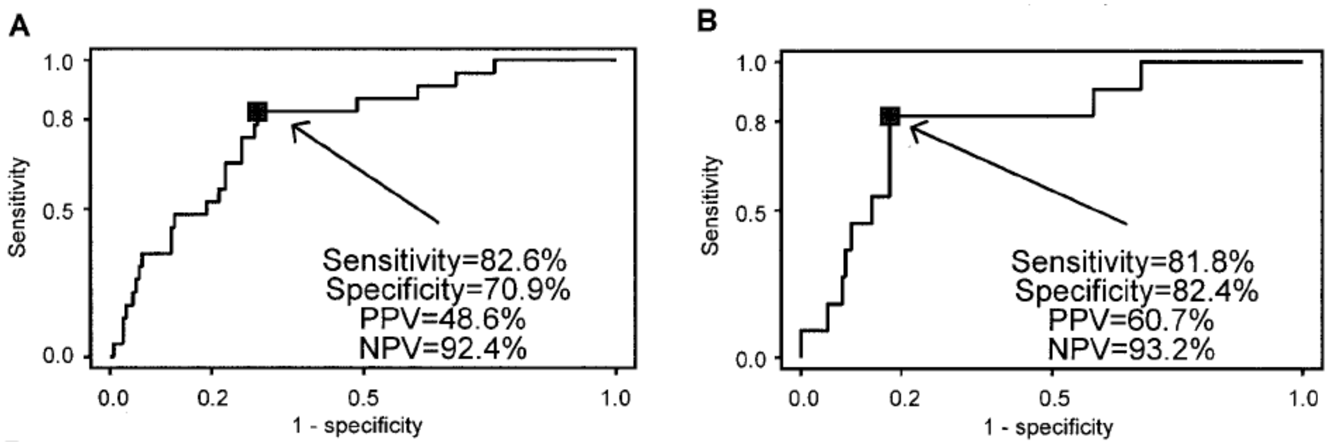

Figure 1. ROC curves for urine NGAL to predict worsening kidney disease using the (A) SLEDAI-2K renal score or (B) BILAG renal score

1.3: Identification of urinary biomarkers for distinguishing subjects with Class IV from Class V lupus nephritis using 2DGE and SELDI-TOF-MS

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 6 children with class IV LN, 7 with class V LN, and 4 with FSGS (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 (2DGE) and SELDI-TOF-MS. We found 2 proteins significantly over-expressed in class IV vs. class V by 2DGE. MALDI-TOF-MS/MS analysis identified these proteins as human serum albumin fragments (25kDa) and α-1-B glycoprotein (60kDa). In SELDI-TOF-MS, we used four different types of ProteinChips and analyzed the spectra with ProteinChip Data Manager 3.07. The most robust and reproducible peaks are shown in Table 1. These define a signature of urinary biomarkers that clearly distinguish between class IV and class V LN. These findings have important implications not only for biomarker discovery, but also for differential pathogenic mechanisms for LN subclasses.

Table 1: SELDI-TOF-MS peaks in LN

Chip	Class IV vs V *	Control vs class IV **	Control vs Class V **
CM 10	7807	3273, 3323	
NP 20	3266, 3278	3936, 4270, 4478, 7787, 23119	23119
H 50	3816, 3876, 4247, 5835, 9075, 9452, 16673	3876, 6796, 16134, 25835, 28101	4475, 4631, 7634, 11830, 11958, 13080, 47905
IMAC 30	4349, 4639, 4702, 8846	15096, 15298, 66411, 138089, 148232	7035, 15096, 15298

* Peaks (Da) with fold change >2, ** Peaks (Da) with fold change > 10

Identification of the most robustly differentially expressed peaks is expected to be completed in the upcoming year.

1.4: 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. Urine samples from 6 children with class IV LN and 7 with class V LN were studied. All samples were collected within 60 days of a kidney biopsy. Urinary profiling was performed using NMR- and MS-based metabonomics at Miami University, in the laboratory of Dr. Michael Kennedy. Initial spectra and profiles obtained show significant differences between patients with Class IV versus Class V SLE nephritis. Identification of the most robustly differentially expressed metabolites is expected to be completed in the upcoming year.

Research Accomplishments for Task 2:

To identify biomarkers predictive of SLE nephritis using advanced proteomic profiling:

2.1: Advanced Proteomics in Lupus Nephritis

This has been 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 will provide ultra-high resolution/mass accuracy protein identification. These studies are expected to be completed in the upcoming year.

KEY RESEARCH ACCOMPLISHMENTS

- Completion of subject recruitment
- Validation of NGAL as a predictive urinary biomarker for impending worsening of SLE disease activity
- Identification of a urinary biomarker signature that distinguish between class IV and class V LN
    - albumin fragments (25kDa) and α-1-B glycoprotein (60kDa) by 2D gel electrophoresis
    - 30 additional proteins by SELDI-TOF-MS

REPORTABLE OUTCOMES

This section contains information on published journal manuscripts, and abstracts submitted (which represent journal manuscripts in preparation). Also included is information on planned participation in technical meetings.

PUBLICATION:

C Hinze, M Suzuki, M Klein-Gitelman, M Passo, J Olson, N Singer, K Haines, K Onel, K O'Neil, E Silverman, L Tucker, J Ying, P Devarajan, H Brunner. Neutrophil Gelatinase-Associated Lipocalin Anticipates the Course of Global and Renal Childhood-Onset Systemic Lupus Erythematosus Disease Activity. Arthritis and Rheumatism 60:2772-2781, 2009. Manuscript included in Appendix.

ABSTRACTS SUBMITTED:

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. Abstract submitted to the Annual Meeting of the American Society of Nephrology, 2009. Selected for oral presentation at the American College of Rheumatology Annual Meeting, and for poster presentation at the American Society of Nephrology meeting. Abstract included in Appendix.

PRESENTATION AT MILITARY RESEARCH FORUM:

Devarajan P. Validation of a novel biomarker panel for active lupus nephritis. Abstract submitted to and 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.

CONCLUSION

Thus far, 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 (25kDa) and α-1-B glycoprotein (60kDa) identified by 2D gel electrophoresis, and approximately 30 additional proteins by SELDI-TOF-MS. Additional proteomic profiling studies using NMR- and MS-based metabonomics as well as LC/MS based protein profiling using Thermo LTQ FT-ICR have been initiated.

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.

The following work is planned to be completed during the next reporting period:

1. Identification and characterization of proteins differentially expressed in lupus sub-classes, as revealed by proteomic profiling with SELDI-TOF-MS and 2D Gel Electrophoresis

2. Identification and characterization of metabolites differentially expressed in lupus sub-classes, as revealed by metabolomics

3. Completion of advanced urinary proteomic profiling of lupus subjects with Thermo LTQ FT-ICR, and identification and characterization of proteins differentially expressed in lupus sub-classes, as revealed by this advanced proteomic technique

Although the work has progressed very well, we must remain cognizant of potential problems in the future, and we must be prepared to address these problems, as summarized below:

(a) Current problems that may impede performance

- Initial SELDI-TOF-MS studies have revealed more than 30 proteins that are differentially expressed in Class IV versus Class V lupus nephritis. This number is larger than initially anticipated. We will need to come up with a scientifically rational way of carefully prioritizing these markers, in order to identify those that are most worthy of further identification and validation. We will also need to perform additional SELDI-TOF-MS experiments on additional samples, in order to establish whether the observed differences are consistent in a new set of samples.
- Initial 2 dimensional gel electrophoresis (2DGE) and SELDI-TOF-MS have identified only 2 proteins significantly over-expressed in class IV vs. class V human serum albumin fragments (25kDa) and α-1-B glycoprotein (60kDa). This number is smaller than initially anticipated.

(b) Anticipated problems

- Initial metabolomic spectra and profiles obtained have shown significant but inconsistent differences between patients with Class IV versus Class V SLE nephritis. Analysis of this data is likely to be challenging. Our collaborator (Dr. Michael Kennedy) is establishing algorithms for efficient and accurate analysis of the data
- Initial LC/MS based protein profiling of urine from SLE patients using Thermo LTQ FT-ICR has proven to be more expensive than initially anticipated. We are working with our collaborators at the Applied Biotechnology Branch, Air Force Research Lab, Wright-Patterson Air Force Base, to accomplish as much of this aim as possible within the budget provided

APPENDIX

1. Manuscript published in Arthritis and Rheumatism, 2009

2. Abstract submitted to American College of Rheumatology Annual Meeting, and to the American Society of Nephrology Meeting, 2009

ARTHRITIS & RHEUMATISM
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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

Claas H. Hinze,[1] Michiko Suzuki,[1] Marisa Klein-Gitelman,[2] Murray H. Passo,[1] Judyann Olson,[3] Nora G. Singer,[4] Kathleen A. Haines,[5] Karen Onel,[6] Kathleen O'Neil,[7] Earl D. Silverman,[8] Lori Tucker,[9] Jun Ying,[10] Prasad Devarajan,[1] and Hermine I. Brunner[1]

***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.**

Supported by the NIH (clinical research grant P60-AR-47784 from the National Institute of Arthritis and Musculoskeletal and Skin Diseases). Dr. Devarajan's work was supported by the NIH (grants R01-DK-069749, R01-DK-53289, P50-DK-52612, and R21-DK-070163 from the National Institute of Diabetes and Digestive and Kidney Diseases) and by the Department of Defense (grant PR064328). Dr. Brunner's work was supported by a grant from the Alliance for Lupus Research.

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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.

Address correspondence and reprint requests to Hermine I. Brunner, MD, MSc, Cincinnati Children's Hospital Medical Center, University of Cincinnati, William S. Rowe Division of Rheumatology, E 4010, 3333 Burnet Avenue, Cincinnati, OH 45229-3039. E-mail: hermine.brunner@cchmc.org.

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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.

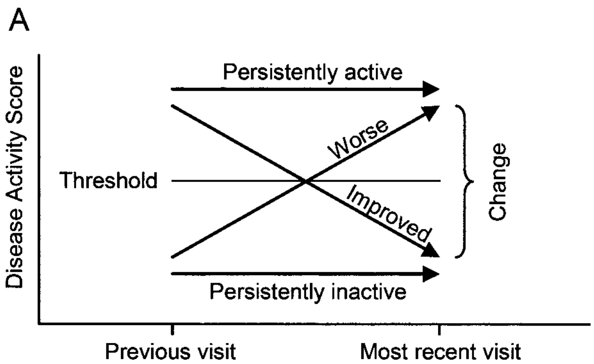
Childhood-onset SLE disease activity measures. At every study visit, global SLE disease activity was measured using 3 separate tools, as follows. The British Isles Lupus Assessment Group (BILAG) index (16) measures disease activity in 8 separate organ systems. While it was designed initially to reflect 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) Time −2	Time −1	Time 0	*P* 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

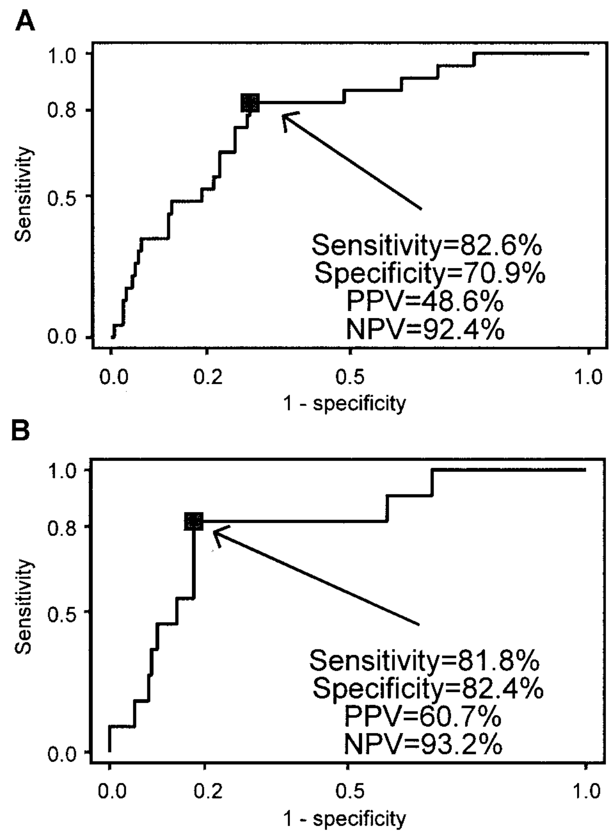


Figure 2. Receiver operating characteristic curves plotted by connecting sensitivity/specificity points under all possible probabilities of worsening of renal disease activity. Shown are the sensitivity and specificity of the predicted probability of worsening, estimated from multivariate logistic regression, 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.

Study conception and design. Hinze, Suzuki, Devarajan, Brunner.

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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Title: Urinary biomarkers for distinguishing subjects with class IV from Class V lupus nephritis

M Suzuki, *[1], M Bennett[1], L Das[1], K Hanes[2], M Klein-Gittelman[3], J Olson[4], K Onel[5], K O'neil[6], E Silverman[7], L Tucker[8], N Singer[9], M Wyder[10], K Greis[10], H Brunner[1] and P Devarajan[1]. [1]Cincinnati Children's Hospital; [2]Hackensack University; [3]Children's Memorial Hosp, Chicago; [4]Medical College of Wisconsin; [5]University of Chicago; [6]Oklahoma University; [7]Hospital for Sick Children,University of Toronto; [8]British Columbia Children's Hospital, Vancouver; [9]Case Western University, Cleveland and [10]University of Cincinnati.

Body: The ISN/RPS class IV and V Lupus nephritis (LN) show different histological features and differ in prognosis. In this study we aimed to identify non-invasive biomarkers which differentiate between class IV and V LN. Urine samples from 6 children with class IV LN, 7 with class V LN, and 4 with FSGS (control) were studied. All LN samples were collected within 60 days of a kidney biopsy. Subjects with overlapping features were not included. Two complementary proteomic methods were employed: 2 dimensional gel electrophoresis (2DGE) and SELDI-TOF-MS. We found 2 proteins significantly over-expressed in class IV vs. class V by 2DGE. MALDI-TOF-MS/MS analysis identified these proteins as human serum albumin fragments (25kDa) and α-1-B glycoprotein (60kDa). In SELDI-TOF-MS, we used four different types of ProteinChips and analyzed the spectra with ProteinChip Data Manager 3.07. The most reproducible peaks are shown in the Table. These define a signature of urinary biomarkers that clearly distinguish between class IV and class V LN. These findings have enormous implications not only for biomarker discovery, but also for differential pathogenic mechanisms for LN subclasses.

SELDI-TOF-MS peaks in LN

	Class IV vs V *	Control vs class IV **	Control vs Class V **
CM 10	7807	3273, 3323	
NP 20	3266, 3278	3936, 4270, 4478, 7787, 23119	23119
H 50	3816, 3876, 4247, 5835, 9075, 9452, 16673	3876, 6796, 16134, 25835, 28101	4475, 4631, 7634, 11830, 11958, 13080, 47905
IMAC 30	4349, 4639, 4702, 8846	15096, 15298, 66411, 138089, 148232	7035, 15096, 15298

* Peaks (Da) with fold change >2, ** Peaks (Da) with fold change > 10