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TRANSLATIONAL RESEARCH

Successful Urine Multiplex Bead Assay to Measure Lupus Nephritis Activity

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Introduction: Lupus nephritis (LN) confers a poor prognosis, mainly from lack of effective laboratory tests to diagnose and to evaluate therapies. We have previously shown that a set of 6 urinary biomarkers (NGAL, KIM-1, MCP-1, adiponectin, hemopexin, and ceruloplasmin) are highly sensitive and specific to identify adult and pediatric patients with active LN using renal biopsy as reference standard. Using these combinatorial urinary biomarkers, the Renal Activity Score for Lupus (RAIL) score was established, with biomarkers measured by enzyme-linked immunosorbent assay (ELISA). To enhance clinical utility of the biomarkers and RAIL, we tested the performance of RAIL with biomarkers measured by ELISA to that of biomarkers measured by the bead multiplex method, hypothesizing that the multiplex bead method would be comparable.

Methods: Spot urine samples (n = 341) of 46 patients aged 20 to 73 years with or without LN were used. Samples were assayed both by ELISA and multiplex using LUMINEX. RAIL scores and biomarker quantities were assessed for agreement with intraassay correlation coefficients and compared using Bland−Altman and regression.

Results: Biomarker measurement by LUMINEX was successful for NGAL, KIM-1, MCP-1, and adiponectin, but not for ceruloplasmin and hemopexin. There was good agreement of the RAIL obtained from these 4 biomarkers, irrespective of assay method (intraclass correlation coefficient [ICC] = 0.78, 95% confidence interval [CI] = 0.78−0.82). The RAIL scores from 4 biomarkers further correlated with those when considering all 6 biomarkers (ICC = 0.97, 95% CI = 0.96−0.98).

Conclusion: The LUMINEX platform allows for the convenient and simultaneous measurement of 4 RAIL biomarkers. RAIL scores considering only these 4 biomarkers may be sufficient to accurately capture LN activity.

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Kidney disease secondary to lupus nephritis (LN) constitutes a major individual and public health problem. Lupus nephritis occurs in about 50% of patients with systemic lupus erythematous (SLE; lupus).[1] Patients with LN have a 26-fold increased risk of death, and an annual health care cost (in 2006) estimated to be between $43,000 and $107,000 per patient.[2] Death from kidney disease in SLE occurs in 5% to 25% of patients with proliferative LN within 5 years of diagnosis, and 10% of patients with LN will develop end-stage renal disease (ESRD).[1,3] Poor prognosis in LN appears to be associated with a lack of laboratory and clinical tests to diagnose LN early and to evaluate response to therapy effectively. These deficiencies also make evaluating efficacy of new therapies challenging.[4] Traditional laboratory measures, such as proteinuria, serum creatinine, urinalysis, and urine sediment will classify patients incorrectly 30% to 40% of the time compared to biopsy[5]; complement levels and anti-dsDNA antibody titers are not effective for predicting the course of pediatric LN.[6,7] Therefore, invasive kidney biopsies are still required to diagnose and to monitor renal

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inflammation and response to therapy over time.[8] Hence, there is a crucial need for new diagnostic tests that noninvasively, accurately, and rapidly detect response to treatment and predict LN flares.

We have previously described and validated a panel of 6 urinary biomarkers, consisting of neutrophil gelatinase−associated lipocalin (NGAL), monocyte chemoattractant protein−1 (MCP-1/CCL2), kidney injury molecule−1 (KIM-1), ceruloplasmin, adiponectin, and hemopexin. Considering the concentrations of all 6 biomarkers, the Renal Activity Index for Lupus (RAIL) can be calculated, on which a higher score reflects high renal inflammation on biopsy.[9,10] We have shown that the RAIL is >90% accurate in detecting LN activity histologic activity measures from kidney biopsy as reference standard. This work has been replicated in both pediatric and adult patients and to identify those with and without LN. Furthermore, changes in the RAIL score precede clinically observed worsening and response to LN therapy for up to 3 months.[9,11] The RAIL biomarkers in all prior research were assayed using individual enzyme-linked immunosorbent assays (ELISAs). This can be time consuming, laborious, and can make *ad hoc* testing of individual patients cumbersome and costly. As the calculation of the RAIL requires knowledge of the quantities of 6 biomarkers, a convenient multiplex assay will be advantageous, preferable, and clinically actionable. A multiplexed reaction also allows multiple biomarkers to be assayed at the same time, often requiring a smaller amount of sample.[12,13] To move the RAIL score toward clinical usability and efficacy, we compared the urinary biomarkers via the original ELISA assay to the multiplex platform to assess correlation between the two. We hypothesized that the multiplex platform would be suitable to assay biomarker concentrations when compared with ELISA measurements as reference standard. We also hypothesized that the RAIL score composed of only 4 biomarkers (calculated with NGAL, MCP-1, KIM1, and adiponectin), would result in similar detection of high LN activity when compared to the original RAIL score that considers 6 biomarkers.

MATERIALS AND METHODS

Patients

This was a retrospective analysis performed on banked urine samples that were obtained from 46 adults who participated in a clinical trial sponsored by Medimmune LLC. Exclusion criteria for this study included active severe SLE-driven renal disease or unstable renal disease prior to screening. They had completed induction therapy and were on stable steroid doses. However, there were longitudinal samples collected and they were monitored for drug treatment efficacy or failure, including LN flare. Because of disease heterogeneity, kidney biopsy samples were not collected.

Besides random spot urine samples from these patients with or without LN, we obtained patient demographics and the results of routine laboratory testing. The SLE disease activity was measured according to the British Isles Lupus Assessment Group (BILAG).[14] The Renal BILAG scores were converted from the letter grading (A−E), with A denoting worse disease, to numeric (A = 12, B = 8, C = 1, D and E = 0) for ease of data analysis, as previously described.[7] The study was approved by the Institutional Review Board of the Cincinnati Children's Hospital Medical Center (CCHMC) and the institutional review boards of all other participating centers, with informed consent obtained prior to any study-related procedures (IRB # 2017-0585).

Sample Storage and Processing

Following sample collection, urinary specimens were stored at 4 °C within 1 hour of collection. They were then spun down and frozen at −80 °C. The urine underwent a single thaw cycle prior to processing. Urine biomarkers were quantified using commercial ELISA kits and multiplex platform. The following biomarkers were measured: adiponectin, ceruloplasmin, KIM-1, MCP-1, NGAL, and hemopexin.

ELISA

Unless stated otherwise, biomarkers were quantified using commercial ELISA kits as per the manufacturer's instructions, and a 4-parameter logistic curve was used to fit the standard curve. Human NGAL was performed using a commercially available assay (BIOPORTO, Hellerup, Denmark, catalog KIT 036RUO); starting dilution 1:500, and the lower limit of detection is 4 pg/ml. Intra- and interassay coefficients of variation were 1.0% and 9.1% respectively. The Human KIM-1 ELISA assay was constructed using commercially available reagents (Duoset DY, R&D Systems, Minneapolis, MN; DY1750B) as described previously[15] (starting dilution 1:1, with a lower limit of detection of 50 pg/ml). Intra- and inter-assay coefficients of variation were 2.0% and 7.8%, respectively. Human MCP-1 was measured via ELISA (R&D Systems, Minneapolis, MN; DCP00, starting dilution of 1:1, with a lower limit of detection 31.2 pg/ml. Intra-assay and interassay were 5.0% and 5.9% respectively. Human adiponectin was measured by commercially available ELISA kit (R&D Systems, Minneapolis, MN; DRP300), diluted 1:1, with a lower limit of detection 3.9 ng/ml. Intra-assay and inter-assay coefficients of variation were 3.7% and 6.8%,

respectively. Human ceruloplasmin was measured by ELISA Kit (Assaypro LLC, St. Charles, MO; EC4201-1), 1:10-20 dilution, with a lower limit of detection 0.156 ng/ml. Intra-assay and inter-assay were 4.1% and 7.1%, respectively. Hemopexin was measured by commercially available ELISA kit (Assaypro LLC, St. Charles, MO; EH2001-1), 1:10 dilution, with a lower limit of detection 6.25 ng/ml. Intra-assay and inter-assay coefficients of variation were 4.7% and 9.2%, respectively.

Multiplex Platform

Biomarkers using multiplex platform (MPL) assays were run during a different freeze−thaw cycle on the same urinary specimens. Multiplex measurements were performed using the Luminex MAGPIX Platform. The following multiplex kits were used: MCP-1 (R&D Systems LUHM279), NGAL (R&D Systems, LHK1757), Adiponectin (Invitrogen, EPX01A-12032-901), KIM-1 (made in-house using R&D kit DY1750), Human Hemopexin (Abcam, #51515), and Human Ceruloplasmin (Assaypro, Cat. #11261-05011).

Statistical Analysis

The 2 methodologies, ELISA and multiplex assays, were compared for each of the urinary biomarkers. The urinary biomarkers were then applied to the Adult RAIL (A-RAIL) algorithm, using the following formula: −5.05 − 00.56*(NGAL) + 0.12*(MCP-1) + 0.88*(Adiponectin) +0.01*(hemopexin) − 0.02*(KIM-1) − 0.29*(ceruloplasmin).[9] This algorithm has been validated for adults, where a result of greater than or equal to −1.92 correlates with high levels of renal inflammation on biopsy, meaning a NIH-AI score >10.[10]

The reliability or agreement of the individual urinary biomarkers as well as the RAIL score obtained from each methodology were assessed using intraclass correlation coefficients (ICC) obtained from a 2-way mixed effects model. An ICC of <0.5 represents poor reliability, between 0.5 and 0.75 represents moderate reliability, 0.75 to 0.9 indicates good reliability, and >0.9 indicates excellent reliability.[16]

Although ICC is the primary analytical method for assessing reliability, it is a summary measure. To display individual data points and their relationship with each other, we also report correlations (scatter plot) and Bland−Altman plots. A Pearson correlation coefficient was obtained along the scatter plot, with r of 0.1 to 0.39 representing weak correlation, 0.4 to 0.69 representing moderate correlation, 0.7 to 0.89 representing strong correlation, and >0.9 representing very strong correlation.[17] In addition, we used a Bland−Altman plot to display the individual data points as well as the mean difference between the 2 methods. In this plot, the differences between the 2 methods for a given urinary biomarker are used in the y-axis, whereas the average of those measurements is used in the x-axis. For each urinary biomarker, the mean difference, or bias, along the 95% limits of agreement will be displayed.[18] In addition, the percentage of values within the 95% limits of agreement will be reported.

Finally, using ELISA methodology, a modified RAIL score was created using only 4 biomarkers. Taking hemopexin and ceruloplasmin to 0, the modified RAIL score formula was as follows: −5.05 − 0.56*(NGAL) + 0.12*(MCP-1) + 0.88*(Adiponectin) − 0.02*(KIM-1). The agreement between this modified RAIL score and the original RAIL score were evaluated using an ICC. All urine biomarker levels are reported after natural log transformation to normalize their distribution when considered in the RAIL or in the presented analyses, as previously described.[9] The clinical status of the patients were blinded to the performers of the ELISA and MPL assays, but status was unblinded for analysis. Statistical analyses were performed using R Foundation for Statistical Computing software, version 4.0.2.

RESULTS

Patients and Samples

Flow of participants are shown in supplementary Figure S1. There were 46 patients, amounting to 341 samples. Because of inadequate standard curve creation with 1 ELISA run for adiponectin, 50 samples were excluded in the adiponectin and RAIL analysis, resulting in a total of 291 samples included for that assessment of method agreement.

Overall patient characteristics are shown in Table 1, also stratified by BILAG score. The average age of the patients was 41 years, ranging from 20 to 73 years, and 61% (28/46) of the patients were female. We lacked clinical information from 11 patients, amounting to 159 samples; however they were not excluded from the analysis. Overall, the patients had normal renal function, with an average glomerular filtration rate (GFR) of 96.5 ml/min per 1.73 m^2. The majority of patients lacked active LN; as such, 113 of 341 patients (33%) had a Renal BILAG score of D, and 39 of 341 patients (11.44%) had a Renal BILAG score of E. The samples from patients with higher BILAG scores (A, B) still had normal renal function with average GFR 92.80 and 99.13 ml/min per 1.73 m^2, respectively. This group had mild proteinuria, with average urine-to-protein ratio (UPCr) of 1.05 mg/mg and 0.45 mg/mg, respectively. Even in the patients with clinically active renal disease, the average RAIL score by ELISA was −6.73 (BILAG A) and −6.41 (BILAG B), that is, well below the cutoff for

Table 1. Patient characteristics (N = 341)

		A	B	C	D	E	N/A	
	Overall	n = 2 (0.6%)	n = 25 (7.33%)	n = 3 (0.9%)	n = 113 (33%)	n = 39 (11.44%)	n = 159 (46.63%)	*P* value
GFR, ml/min per 1.73 m^2, mean (SD)	96.52 (20.88)	92.80 (0)	99.13(23.97)	87.30 (7.45)	95.46 (23.12)	98.81 (10.71)		0.776
UPCr, mg/mg, mean (SD)	0.17 (0.18)	1.05 (0)	0.45 (0.21)	0.46 (0.04)	0.12 (0.06)	0.08 (0.07)		2×10^{-16}
dsDNA, IU/ml, mean (SD)	124.48 (168.74)	57.00 (0)	371.72 (271.26)	185.00 (39.84)	80.65 (96.12)	91.79 (119.49)		8.14×10^{-16}
C3, mg/dl, mean (SD)	91.65 (36.61)	38.00 (0)	73.38 (17.23)	40.13 (13.57)	99.47 (35.58)	87.41 (40.93)		0.000115
C4, mg/dl, mean (SD)	18.13 (10.28)	5.00 (0)	13.50 (4.71)	9.40 (4.33)	17.55 (10.16)	24.13 (10.85)		4.07×10^{-5}
Complete RAIL score (by ELISA); mean (SD)	- 6.81 (1.22)	-6.73 (0.96)	-6.41 (1.12)	-5.82 (0.43)	-6.75 (1.20)	-7.17 (1.24)		0.135

ELISA, enzyme-linked immunosorbent assay; GFR, glomerular filtration rate; RAIL, Renal Activity Score for Lupus; UPCr, urine-to-protein ratio.

high renal inflammation of −1.92. The patient with the highest RAIL score in the study for whom clinical information was available was in BILAG Group B (−4.11), and the overall the highest RAIL score was −3.83. A RAIL score exceeding −1.92 reflects high inflammation on renal biopsy, or a NIH-AI score >10. Table 2 demonstrates the differences in the individual biomarkers by BILAG score. The majority of the biomarkers differed by BILAG score, with higher BILAG class having higher levels than the lower classes. The exceptions were NGAL measured by MPL, KIM-1 measured by MPL, and adiponectin measured by ELISA.

The multiplex platform was incapable of quantitating hemopexin and ceruloplasmin. Specifically, we were unable to detect the high levels of ceruloplasmin and hemopexin that were present in the urine samples by ELISA. This prompted the exploration of a modified RAIL score, which omitted ceruloplasmin and hemopexin.

NGAL

NGAL levels measured by ELISA ranged from −1.26 to 8.28 ng/ml (mean ± SD = 3.0 ± 1.44 ng/ml). Luminex values ranged from −0.10 to 4.12 ng/ml (mean ± SD = 2.31 ± 0.88 ng/ml). As shown in Table 3, the ICC was 0.69 (95% CI = 0.63−0.74), supporting moderate agreement between the 2 tests. The scatter plot and Bland−Altman plot are shown in Figure 1a and b. Overall, NGAL demonstrated strong correlation, with an r value of 0.78. The multiplex assay for NGAL appeared to have a ceiling effect, noted for values of NGAL detected by multiplex when high amounts were detected by ELISA, as shown in Figure 1a. The Bland−Altman plot showed that there was detectable mean difference of 0.669 (95% CI = 0.57−0.77), suggesting that the ELISA was consistently higher than the multiplex assay (Figure 1b). However, the majority of the values (96%) were within the 95% limits of agreement.

KIM-1

KIM-1, measured in picograms per milliliter (pg/ml), ranged from 2.38 to 8.44 by ELISA (mean ± SD = 6.19 ± 1.15 pg/ml) and −2.45 to 8.48 (mean ± SD = 6.14 ± 1.37 pg/ml) when using the MPL assay. The ICC was 0.86 (95% CI = 0.83−0.88) (Table 3), supporting good agreement between the 2 tests. The scatter plot and Bland-Altman plots are shown in Figure 2a and b. There was minimal detectable mean difference between the 2 methodologies as evidenced by the Bland−Altman (Figure 2b), with a mean difference of

Table 2. Biomarker mean results by BILAG class (N = 341)

	A (n = 2)	B (n = 25)	C (n = 3)	D (n = 113)	E (n = 39)	N/A (n = 159)	*P* value
NGAL, ng/ml, mean (SD)	2.66 (1.98)	4.03 (1.22)	1.87 (2.03)	2.84 (1.37)	3.52 (1.45)	2.80 (1.41)	0.00016
ELISA	2.15 (0.93)	2.54 (0.47)	1.60 (1.24)	2.20 (0.82)	3.52 (0.57)	2.33 (1.00)	0.232
MPL							
KIM-1, pg/ml; mean (SD)	5.78 (1.02)	6.82 (0.67)	5.95 (0.63)	5.97 (1.35)	5.99 (1.20)	6.30 (0.99)	0.01
ELISA	5.78 (0.78)	6.75 (0.71)	6.13 (0.48)	5.87 (1.78)	6.19 (1.10)	6.24 (1.15)	0.0689
MPL							
MCP-1, pg/ml, mean (SD)	5.53 (0.53)	5.47 (0.75)	4.69 (0.85)	4.60 (1.73)	4.89 (1.20)	5.20 (0.96)	0.00231
ELISA	5.26 (1.04)	5.48 (0.74)	4.89 (0.81)	4.60 (1.90)	4.75 (1.33)	5.14 (0.93)	0.00978
MPL							
Adiponectin, ng/ml, mean (SD)	1.71 (0.10)	1.69 (0.18)	1.53 (0.17)	1.55 (0.31)	1.56 (0.26)	1.63 (0.23)	0.0609
ELISA	1.54 (4.82)	4.11 (0.59)	2.88 (0.61)	2.49 (1.33)	2.78 (0.99)	2.27 (1.44)	7.03×10^{-8}
MPL							
Ceruloplasmin, ng/ml, mean (SD) ELISA	8.80 (4.01)	8.58 (1.05)	7.34 (0.16)	6.77 (1.09)	6.80 (1.02)	7.05 (0.86)	1.44×10^{-13}
Hemopexin, ng/ml, mean (SD) ELISA	6.23 (1.87)	7.21 (0.42)	6.51 (0.47)	5.91 (1.06)	5.85 (1.01)	6.29 (0.94)	6.54×10^{-8}

BILAG, British Isles Lupus Assessment Group; ELISA, enzyme-linked immunosorbent assay; KIM-1, kidney injury molecule−1; MCP-1, monocyte chemoattractant protein−1; MPL, multiplex platform; NGAL, neutrophil gelatinase−associated lipocalin.

Table 3. Intraclass correlation coefficient (ICC) values

Biomarker	R = ICC	95% Lower CL	95% Upper CL
NGAL	0.69	0.63	0.74
KIM-1	0.86	0.83	0.88
MCP-1	0.93	0.91	0.94
Adiponectin	0.78	0.74	0.82
Modified versus original RAIL	0.68	0.62	0.74
Original RAIL MPL versus ELISA	0.97	0.96	0.98

CL, confidence limit; ELISA, enzyme-linked immunosorbent assay; KIM-1, kidney injury molecule−1; MCP-1, monocyte chemoattractant protein−1; MPL, multiplex platform; NGAL, neutrophil gelatinase−associated lipocalin; RAIL, Renal Activity Score for Lupus.

0.047 (95% CI = −0.03 to 0.12), and 97% of values were within the 95% limits of agreement.

MCP-1

MCP-1, measured in picograms per milliliter (pg/ml), ranged from −0.65 to 7.79 (mean ± SD = 4.99 ± 1.31 pg/ml) by ELISA and −1.35 to 8.16 (mean ± SD = 4.94 ± 1.39 pg/ml) when using the MPL assay. The ICC was 0.93 (95% CI = 0.91−0.94) (Table 3), supporting excellent agreement between the 2 tests. The scatter plot and Bland−Altman plots are shown in Figure 3a and b. Overall, MCP-1 demonstrated very strong correlation throughout the multiplex detection range (Figure 3a). There was minimal difference between the 2 tests, as shown by the Bland−Altman (Figure 3b), with a mean difference of 0.046 (95% CI = −0.01 to

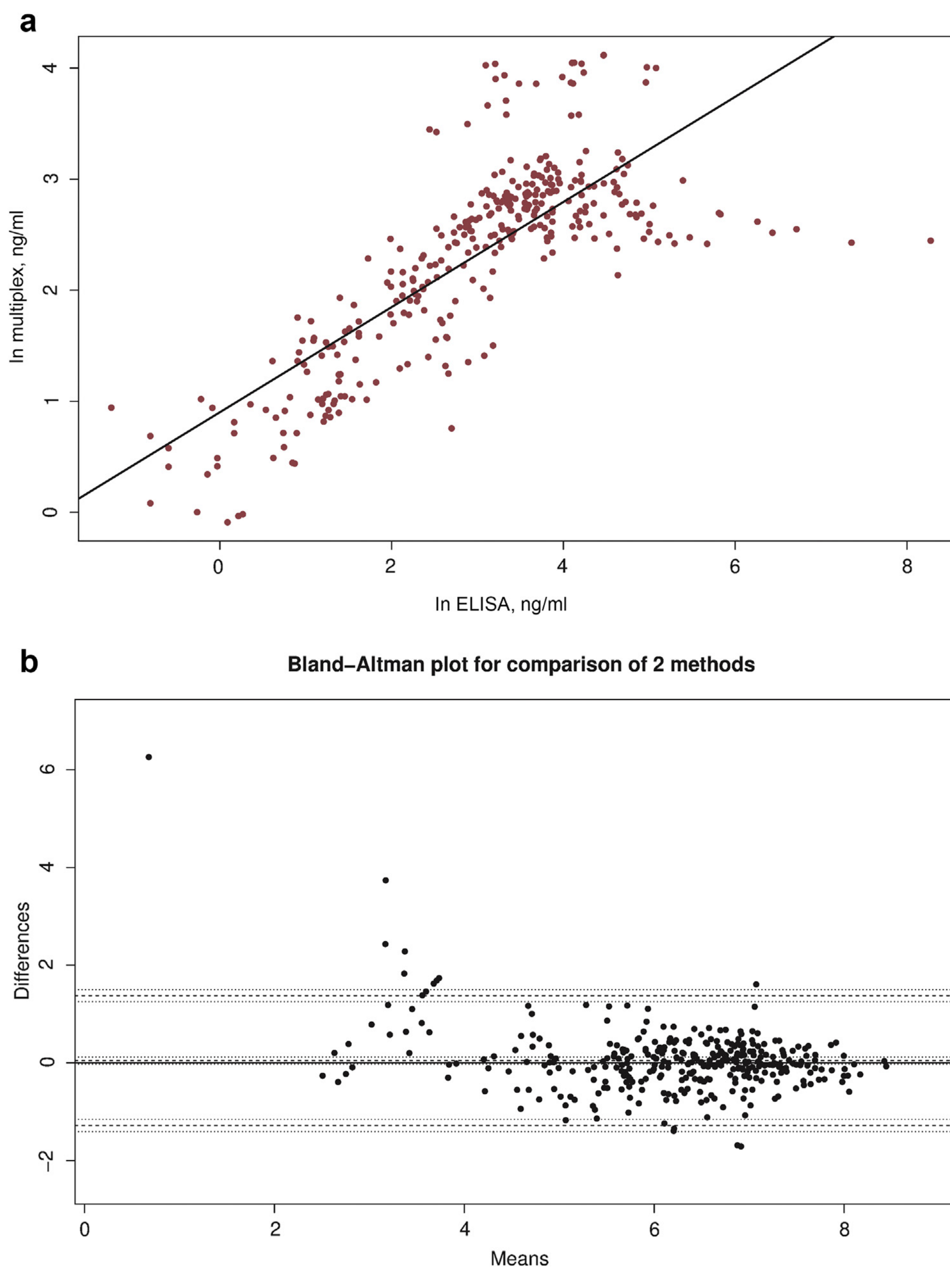

Figure 1. (a) Neutrophil gelatinase−associated lipocalin (NGAL) scatter plot. Comparison of enzyme-linked immunosorbent assay (ELISA) and multiplex for NGAL, with R value of 0.78. (b) NGAL Bland−Altman plot. Mean difference of 0.669, denoted by central line (95% confidence interval = 0.57−0.77). In all, 96% of differences will be between −1.17 and 2.50 (the 95% limit of agreement denoted by lines).

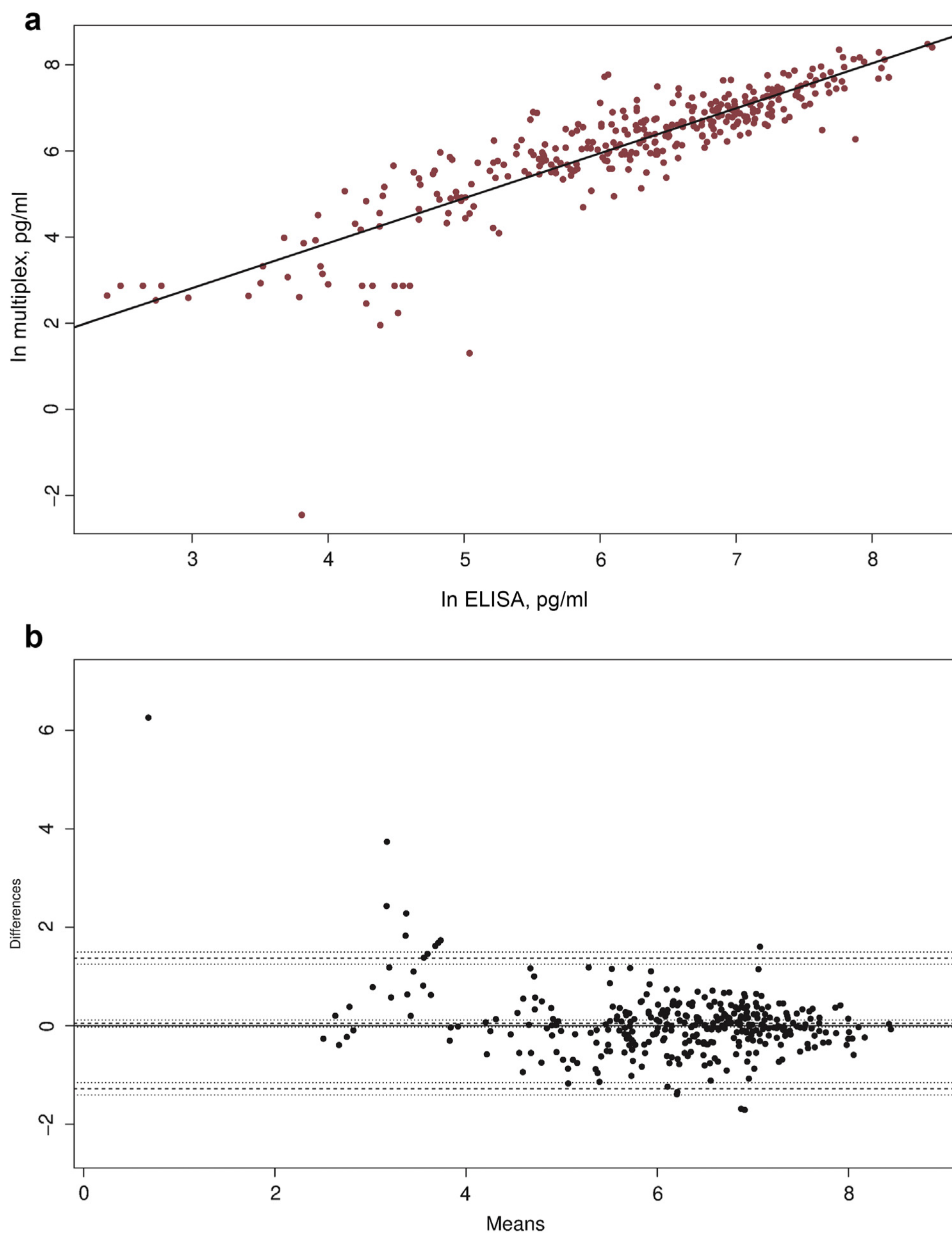

Figure 2. (a) Kidney injury molecule–1 (KIM-1) scatter plot. Comparison of enzyme-linked immunosorbent assay (ELISA) and multiplex for KIM-1, with R value of 0.87. (b) KIM-1 Bland–Altman plot. Mean difference of 0.047, denoted by central line with 95% confidence interval −0.03 to 0.12. In all, 97% of differences will be between −1.28 and 1.38 (the 95% limit of agreement denoted dashed lines).

0.1), and 96% of values were within the 95% limits of agreement.

Adiponectin

Adiponectin levels (in nanograms per milliliter [ng/ml]) by ELISA ranged from −1.05 to 5.37 (mean ± SD =1.61 ± 0.26 ng/ml) and from −3.36 to 5.24 (mean ± SD = 2.54 ± 1.41 ng/ml) when using the MPL assay. The ICC was 0.78 (95% CI = 0.74–0.82) (Table 3), supporting good agreement between the 2 tests. Overall, adiponectin demonstrated strong correlation throughout the multiplex detection range, although there is evidence of a flooring effect for the MLP assay to capture the lower end of the ELISA range (as shown in Figure 4a). There was some negative bias as shown by the Bland–Altman (Figure 4b), with a negative mean difference of −0.87 (95% CI = −0.97 to −0.77), suggesting that the multiplex assay values were consistently higher than the ELISA assay values and that 96% of the values were within the 95% limits of agreement.

Modified RAIL Score ELISA Versus Multiplex

The RAIL scores were then calculated using the formula listed above, using NGAL, KIM-1, MCP-1, and adiponectin. A total of 291 samples were included in this analysis. The Modified RAIL scores using the ELISA ranged from −9.562 to −1.742 (mean ± SD = −4.82 ±

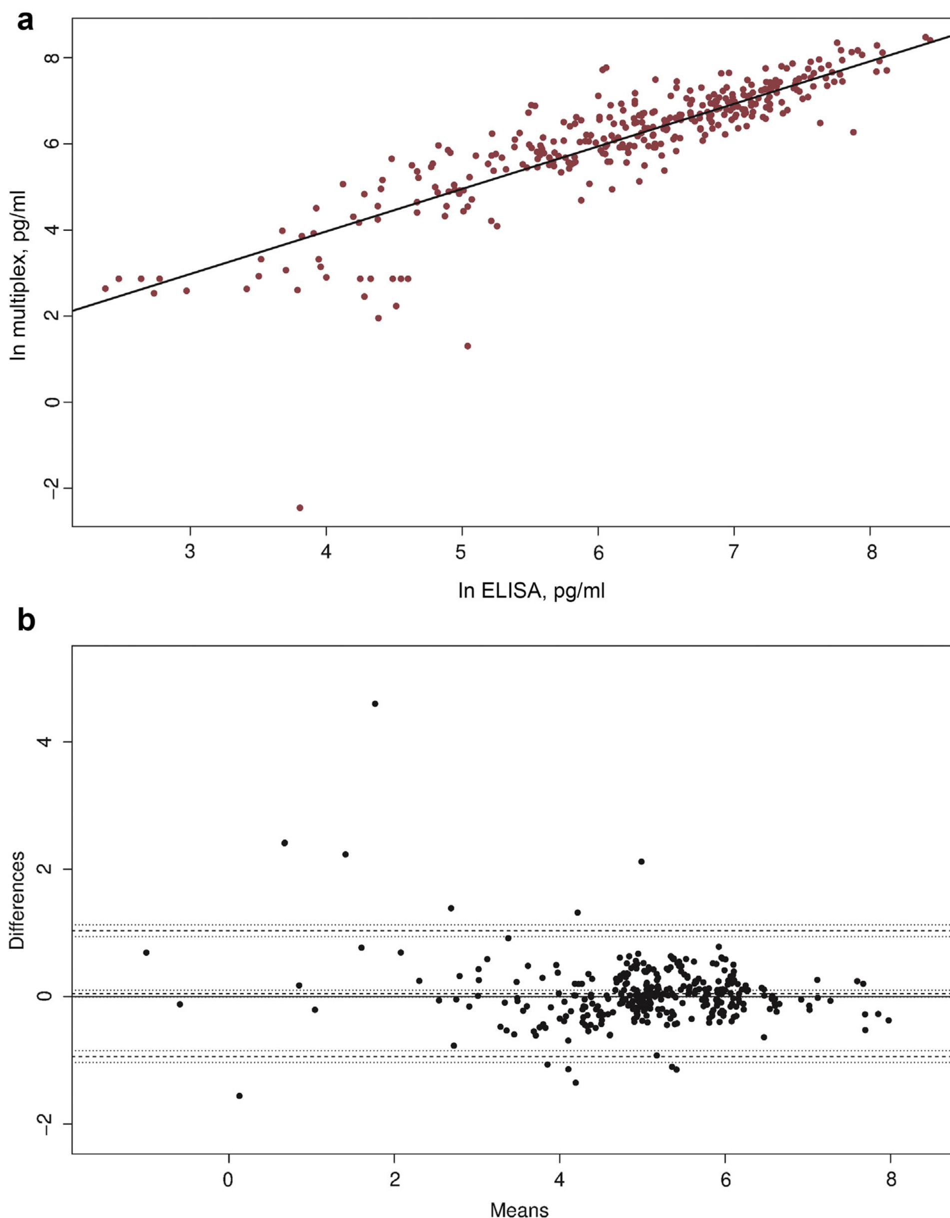

Figure 3. (a) Monocyte chemoattractant protein−1 (MCP-1) scatter plot. Comparison of enzyme-linked immunosorbent assay (ELISA) and multiplex for MCP-1, with R value of 0.93. (b) MCP-1 Bland−Altman plot. Mean difference of 0.046, denoted by the central line, with 95% confidence interval of −0.01 to 0.1. In all, 96% of mean differences will be between −0.94 and 1.03 (the 95% limit of agreement denoted by dashed lines).

1.26). The Modified RAIL scores using the MPL assay ranged from −8.776 to −1.056 (mean ± SD = −3.64 ± 1.15). The ICC was 0.68 (95% CI = 0.62−0.74) (Table 3), supporting good consistency between the 2 tests. As shown in Figure 5a and b, the comparison of the modified *RAIL* score via ELISA versus MPL using only 4 urinary biomarkers demonstrated good correlation (R = 0.65; $P = 2 \times 10^{-16}$). Of note, variability between the modified RAIL scores (ELISA, MPL) tended to increase toward the negative end range of the scales (Figure 5a). There was a mean difference of −1.14 per the Bland−Altman (Figure 5b), suggesting a consistently lower measurement by ELISA compared to MPL. Finally, about 95% of values calculated by Bland−Altman fell between the 95% limits of agreement lines.

Modified Versus Original RAIL Score

Finally, using the ELISA results, RAIL scores using the biomarkers for which the MPL bead assay was successful (NGAL, KIM-1, MCP-1, and adiponectin), were calculated and compared to the original RAIL scores, using all 6 biomarkers (NGAL, KIM-1, MCP-1, adiponectin, hemopexin, and ceruloplasmin). As shown in Figure 6a, the Modified RAIL score ranged from −9.562 to −1.742 (mean ± SD = −4.82 ± 1.26). The original RAIL score ranged from −12.01 to −3.831 (mean ±

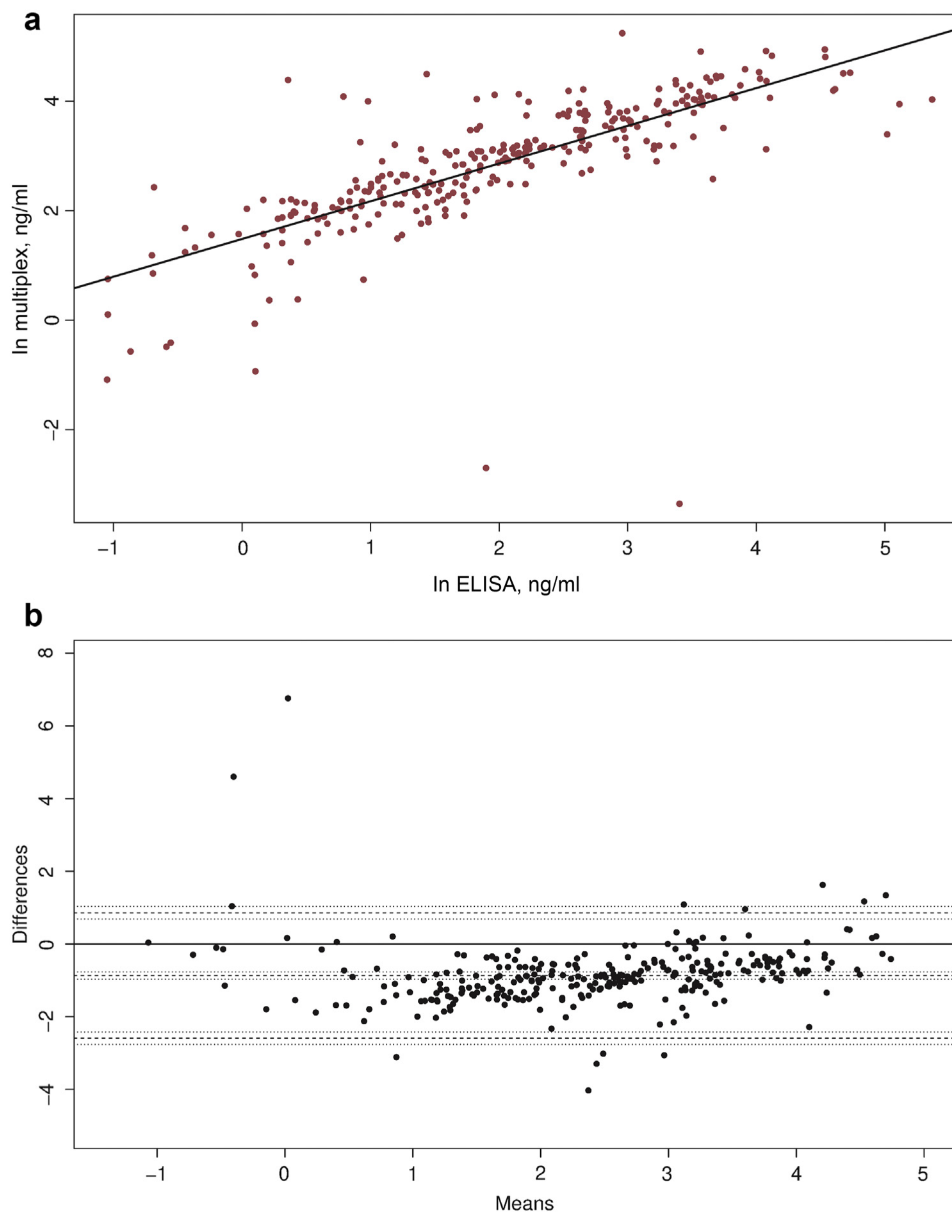

Figure 4. (a) Adiponectin scatter plot. Comparison of enzyme-linked immunosorbent assay (ELISA) and multiplex for adiponectin, with R value of 0.75. (b) Adiponectin Bland−Altman plot. Mean difference of −0.87, denoted by middle line, with 95% confidence interval of −0.97 to −0.77. In all, 96% of mean differences will be between −2.59 and 0.85 (the 95% limits of agreement denoted by dashed lines).

SD = −6.81 ± 1.22). The ICC was excellent at 0.97 (95% CI = 0.96−0.98) (Table 3). The regression correlation coefficient was 0.97, suggesting excellent correlation between a RAIL score based on only 4 urinary biomarkers and the original RAIL score based on all 6 biomarkers. On Bland−Altman analysis (Figure 6b), the modified RAIL score exhibited a higher value of ~2 (based on a mean difference of 1.98), consistent with the absence of 2 biomarkers in the formula. In all, 94% of values were within the 95% confidence intervals of the limits of agreement.

DISCUSSION

We report a high level of congruency among 4 of the 6 urine biomarkers considered in the RAIL when assayed on the LUMINEX platform as opposed to ELISAs. Considering only the 4 RAIL biomarkers that we successfully multiplexed, the RAIL scores of the 2 modalities had an ICC of 0.784, supporting good agreement between the 2 tests. Furthermore, the ICC between the modified RAIL with 4 biomarkers (NGAL, KIM-1, MCP-1, and adiponectin) and original RAIL with 6 biomarkers was excellent, at 0.97. With this reliability between the multiplex assay and ELISA, the RAIL score will be more clinically accessible and with a reasonable turnaround time to make clinical decisions. This raises the possibility that a modified RAIL score could suffice to identify LN activity over time. Further research is needed to evaluate the ability of this modified

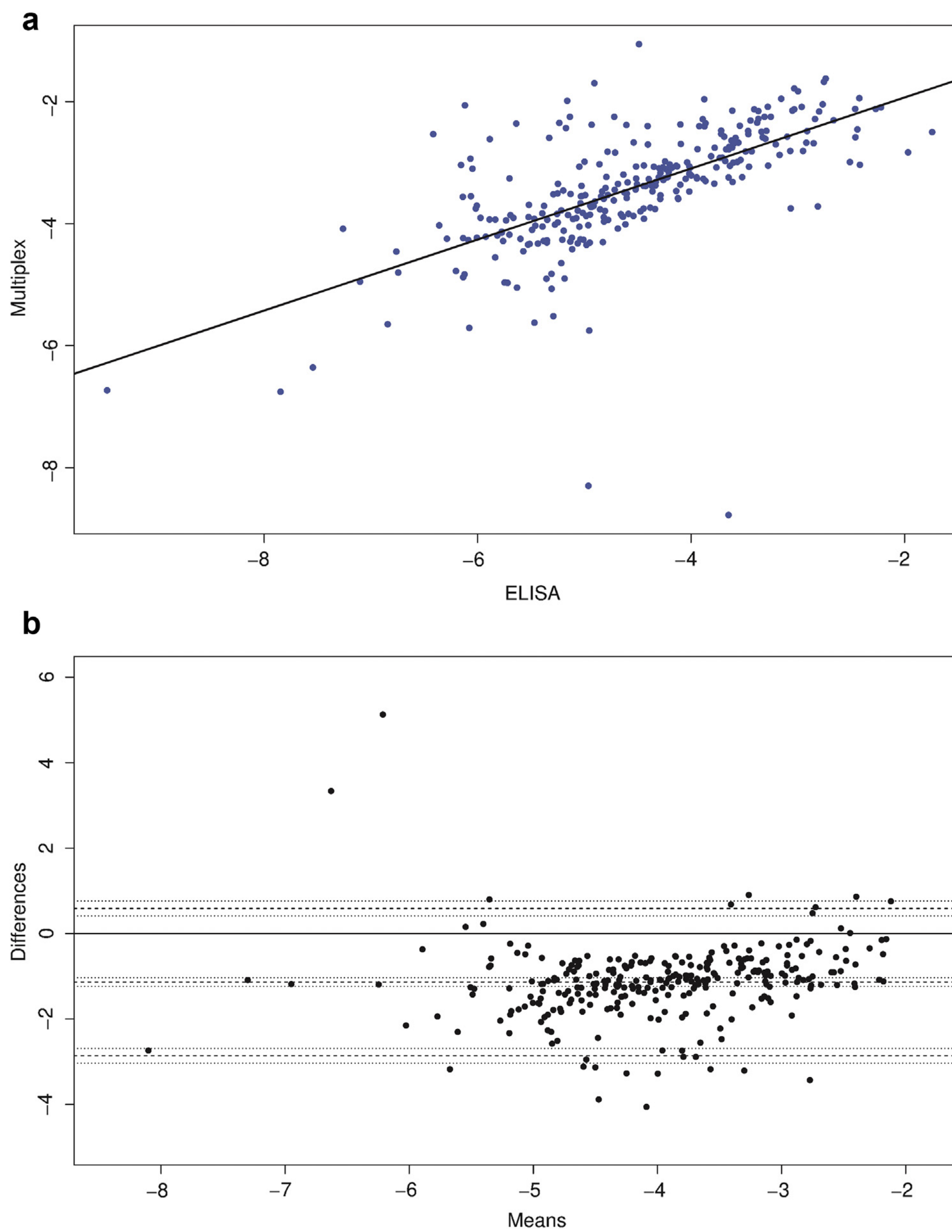

Figure 5. (a) Modified Renal Activity Score for Lupus (RAIL) score scatter plot. Comparison of enzyme-linked immunosorbent assay (ELISA) and multiplex for RAIL score calculated with 4 urinary biomarkers (excludes hemopexin and ceruloplasmin), with R value of 0.65. (b) Modified RAIL score Bland−Altman Plot. Bland−Altman Plot for RAIL score calculated with 4 urinary biomarkers. Mean difference of −1.14, denoted by dashed lines, with 95% confidence interval of −1.24 to 1.03. In all, 95% of values fall between −2.86 and 0.76 (the 95% limits of agreement denoted by the dashed lines).

RAIL score to predict high renal inflammation on kidney biopsy. However, these preliminary data are promising, as the modified RAIL score appears to be a robust tool to use RAIL testing in daily clinical practice.

There continues to be intensive research in urinary biomarkers for kidney disease, given the inadequacy of current markers, both in lupus and in other diseases.[7,19–21] As shown by Devarajan *et al.*, serum creatinine was a problematic and often inaccurate measure of renal injury, and urine NGAL was introduced as a biologically appropriate marker of injury.[22] It has since been instituted clinically, successfully detecting acute kidney injury (AKI) prior to increase in serum creatinine. Of particular importance, the test is point of care, allowing it to be run rapidly to ensure clinical utility.[23] Tengstrand *et al.* performed multiplexed assay for urinary biomarkers in drug-induced kidney injury in rats. Their biomarkers including KIM-1 and NGAL, among others. They found the multiplexed assays efficient to quantify the biomarkers, saving time and costs with overall analysis. Future directions would be working toward configuring the panel toward human analysis.[19]

Urinary biomarkers in LN specifically are also an active field of study. Landolt-Marticorena *et al.*

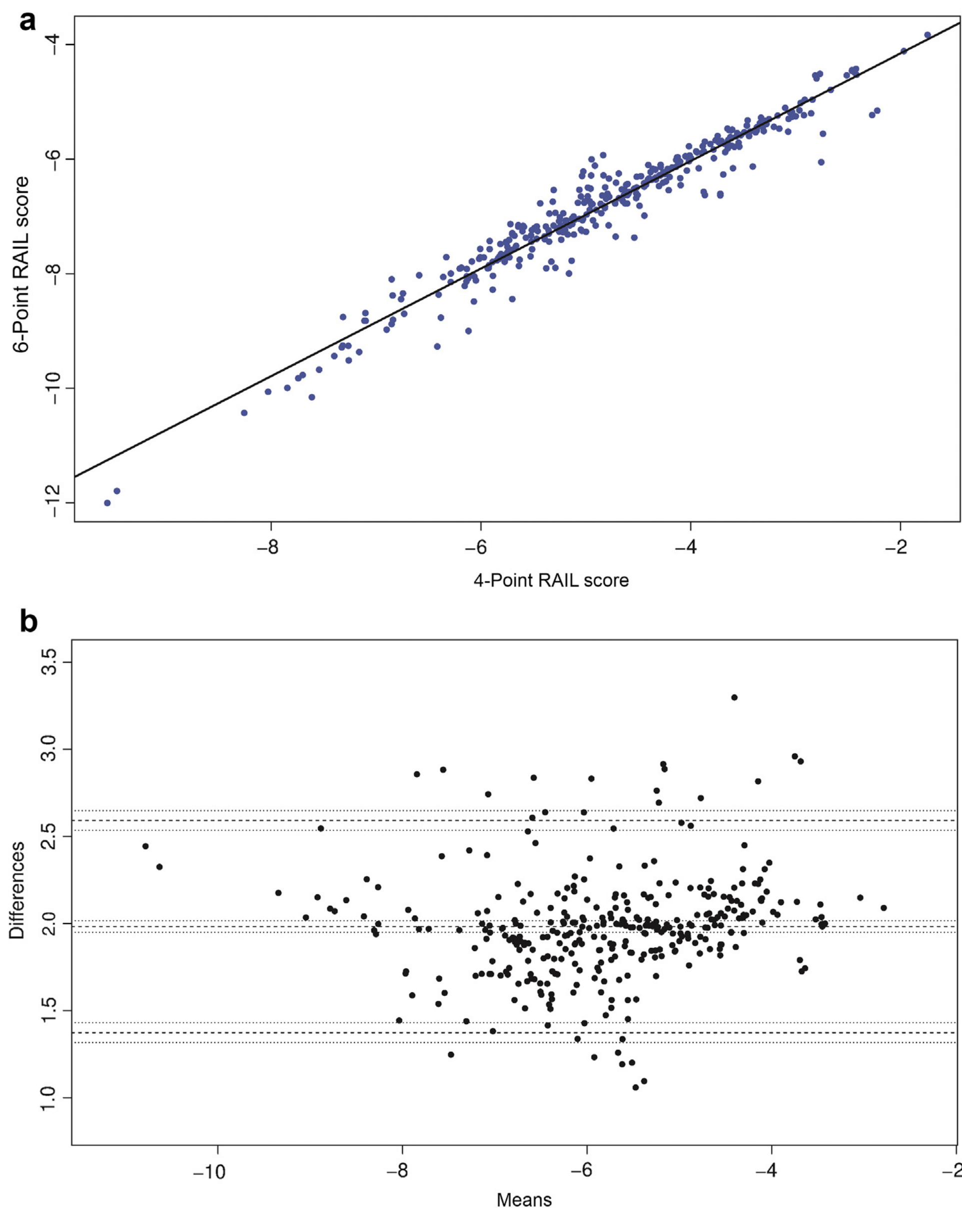

Figure 6. (a) Scatter plot comparison of modified versus original Renal Activity Score for Lupus (RAIL) score. RAIL scores calculated with 4 (excludes hemopexin and ceruloplasmin) versus 6 urinary biomarkers via enzyme-linked immunosorbent assay (ELISA) methodology, with R value of 0.97. (b) ELISA Bland−Altman Plot. Comparison of modified versus original RAIL score. RAIL scores calculated with 4 (excludes hemopexin and ceruloplasmin) versus 6 urinary biomarkers via ELISA methodology. Mean difference of 1.98, denoted by dashed line (95% confidence interval = 1.95−2.2). In all, 94% of the values fell between 1.37 and 2.59 (the 95% limits of agreement denoted by the dashed lines).

published on urinary biomarkers distinguishing active LN patients and patients with SLE without nephritis, which were identified using multiplex technology and had excellent correlation with biopsy activity. Their panel included NGAL, MCP-1, KIM-1, and adiponectin, but did not include hemopexin or ceruloplasmin. They did note that some of their samples had biomarkers that outperformed the same biomarkers in other studies that used ELISA but did not attribute it to the multiplex system.[24] Smith *et al*. have also proposed urinary biomarker panel measured by ELISA with excellent predictive ability of kidney histopathology. However, the group acknowledges the need for further validation as well as the need to transition to point-of-care testing for their biomarkers.[25,26]

There were several limitations to this study. First, it was limited by the number of patients with severe LN, in whom the RAIL score has been validated. In addition, there were no histological data available for comparison of the clinical renal disease activity. When comparing the RAIL score on the 2 different platforms, more differences are noted in the more negative range, when patients do not have renal disease or do not have active renal disease. Given this, it likely does not hold much clinical significance, but will require further study. Next, the samples were run on the respective

platforms at 2 different time points. Stability of the urinary biomarkers in the RAIL formula was previously established, but there was note of minimal degradation of the proteins over time.[27] The ELISA samples were run first, followed by multiplex assay, and it is unclear whether this led to some of the bias seen, particularly with NGAL and adiponectin. With NGAL, there also appeared to be dilutional factors needed for the multiplex modality, particularly when ELISA detected NGAL >500 ng/ml. For NGAL multiplex, the minimum detection was 17 to 63.1 pg/ml, with no specific upper limit of detection listed on the manufacturer's website. Further evaluation will be required the assess the MPL's ability to detect NGAL in high quantities in the urine.

This study holds promise in the feasibility of using the multiplex platform to calculate the RAIL score. Future directions include repeating the multiplex assays in a new cohort of patients, particularly those with active renal inflammation. In addition, further assessment is needed with the modified RAIL score to assay its ability in detecting high renal inflammation to determine whether it is an acceptable replacement for the original formula. Correlating with clinical parameters will help finalize the preparation of RAIL for clinical use.

DISCLOSURE

HIB, MB, and PD are co-inventors on patents submitted for the use of RAIL biomarkers in lupus nephritis. HIB: Speaking fees for Novartis and Roche (both >$10,000) and GlaxoSmithKline (<$10,000); Consultancies/honoraria (<$10,000): AbbVie, Astra Zeneca-Medimmune, Biogen, Boehringer, Bristol-Myers Squibb, Celgene, Eli Lilly, EMD Serono, Genzyme, GlaxoSmithKline, F. Hoffmann-La Roche, Merck, Novartis, R-Pharm, Sanofi. The Cincinnati Children's Hospital, where HBR works as a full-time public employee, has received contributions (>$10,000 each) from the following industries in the past 3 years: Bristol-Myers Squibb, Eli Lilly, GlaxoSmithKline, F. Hoffmann-La Roche, Janssen, Novartis, and Pfizer. This funding has been reinvested for the research activities of the hospital in a fully independent manner, without any commitment to third parties. HIB's time is supported by supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health (grant P30-AR-076316). This work was funded by an Innovation Award from the Cincinnati Children's research Foundation awarded jointly to PD and HIB. All the other authors declare no competing interests.

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SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Figure S1. Flow chart for patient and sample inclusion.

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