ACR Open Rheumatology
Vol. 7, No. 5, May 2025, e70044
DOI 10.1002/acr2.70044

American College of Rheumatology
Empowering Rheumatology Professionals

Urinary V-Set Ig Domain-Containing Protein 4 and Immune Complexes for Tracking Lupus Nephritis and Renal Pathology

Aygun Teymur,[1] Chenling Tang,[1] Fariz Nazir,[1] Neda Ostadnejad,[1] Qi Cai,[2] Ramesh Saxena,[2] and Tianfu Wu[1]

Objective. This study aims to investigate whether V-set Ig domain-containing protein 4 (VSIG4; also known as complement receptor of the Ig superfamily [CRIg]) forms immune complexes (ICxs) with IgG and complement component 3 (C3) in the kidneys of patients with lupus nephritis (LN) and to assess the potential of urinary VSIG4 and VSIG4-ICx as noninvasive biomarkers of LN.

Methods. Immunofluorescent staining was employed to detect the deposition of VSIG4 (CRIg), IgG, and C3 in kidney tissue. Urine samples from 102 patients with LN, 51 healthy controls (HCs), and 13 patients with chronic kidney disease (CKD) were analyzed via enzyme-linked immunosorbent assay for VSIG4-ICx and free-form VSIG4.

Results. Immunofluorescence costaining demonstrated the colocalization of VSIG4, IgG, and C3 in the kidneys of those with LN and elevated VSIG4 protein expression in the glomeruli regions in LN. Compared with HCs and those with CKD, patients with LN exhibited significantly elevated levels of urinary VSIG4 in both free form and ICx. Urinary VSIG4-ICx correlated with clinical parameters, including the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) ($R = 0.55$, $P < 0.0001$), renal SLEDAI ($R = 0.52$, $P < 0.0001$), estimated glomerular filtration rate (-0.5, $P < 0.001$), activity index ($R = 0.25$, $P < 0.05$), chronicity index ($R = 0.32$, $P < 0.05$), complement C3 ($R = -0.33$, $P < 0.05$), and complement C4 ($R = -0.31$, $P < 0.05$). The strong association of the urinary VSIG4-ICx with disease activity metrics and histopathologic evidence underscores its potential for clinical utility in diagnosing and monitoring LN.

Conclusion. VSIG4-ICx shows promise as a novel urine biomarker for LN, with potential utility for diagnosis and disease monitoring.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic autoimmune condition that can progress to a more severe manifestation, lupus nephritis (LN), potentially leading to kidney deterioration.[1] The development of LN is primarily initiated by forming immune complexes (ICxs), which subsequently activate an immune response within the renal system, leading to kidney damage.[2] Although LN commonly manifests through immune deposits, current evidence predominantly points to the glomerular accumulation of IgG autoantibodies as the initial phase in its development.[1] It has been discovered that glomerular ICxs are formed in place through secondary attachment to nucleosomes released by renal cells.[3] In direct immunofluorescence studies, IgG deposition is found in more than 90% of cases, whereas complement component 3 (C3) and complement C1q depositions are observed in approximately 80% of cases.[4] Several other studies have also found a pattern of glomerular Ig and complement deposits in renal tissues of kidney diseases.[5–7]

Recently, exploring noninvasive methods for diagnosing diseases and monitoring their progression has gained significant attention. Proteinuria, urinary protein-to-creatinine (Cr) ratio (uPCR), Cr clearance, anti–double-stranded DNA, and complement levels are current laboratory markers used to help diagnose patients with LN.[8] However, these parameters are suboptimal in specificity and sensitivity for identifying renal activity and damage that can occur

Supported by the NIH (grant R01-AG-062987 awarded to Dr Wu).
[1]Aygun Teymur, PhD, Chenling Tang, PhD, Fariz Nazir, BS, Neda Ostadnejad, MS, Tianfu Wu, PhD: University of Houston, Houston, Texas; [2]Qi Cai, MD, PhD, Ramesh Saxena, MD, PhD: University of Texas Southwestern Medical Center, Dallas.
Additional supplementary information cited in this article can be found online in the Supporting Information section (https://acrjournals.onlinelibrary.wiley.com/doi/10.1002/acr2.70044).

Author disclosures are available online at https://onlinelibrary.wiley.com/doi/10.1002/acr2.70044
Address correspondence via email to Tianfu Wu, PhD, at Twu13@central.uh.edu.
Submitted for publication October 14, 2024; accepted in revised form March 27, 2025.

in LN. Thus, there is a clear need for new biomarkers capable of distinguishing lupus renal activity, predicting renal flares, and monitoring treatment responses and disease progression.

Our previous studies discovered that serum V-set Ig domain-containing protein 4 (VSIG4) was significantly elevated in patients with LN and could reflect the renal pathology activity index (AI), particularly on crescent formation and hyaline deposits.[9] VSIG4, also known as complement receptor of the Ig superfamily (CRIg), a type I transmembrane receptor, can bind C3b and iC3b in the complement alternative pathway and significantly inhibits macrophage activation.[10] This receptor binds to complement C3 fragments and inhibits the activation of cytotoxic T lymphocytes,[11] and circulating iC3b and C3 are associated with lupus disease activity.[12] Recombinant VSIG4 reduced murine LN and cutaneous disease.[13] Given the involvement of VSIG4 in the complement system and LN, we examined if VSIG4 colocalizes with C3 and IgG to form pathogenic ICxs within the renal sections of patients with LN and if VSIG4 or VSIG4-ICx has the potential as a noninvasive urinary biomarker of LN.

PATIENTS AND METHODS

Patient and clinical data collection. In this study, urine samples from 102 patients with LN, 51 healthy controls (HCs), and 13 patients with chronic kidney disease (CKD; including diabetic nephropathy, IgA nephropathy, and hypertensive nephropathy) were used to determine VSIG4 levels. Specifically, 83 patients with active LN (LNA) and 19 with inactive LN (LNI) were used to differentiate within the LN cohort. LNA is defined as having an SLE Disease AI (SLEDAI) score of >4 and renal SLEDAI (rSLEDAI) score of >0, as previously established in the literature.[14] Patients with LNI are defined as having a SLEDAI score of ≤4 and rSLEDAI score equal to 0 as described previously.[9,15] The detailed demographics and clinical information are summarized in Supplementary Tables 1 and 2.

Patients were further stratified into LN classes of 10 patients with mesangial proliferative LN (class II), 23 with focal LN (class III), 10 with diffuse LN (class IV), 26 with mixed LN (class III/IV and V), and 11 with pure membranous LN (class V). These classifications are the current standard for diagnosing patients with LN.[16] Three patients with class V LN were included in the immunofluorescence staining, alongside a control using normal tissue that was collected from nonneoplastic kidney of radical nephrectomy. The median time between kidney biopsy and urine sample collection was 47 days with an interquartile range of 39 days.

Colocalization staining in fixed kidney tissue. Immunofluorescence microscopy was used to assess the colocalization of proteins within human renal biopsy samples. Tissue sections were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned. The sections underwent deparaffinization, rehydration, and heat-induced epitope retrieval in 10 mM sodium citrate buffer (pH 6.0) using a TintoRetriever pressure cooker (Bio SB). The sections were then blocked with 10% normal mouse serum (ab7481; Abcam) in 1% bovine serum albumin in tris-buffered saline and incubated overnight with a mixture of rabbit anti-human VSIG4 antibody (ab252933; Abcam) and goat anti-human C3 antibody (BS-0367G; ThermoFisher). Afterward, the sections were incubated with a mixture of fluorophore-conjugated antibodies: mouse anti-human IgG (209-025-082; Jackson ImmunoResearch), mouse antirabbit IgG (211-605-109; Jackson ImmunoResearch), and mouse antigoat IgG (205-545-108; Jackson ImmunoResearch). The sections were mounted in limonene mounting medium (ab104141; Abcam), and slides were visualized with a Fluoview FV3000 confocal microscope. Thirty fields were randomly selected from five glomeruli structures in each slide for quantification, and images were obtained for quantification and analysis using ImageJ. Renal tissue from three patients with class V LN was examined, with intensities averaged across 30 fields per glomerulus per patient.

Urinary ELISA-based detection of ICx and free-form VSIG4. An in-house enzyme-linked immunosorbent assay (ELISA) kit was developed and optimized to measure the VSIG4-ICx form in human urine. Briefly, monoclonal anti-human VSIG4 antibodies (MAB46461-100; R&D Systems) were coated onto Nunc microwell 96-well microplates (243656; ThermoFisher Scientific) and incubated overnight at 4°C. Afterward, the plate was blocked, and the urine samples, diluted 1:100 in reagent diluent, were then applied to the plates and incubated for 2 hours. Detection involved incubation with an anti-human IgG secondary antibody (109-065-170; Jackson ImmunoResearch), followed by a streptavidin horseradish peroxidase (HRP) conjugate and 3,3',5,5' tetramethylbenzidine (TMB) substrate. The resultant colorimetric signal was measured at an optical density (OD) of 450 nm using an Epoch microplate spectrophotometer (BioTek Instruments), with the blank-corrected values representing the levels of VSIG4-ICxs. Urinary levels were normalized to urinary Cr levels to correct for any influence of dilution, measured using a Cr assay kit (KGE005; R&D Systems). VSIG4-ICx was quantified with the following equation:

$$\text{VSIG4 ICx} = (\text{Sample OD450 - Blank OD450})/(\text{mg urine creatinine}) = \text{AU/mg}$$

The presence of free-form VSIG4 was assessed using a commercially available ELISA kit (LS-F50060-1; LSBio) according to the manufacturer's manual.

Statistical analysis. The data were analyzed, plotted, or visualized using the R programming environment (version 4.3.2) and the 'ggplot2' package for data visualization and representation.[17] All data were log-transformed to normalize the distributions

and stabilize variances across samples. Comparisons of multiple groups were performed using a one-way analysis of variance (ANOVA). The Tukey multiple comparison test was further used to assess the statistical difference between the individual groups (assessed with *P* values). Receiver operating characteristic (ROC) curve analysis was facilitated through the pROC package.[18] Correlation assessments among clinical and pathologic parameters were computed using the Spearman rank correlation coefficient, implemented with the 'corrplot' package.[19] The classification and pathologic parameters used for correlation are derived from the revised classification of LN and modified version of the National Institutes of Health activity and chronicity indices (CIs).[16]

Data sharing and disclosure statements. Clinical data used in this study have been described and summarized in Supplementary Table 1. The raw data from the fluorescent intensity or ELISA measurements and any other data generated from this work will be available upon email request (twu13@central.uh.edu). All experiments involving human participants were conducted in accordance with the institutional review board–approved protocol from the University of Houston (no. STUDY00001299). The clinical samples used in this study were acquired from the existing sample bank at the University of Houston.

RESULTS

VSIG4, IgG, and C3 colocalization in kidney sections via immunofluorescence. Our previous immunohistochemistry experiments revealed that VSIG4 was expressed in the glomeruli of LN.[9] Here, we further investigated whether VSIG4 is colocalized with IgG and C3 to form an ICx in the kidneys of patients with LN. The colocalization of VSIG4 (magenta), IgG (green), and C3 (red) was visualized at different magnifications in patients with LN and normal control patients (Supplementary Figure 1A). In the merged image, we observed colocalization of C3, IgG, and VSIG4 via the overlap of immunostaining, as evidenced by the yellow/orange areas, indicating the deposition of these molecules, specifically in the glomeruli and tubules. No staining was observed in the negative control. Intensity correlation plots revealed the extent of colocalization among VSIG4 and IgG, C3, and IgG, respectively (Supplementary Figure 1B and C). The intensity correlation of VSIG4 and C3 is tightly clustered along the diagonal axis, indicating a high degree of colocalization between VSIG4 and IgG. Similarly, the correlation between C3 and VSIG4 shows a dense cluster along the diagonal axis. The colocalization analysis revealed a high degree of overlap, with the *R* coefficient between VSIG4 and IgG being 0.884 and a similar overlap of 0.716 for VSIG4 and C3 (Supplementary Table 3).

The relative intensity of DAPI, C3, IgG, and VSIG4 was quantified, with C3 and IgG having the highest intensities for LN and the normal control (Figure 1D). There was a significant difference ($P < 0.0001$) between the intensities of LN and normal tissue for VSIG4, as well as a significant difference between IgG intensities ($P = 0.046$). Consistent with expectations based on the abundance of ICxs in LN, IgG, and C3 exhibited the highest intensity levels, indicating their substantial presence and potential involvement in the inflammatory processes in kidney deposition. Despite the lower abundance of VSIG4, its intensity levels are notably substantial, falling close to those of IgG and C3. This observation underscores a significant presence of VSIG4 colocalization with IgG and C3, suggesting that VSIG4 may form an ICx with IgG and C3 in the kidney and contribute to the pathogenesis and development of LN.

Optimization of ELISA for urinary ICx of VSIG4. The experimental design for ELISA detection of VSIG4-ICxs in the urine is outlined in Figure 1A. Anti-VSIG4 antibodies are first immobilized on the surface of the microplate. Urine samples are added, allowing the binding to the capture antibody. Subsequently, biotinylated anti-IgG is introduced to bind to the complex and streptavidin-HRP. Adding the TMB substrate results in a colorimetric reaction catalyzed by HRP, indicating the presence of VSIG4. The ICx contains the VSIG4 antigen and an IgG antibody, further detected by a secondary anti-IgG antibody. The optimization of the secondary antibody dilution for the VSIG4-ICx ELISA was further evaluated (Figure 1B). The dilution series using five patient samples for each dilution provided a gradient to determine the optimal dilution ratio for the anti-human IgG antibody. Notably, a 1:200,000 dilution was selected based on its consistency of responses across samples, as evidenced by the mean ± SD OD readings (2.08 ± 0.466). The lower dilutions showed a reduced signal-to-noise ratio, potentially obscuring subtle differences between samples. Similarly, we evaluated the dilution of the immobilized capture antibody, which ranged from 1:6.25 to 1:800 (Figure 1C). The optimal dilution was determined to be 1:100, resulting in mean ± SD OD readings of 1.62 ± 0.147, indicating a robust and reliable signal. Urine samples were also tested across a dilution range from 1:10 to 1:500 to establish the optimal concentration for the assay (Figure 1D). The optimal dilution was identified as 1:100, yielding mean ± SD OD readings of 1.71 ± 0.119. Arbitrary units were used to quantify the VSIG4-ICx concentration. The selected dilution ratios consistently demonstrated the most reliable variance across LN samples, ensuring accurate and reproducible results.

Quantitative analysis of urinary VSIG4-ICx. The concentration of the VSIG4-ICx in urine was quantitatively assessed across various patient groups: HCs, patients with LN, and patients with CKD. Further stratification of patients with LN into LNI and LNA revealed marked distinctions in log10 normalized VSIG4-ICx levels. Patients with LNA exhibited significantly higher levels of VSIG4-ICx than both the HCs ($P < 0.0001$) and patients with LNI ($P < 0.0001$). In comparison, patients with CKD had

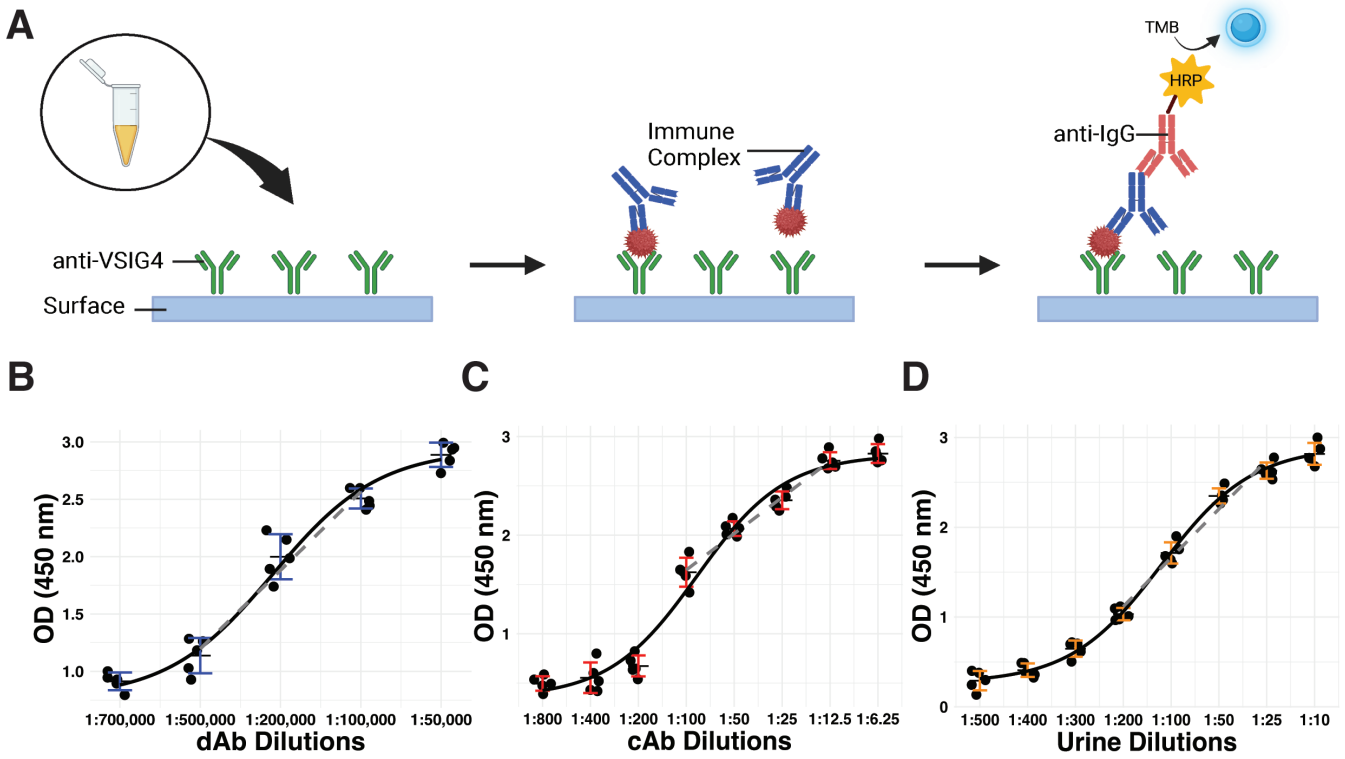

Figure 1. Enzyme-linked immunosorbent assay method for VSIG4 detection and quantification. (A) Schematic representation of the enzyme-linked immunosorbent assay protocol for detecting VSIG4 immune complexes. (B) Standard curve of OD at 450 nm for anti-IgG antibody dilutions ranging from 1:50,000 to 1:700,000. OD values decrease with increasing dilutions, following a four-parameter logistic curve (black line), with error bars representing the SD of replicate measurements. The dashed line highlights a reference linear trendline in the linear response range of the assay. (C) Standard curve of OD at 450 nm against cAb dilutions ranging from 1:6.25 to 1:800. (D) Standard curve of OD at 450 nm against urine dilutions ranging from 1:10 to 1:500 dilutions. cAb, capture antibody; dAb, detection antibody; HRP, horseradish peroxidase; OD, optical density; TMB, 3,3',5,5' tetramethylbenzidine; VSIG4, V-set Ig domain-containing protein 4.

similar values to LNA ($P = 0.46$) (Figure 2A). The LNI group also exhibited significantly higher VSIG4-ICx levels compared with HCs ($P = 0.057$) and significantly lower VSIG4-ICx levels than CKD ($P < 0.001$).

To evaluate the diagnostic potential of VSIG4-ICX as a biomarker for LN, an ROC curve analysis was performed (Figure 2B). The area under the curve (AUC) for LN versus HC was 0.8799 (95% confidence interval [95% CI] 0.82–0.94, $P < 0.001$), indicating robust diagnostic accuracy. Additionally, the comparison between LNA and LNI yielded an AUC of 0.8438, underscoring the biomarker's capability to differentiate disease activity levels. For LNA versus LNI, the AUC was 0.8438 (95% CI 0.72–0.97, $P < 0.001$). The log10 normalized cutoff point for distinguishing between patients with LNA and LNI was identified as 4.117 AU/mg, whereas discriminating between patients with LN and HCs an optimal cutoff point of 3.684 AU/mg was determined (Figure 2B). The density distributions of log10-transformed VSIG4-ICx levels were analyzed to compare their distribution between patients with LN and HCs (Figure 2C). The results demonstrate that VSIG4-ICx levels are elevated in patients with LN, with a greater frequency of higher values compared with the HC group.

Patients were further classified into LN classes in relation to natural log normalized VSIG4-ICx levels (Figure 2D). Mesangial proliferative, focal, diffuse, mixed, and pure membranous LN classes were evaluated. Significant differences were observed between the classes, with pure membranous LN showing the highest VSIG4-ICx levels ($P < 0.0001$) compared with the mesangial proliferative and mixed classes ($P < 0.0001$). No significant differences were found between the diffuse and mixed, diffuse and pure membranous, and mixed and pure membranous classes.

Boxplot analysis was further conducted to compare urinary VSIG4-ICx levels across different medication groups to compare the medication burden on VSIG4-ICx levels (ANOVA $P < 0.001$) (Supplementary Figure 2A). Patients received various combinations of medications, including prednisone, mycophenolate mofetil, azathioprine, and hydroxychloroquine. A significant decrease in VSIG4-ICx levels was observed among the increasing medications groups (Tukey honestly significant difference test $P < 0.05$) (Supplementary Figure 2A). We further stratified patients based on the specific medications they were taking, rather than the total number of medications prescribed (Supplementary Figure 2B). This trend suggests a potential relationship

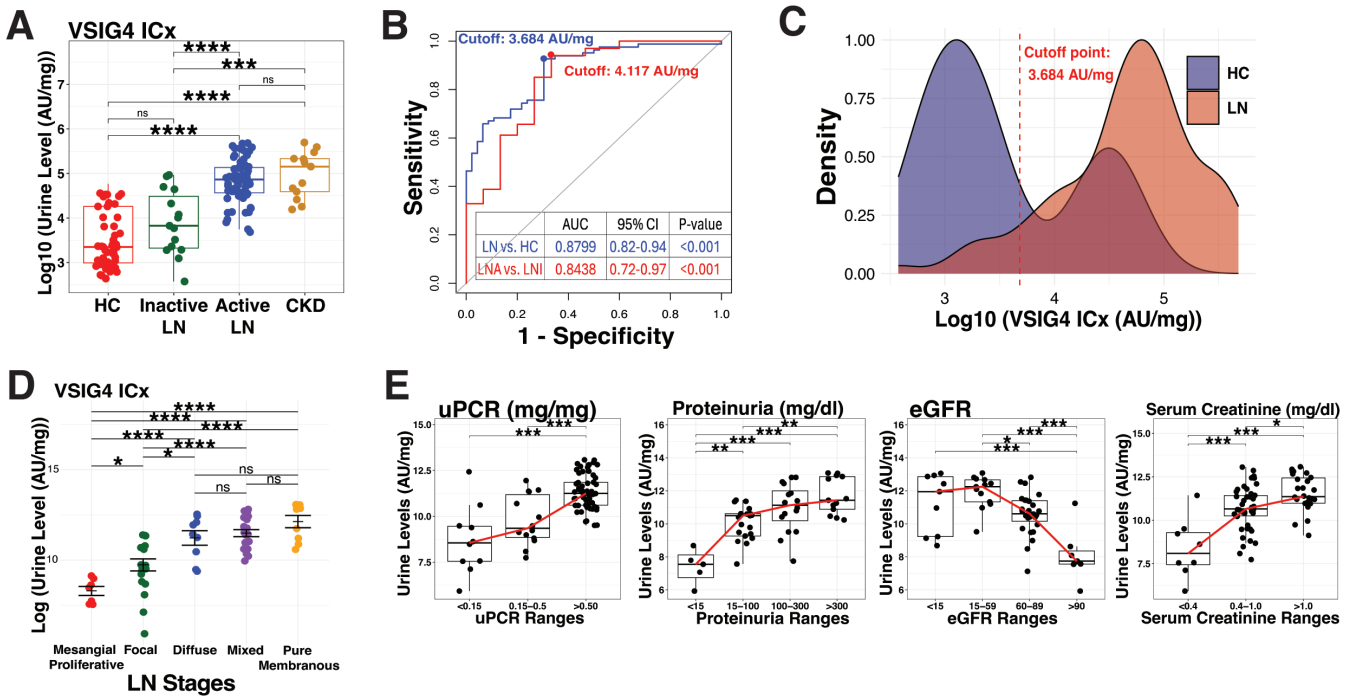

Figure 2. Urinary VSIG4-ICx analysis and diagnostic performance evaluation. (A) Scatter plot depicting the log10 normalized urinary levels of VSIG4-ICx across four groups: HCs, inactive LN, active LN, and CKD. CKD is used as a disease control. Each dot represents an individual participant. Significance is denoted by asterisks (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$). (B) Receiver operating characteristic curves evaluating the sensitivity and specificity of VSIG4-ICx log10 normalized levels in distinguishing patients with LN from HCs and LNI from LNA. Two cutoff values are indicated, with the AUC, 95% CIs, and *P* values listed for each comparison. The red curve represents LN versus HC, and the blue curve represents inactive LN versus LNA. (C) Density plot showing the distribution of VSIG4-ICx log10 normalized levels in HCs (purple) and patients with LN (red), with the optimal cutoff value marked by the red dashed line. The peak of each distribution indicates the most frequent value range for VSIG4-ICx in each group, with patients with LN displaying a right-shifted distribution compared with HCs. (D) Box plot of urinary VSIG4-ICx natural log normalized levels in different LN classes: mesangial proliferative LGN, focal LGN (< 50% glomeruli), diffuse LGN (> 50% glomeruli), mixed LGN, and pure membranous LGN. Statistical significance between classes is indicated. (E) Scatter plots showing the correlation between urinary VSIG4-ICx levels and various clinical parameters: uPCR, serum creatinine, eGFR, and proteinuria levels. Linear regression lines are included, and statistical significance is indicated. 95% CI, 95% confidence interval; AUC, area under the curve; CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; HC, healthy control; ICx, immune complex; LGN, lupus glomerulonephritis; LN, lupus nephritis; LNA, active lupus nephritis; LNI, inactive lupus nephritis; ns, not significant; uPCR, urinary protein-to-creatinine ratio; VSIG4, V-set Ig domain-containing protein 4.

between immunosuppressive therapy and VSIG4-ICx levels, as an increase in immunosuppressive medication use was associated with lower VSIG4-ICx levels.

Lastly, the relationship between VSIG4-ICx levels and various clinical parameters was explored, including uPCR, serum Cr, estimated glomerular filtration rate (eGFR), and proteinuria levels grouped into various ranges currently used in the clinic for diagnosis (Figure 2E). The clinical categories and ranges used are found in Supplementary Table 4. Positive correlations were observed between VSIG4-ICx levels and uPCR ($P < 0.001$), serum Cr ($P < 0.001$), and proteinuria ($P < 0.001$). Conversely, a negative correlation was found with eGFR ($P < 0.001$), indicating higher ICx VSIG4 levels in patients with reduced kidney function.

Correlation of VSIG4-ICx with clinical parameters. Furthermore, the correlations between the levels of VSIG4-ICx/Cr in arbitrary units per milligram and various clinical parameters were evaluated to justify the potential use of this marker in clinical settings. The SLEDAI shows a positive correlation with VSIG4-ICx/Cr levels ($R = 0.55$, $P = 1.1e{-}07$), suggesting that higher levels of VSIG4-ICxs are associated with increased disease activity (Figure 3A). Similarly, the rSLEDAI illustrates a positive correlation between rSLEDAI and VSIG4-ICx/Cr ($R = 0.52$, $P = 4.3e{-}07$) (Figure 3B). Contrastingly, complement C3 levels negatively correlate with VSIG4-ICx ($R = -0.33$, $P = 0.017$), which might indicate that higher ICx levels are associated with lower C3 levels, a common finding in LNA (Figure 3C). A similar inverse relationship is shown with complement C4 and VSIG4-ICx ($R = -0.31$, $P = 0.026$) (Figure 3D). Looking further into the AI, there is a weak positive correlation with the VSIG4-ICx form ($R = 0.25$, $P = 0.065$) and with the CI ($R = 0.32$, $P = 0.065$) as well (Figure 3E and F). Notably, the eGFR, a measure of kidney function, demonstrates a significant negative correlation with VSIG4-ICx/Cr ($R = -0.5$, $P = 0.00031$), indicating that higher

ICx levels may be indicative of decreased renal function (Figure 3G). Finally, there is a strong positive correlation between the measured the uPCR (R = 0.52, P = 1.1e–06) (Figure 3H). These correlations underscore the potential of VSIG4-ICx/Cr as a valuable biomarker for assessing disease activity and severity in LN, with implications for monitoring disease progression and therapeutic response.

Quantitative analysis of free-form VSIG4 levels in urine and its correlation with VSIG4-ICx. In addition to evaluating the levels of VSIG4-ICx, we also assessed the free-form VSIG4 to compare these two forms of VSIG4 in the urine. Using the same cohort of patients, stratification of LN into active and inactive disease states revealed that patients with LNA exhibited significantly higher levels of free-form VSIG4 than the HC group (P < 0.0001), whereas patients with LNI (P <0.05) had not as significant differences (Figure 4A). Additionally, LNI showed significantly higher levels compared with HC (P < 0.05). In contrast, patients with CKD had levels similar to those in the LNA group (P = 0.29). The distribution of free-form VSIG4 levels across various LN classes indicated significant differences. Pure membranous LN exhibited the highest levels of free-form VSIG4 compared with mesangial proliferative (P < 0.0001) and focal LN classes (P < 0.001) (Figure 4B). No significant differences were observed between the lower LN classes.

The diagnostic utility of free-form VSIG4 as a biomarker was further evaluated by ROC analysis. The AUC values indicated a strong potential for free-form VSIG4 to distinguish between patients with LN and HCs (AUC = 0.896, 95% CI 0.84–0.95; P < 0.001), as well as between active and inactive disease states with patients with LN (AUC = 0.6693, 95% CI 0.51–0.83; P = 0.044), with a wider 95% CI and a lower P value (Figure 4C). The normalized cutoffs were found to be 0.0528 ug/mg for LN versus HCs and 0.0708 ug/mg for LNA versus LNI. The density plot illustrated the optimal normalized cutoff between the LN and HC samples and their normalized intensity overlap (Figure 4D).

The correlation between the ICx form and the free form of VSIG4 was evaluated to compare further clinical parameters to justify the diagnostic potential of this protein biomarker. There is a significant positive correlation between urinary free-form and ICx VSIG4 levels (R = 0.5, P = 2.8e–06) (Figure 4E). This indicates

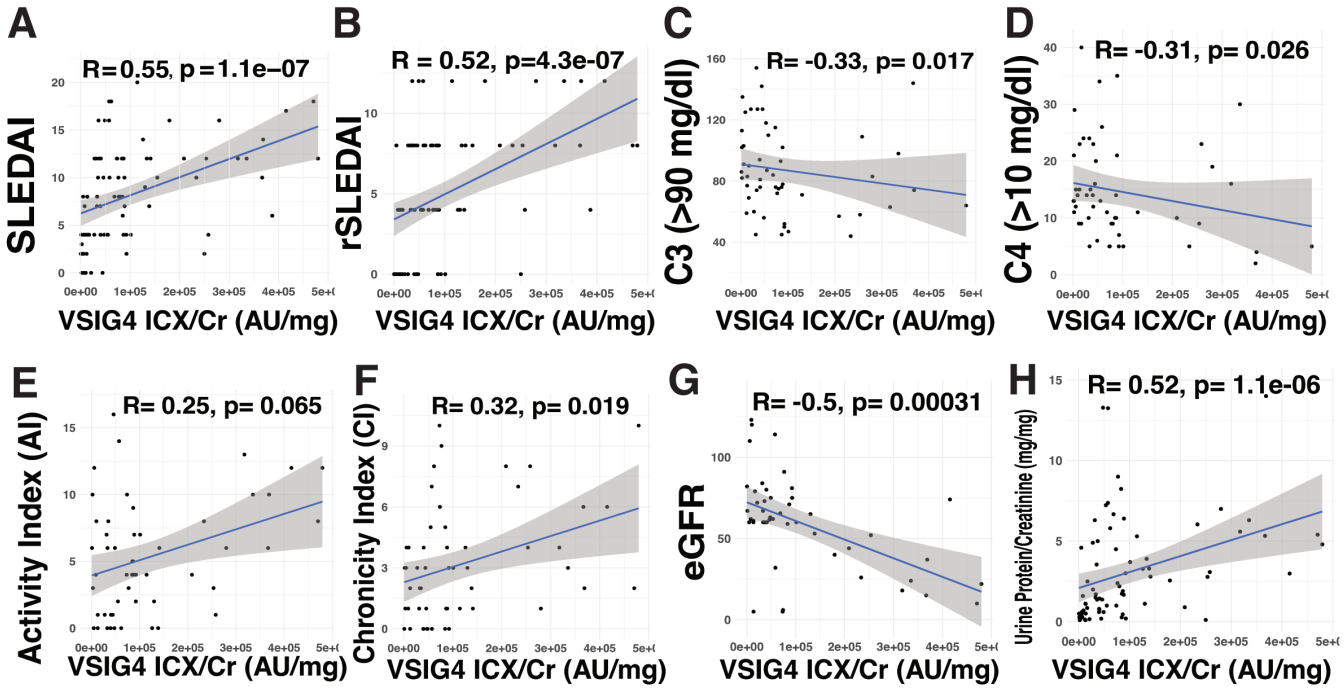

Figure 3. VSIG4-ICX correlation with clinical parameters. (A) Correlation between VSIG4-ICx/Cr and SLEDAI, showing a significant positive relationship (R = 0.55, P = 1.1e–07). (B) Positive correlation was depicted between VSIG4-ICx/Cr and rSLEDAI (R = 0.52, P = 4.3e–07). (C) Inverse correlation was shown between VSIG4-ICx/Cr and serum complement C3 levels (>90 mg/dl), with R = –0.33 (P = 0.017). (D) Similar negative correlation between VSIG4-ICx/Cr and serum complement C4 levels (>10 mg/dl), with R = –0.31 (P = 0.026). (E) There is a trend toward a positive correlation between VSIG4-ICx/Cr and activity index. However, it was not statistically significant (R = 0.25, P = 0.065). (F) Positive correlation was shown between VSIG4-ICx/Cr and chronicity index, with R = 0.32 (P = 0.019). (G) Significant inverse correlation between VSIG4-ICx/Cr and eGFR, with R = –0.5 (P = 0.00031). (H) Positive correlation between VSIG4-ICx/Cr and urinary protein/Cr ratio (mg/mg), with R = 0.52 (P = 1.1e–06). Blue lines represent the linear regression fit to the data points, with shaded areas indicating the 95% confidence interval for the fit. Each point represents an individual patient sample. P values are reported using the Spearman correlation. C3, component 3; Cr, creatinine; eGFR, estimated glomerular filtration rate; ICx, immune complex; rSLEDAI, renal Systemic Lupus Erythematosus Disease Activity Index; SLEDAI, Systemic Lupus Erythematosus Disease Activity Index; VSIG4, V-set Ig domain-containing protein 4.

that as the concentration of VSIG4-ICx/Cr increases there is also an increase in VSIG4/Cr levels.

A heatmap that represents the Spearman correlation of urinary free form along with ICx VSIG4 was used to identify which form of VSIG4 generates better diagnostic capability (Figure 4F). Both urinary free-form VSIG4 and urinary VSIG4-ICx showed various degrees of correlation with clinical and histopathological parameters, ranging from strong positive (red) to strong negative (blue) correlation. For urinary free-form VSIG4, a significant positive correlation was observed with serum VSIG4, SLEDAI, rSLEDAI, CI, interstitial inflammation, uPCR, and serum Cr, among others, with varying levels of statistical significance denoted by asterisks (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$). In contrast, urinary VSIG4-ICx displayed a stronger correlation with the same clinical parameters and additional parameters such as cellular crescents and interstitial fibrosis. These results suggest that urinary VSIG4, in both free-form and ICx-associated forms, is linked with key renal function and pathology indicators.

Correlation of free-form VSIG4 levels with clinical parameters and association with ICx. To further evaluate the potential role of the free-form VSIG4 as a biomarker for disease activity, the correlation between different clinical parameters was also explored within patients with LN to identify if there is a reflection in renal pathology (Supplementary Figure 3). Urinary free-form VSIG4 levels were normalized to Cr levels (VSIG4/Cr). SLEDAI and rSLEDAI displayed strong positive correlations, with correlation coefficients of 0.63 and 0.63, respectively, with highly

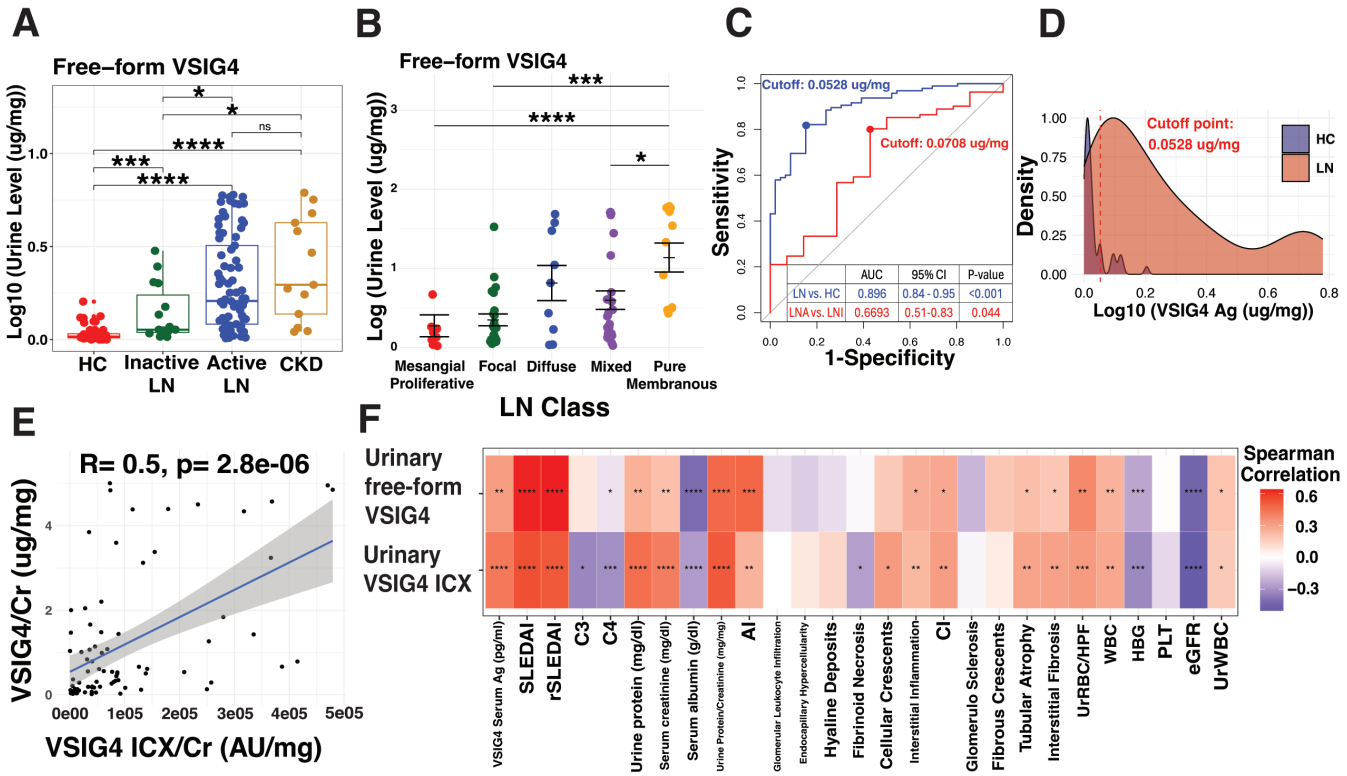

Figure 4. Urinary free-form VSIG4 analysis and correlation with VSIG4-ICx. (A) Box plot showing log10 normalized levels of free-form VSIG4 (ug/mg) in urine across different patient groups: HCs, LNI, LNA, and CKD. Significance is denoted by asterisks (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$). (B) Box plot of log normalized urinary VSIG4 levels in different LN classes: mesangial proliferative LGN, focal LGN (< 50% glomeruli), diffuse LGN (> 50% glomeruli), mixed LGN, and pure membranous LGN. Statistical significance between classes is indicated. (C) Receiver operating characteristic curves for diagnosing LN using free-form log10 normalized VSIG4 levels. The blue line represents the receiver operating characteristic curve for LN versus HC patients (AUC = 0.896) with a cutoff value of 0.0528 ug/mg, and the red line represents the curve for patients with active versus inactive LN (AUC = 0.6693) with a cutoff of 0.0708 ug/mg. The AUC, 95% CI, and *P* value for each comparison are indicated. (D) Density plots comparing the distribution of log10 normalized free-form VSIG4 levels between HC and LN, with a vertical line marking the optimal cutoff value (0.0528 ug/mg) for distinguishing between the two groups, as indicated by the red line. (E) Correlation between urinary unbound VSIG4 (y axis) and ICx VSIG4 (x axis) using the Spearman correlation, with $R = 0.5$ and $P = 2.8e{-}06$. (F) Heatmap showing a correlation between urine levels of free-form and ICx VSIG4 with clinical parameters of LN. According to the Spearman correlation coefficient values, the map is color-coded, ranging from purple (negative correlation) to red (positive correlation). 95% CI, 95% confidence interval; AI, activity index; AUC, area under the curve; CI, chronicity index; CKD, chronic kidney disease; Cr, creatinine; HBG, Hemoglobin; HC, healthy control; UrRBC/HPF, Urinary white blood cells/high power field; ICx, immune complex; LGN, lupus glomerulonephritis; LN, lupus nephritis; LNA, active lupus nephritis; LNI, inactive lupus nephritis; PLT, Platelet count; rSLEDAI, renal Systemic Lupus Erythematosus Disease Activity Index; SLEDAI, Systemic Lupus Erythematosus Disease Activity Index; UrWBC, Urinary white blood cells; VSIG4, V-set Ig domain-containing protein 4; WBC, White blood cells.

significant P values ($P < 0.0001$). In contrast, C3 and C4 levels ($R = 0.082$, $R = -0.081$) indicate no significant correlation between VSIG4/Cr levels and the complement components. Moreover, the AI correlation ($R = 0.49$, $P < 0.0001$) suggests that higher VSIG4 protein levels may be associated with increased AI. In contrast, the CI ($R = 0.28$, $P = 0.035$) also positively correlates with VSIG4. There is also a strong negative correlation with eGFR ($R = -0.44$, $P < 0.001$), indicating a similar result as the ICx levels, where higher levels of VSIG4 are associated with deteriorating kidney function. Lastly, uPCR levels were positively correlated with free-form VSIG4 levels ($R = 0.48$, $P < 0.0001$).

Urinary VSIG4 in tracking disease progression. We further examined the potential for both forms of VSIG4 to track disease progression or any flares in LN using longitudinally collected urine samples (mean visit interval of 5.5 months). The trends of the free form and ICx form of VSIG4 were evaluated, as well as two disease activity scores, SLEDAI and rSLEDAI, across multiple time points for six patients (Figure 5). The parameters were tracked over time to assess the correlation with LNA (indicated by triangles) and LNI (indicated by circles). Most patients demonstrated a parallel decline in VSIG4 levels and disease activity scores, the ability of free-form and ICx-form VSIG4 in the urine may track the changes in disease activity.

DISCUSSION

Renal deposits of Igs and complement in glomeruli have been indicated to cause kidney injury and further deterioration in LN.[20] Renal biopsy remains the gold standard for assessing the extent of active inflammation and long-term damage in the kidney, and it aids in determining the appropriate therapeutic effect for patients with LN.[21] The complement system is vital to the innate immune system's humoral component and initiates a specific immune response.[22] However, when triggered or controlled improperly, complement activation can lead to inflammation and harm to tissues.[23] Specifically, complement C3b functions as an opsonin, facilitating the clearance of ICxs, which may explain why the VSIG4-IgG complex is targeted by C3, forming a larger complex.[24] Previous studies have demonstrated that ICx deposition in tissue can activate the complement system, further propagating inflammatory cascades through the generation of proinflammatory mediators, ultimately leading to renal inflammation.[22]

Given that VSIG4 is a receptor of C3b and has been identified as novel biomarker of LN,[9] we were interested to investigate if VSIG4 could form a larger ICx with C3 and IgG, which could indicate renal pathology of LN. As a specific complement receptor of macrophages, VSIG4 was found to participate in complement and coagulation cascade pathways.[25,26] Also, VSIG4 (or CRIg) was reported to reduce proteinuria and improve kidney pathology

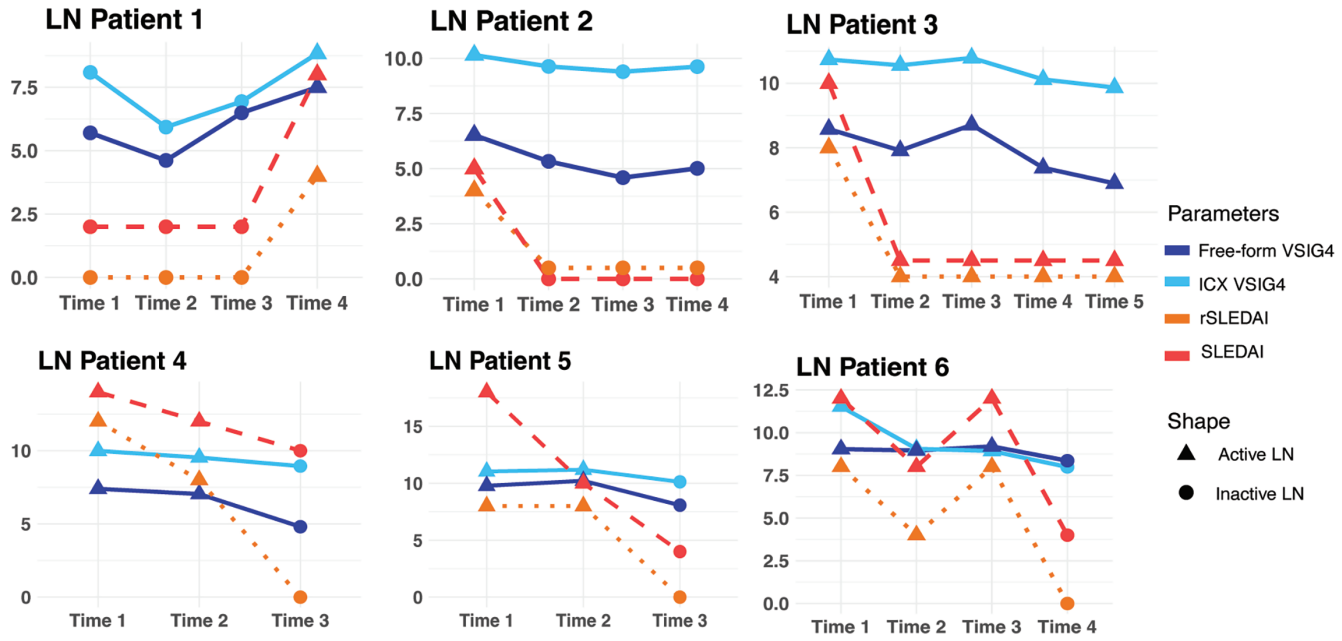

Figure 5. Urinary VSIG4 reflects disease progression in longitudinally collected samples from patients with LN. The graph presents longitudinal measurements of disease activity and log normalized biomarker levels in six patients with LN. Circles represent inactive LN states, whereas triangles denote active LN states. The light blue lines indicate the log normalized levels of ICx VSIG4, and the dark blue lines represent free-form VSIG4 levels. Disease activity is assessed by the dotted orange lines showing scores from the rSLEDAI (not normalized), and the dashed red lines depict the SLEDAI scores (not normalized). The data are captured over different time points to track the progression or remission of the disease in individual patients. ICx, immune complex; LN, lupus nephritis; rSLEDAI, renal Systemic Lupus Erythematosus Disease Activity Index; SLEDAI, Systemic Lupus Erythematosus Disease Activity Index; VSIG4, V-set Ig domain-containing protein 4.

in mice with lupus.[13,27] VSIG4 was also found inhibiting T cell proliferation and interleukin 2 production in both murine and human T cells.[28] Our observations of VSIG4 colocalization with IgG and C3 indicate that VSIG4 likely plays an active role in modulating the immune response. It is plausible that VSIG4 functions to regulate complement activation in renal tissue by binding to C3b, thereby mitigating excessive complement-mediated inflammation. However, the extensive formation of ICxs may overwhelm the regulatory capacity of VSIG4, resulting in unchecked complement activation. This uncontrolled activation could contribute to renal tissue injury, as the sustained inflammation and complement activation drive tissue damage and promote the shedding of ICxs into the urine. It is important to note that although the colocalization of VSIG4, IgG, and C3 within renal tissues indicates their immunogenic interplay in LN, the precise mechanisms through which these proteins contribute to kidney damage warrant further investigation.

Urinary ICxs were predominantly detected in patients with severe crescentic glomerulonephritis and in patients with membranoproliferative glomerulonephritis.[29] The accumulation of ICxs within the kidneys may drive their secretion into the urine, which could explain the elevated urinary VSIG4-ICx levels in LN. It seems that urinary VSIG4-ICx elevation is not exclusive to LN but may also occur in CKD because of similar mechanisms of ICx accumulation and renal injury, further highlighting its potential as a marker of kidney damage. In membranous LN, the predominant isotypes deposited in the kidneys were IgG1 and IgG3, along with significant C3 and C4 complement deposition.[30] This pattern suggests a strong involvement of the classical complement pathway, which may contribute to the progressive kidney damage seen in membranous LN. However, significant differences were not found in urinary VSIG4-ICx among patients with class III, IV, and V LN in this limited study.

Furthermore, our analysis revealed urinary VSIG4 responses to the number of medications taken by patients indicating a promising use of urinary VSIG4 as a biomarker in clinical practice or clinical trials for LN. This observation opens the possibility that certain medications used to manage LN may modulate the formation or clearance of ICxs, thereby affecting urinary VSIG4-ICx levels. Specifically, drugs that influence ICx formation or complement activation, such as mycophenolate mofetil, could indirectly alter VSIG4-ICx levels either by reducing ICx burden in renal tissues or by impacting VSIG4 expression on macrophages. Our results also suggest that ICx-bound VSIG4, compared with free-form VSIG4, may serve as a more precise marker in distinguishing between different disease states in LN. This is likely because ICx-bound VSIG4 reflects active complement involvement and ICx deposition in the kidney, directly linking the biomarker to underlying disease mechanisms.

Notably, when examining the relationship between C3 and C4 blood levels versus urinary VSIG4-ICx, a negative correlation was observed, as blood C3 and C4 levels were decreased in LN, which aligns with findings from numerous other studies.[31–33] This depletion of complement proteins in the bloodstream is primarily due to their activation and consumption within affected tissues, particularly the kidneys, where ICx deposition drives local complement activation. This consumption leads to reduced circulating levels of these complement proteins in the blood. Notably, the longitudinal data, although collected from a small subset of patients, highlights the potential use of urinary VSIG4-ICx as a reliable biomarker for tracking disease progression and flare-ups in LN over time, offering a noninvasive tool for monitoring disease status.

Study limitations include the need for further elucidation of the precise mechanisms underlying VSIG4's contribution to ICx formation and kidney damage. Although longitudinal assessments highlighted the potential of VSIG4 as a biomarker for tracking disease progression, this study's relatively short follow-up period with a small cohort warrants longer-term investigations to validate its efficacy over extended periods. The possibility that the ICx environment varies between different types of LN highlights the need for a more comprehensive evaluation of tissue expression across the distinct LN classes. In summary, this may represent the first attempt to demonstrate that VSIG4 forms an ICx in the kidney of LN, and urinary VSIG4 and ICx have great potential to reflect the clinical status of LN, including renal pathology.

AUTHOR CONTRIBUTIONS

All authors contributed to at least one of the following manuscript preparation roles: conceptualization AND/OR methodology, software, investigation, formal analysis, data curation, visualization, and validation AND drafting or reviewing/editing the final draft. As corresponding author, Dr Wu confirms that all authors have provided the final approval of the version to be published, and takes responsibility for the affirmations regarding article submission (eg, not under consideration by another journal), the integrity of the data presented, and the statements regarding compliance with institutional review board/Declaration of Helsinki requirements.

REFERENCES

1. Lech M, Anders HJ. The pathogenesis of lupus nephritis. J Am Soc Nephrol 2013;24(9):1357–1366.
2. Mohan C, Putterman C. Genetics and pathogenesis of systemic lupus erythematosus and lupus nephritis. Nat Rev Nephrol 2015;11(6):329–341.
3. Mortensen ES, Rekvig OP. Nephritogenic potential of anti-DNA antibodies against necrotic nucleosomes. J Am Soc Nephrol 2009;20(4):696–704.
4. Weening JJ, D'Agati VD, Schwartz MM, et al; International Society of Nephrology Working Group on the Classification of Lupus Nephritis; Renal Pathology Society Working Group on the Classification of Lupus Nephritis. The classification of glomerulonephritis in systemic lupus erythematosus revisited. Kidney Int 2004;65(2):521–530.
5. Tan Y, Song D, Wu LH, et al. Serum levels and renal deposition of C1q complement component and its antibodies reflect disease activity of lupus nephritis. BMC Nephrol 2013;14(1):63.
6. Muso E, Sekita K, Doi T, et al. Immunopathological correlation between mesangial C3d-deposition and C3d-fixing circulating immune complexes in lupus nephritis. Clin Immunol Immunopathol 1984;32(3):351–358.

7. Haas M. IgG subclass deposits in glomeruli of lupus and nonlupus membranous nephropathies. Am J Kidney Dis 1994;23(3):358–364.
8. Mok CC. Biomarkers for lupus nephritis: a critical appraisal. J Biomed Biotechnol 2010;2010:638413.
9. Tang C, Zhang S, Teymur A, et al. V-set immunoglobulin domain-containing protein 4 as a novel serum biomarker of lupus nephritis and renal pathology activity. Arthritis Rheumatol 2023;75(9): 1573–1585.
10. Liu B, Cheng L, Gao H, et al. The biology of VSIG4: implications for the treatment of immune-mediated inflammatory diseases and cancer. Cancer Lett 2023;553:215996.
11. He JQ, Wiesmann C, van Lookeren Campagne M. A role of macrophage complement receptor CRIg in immune clearance and inflammation. Mol Immunol 2008;45(16):4041–4047.
12. Kim AHJ, Strand V, Sen DP, et al. Association of blood concentrations of complement split product iC 3b and serum C3 with systemic lupus erythematosus disease activity. Arthritis Rheumatol 2019;71(3): 420–430.
13. Lieberman LA, Mizui M, Nalbandian A, et al. Complement receptor of the immunoglobulin superfamily reduces murine lupus nephritis and cutaneous disease. Clin Immunol 2015;160(2):286–291.
14. Bombardier C, Gladman DD, Urowitz MB, et al; The Committee on Prognosis Studies in SLE. Derivation of the SLEDAI. A disease activity index for lupus patients. Arthritis Rheum 1992;35(6):630–640.
15. Tang C, Fang M, Tan G, et al. Discovery of novel circulating immune complexes in lupus nephritis using immunoproteomics. Front Immunol 2022;13:850015.
16. Bajema IM, Wilhelmus S, Alpers CE, et al. Revision of the International Society of Nephrology/Renal Pathology Society classification for lupus nephritis: clarification of definitions, and modified National Institutes of Health activity and chronicity indices. Kidney Int 2018;93(4): 789–796.
17. Wickham H. ggplot2. Wiley Interdiscip Rev Comput Stat 2011;3(2): 180–185.
18. Robin X, Turck N, Hainard A, et al. pROC: an open-source package for R and S+ to analyze and compare ROC curves. BMC Bioinformatics 2011;12(1):77.
19. Wei T, Simko V, Levy M, Xie Y, Jin Y, Zemla J. Package 'corrplot'. Stat. 2017;56(316):e24. Accessed October 14, 2024.
20. Koffler D, Agnello V, Carr RI, et al. Variable patterns of immunoglobulin and complement deposition in the kidneys of patients with systemic lupus erythematosus. Am J Pathol 1969;56(3):305–316.
21. Brachemi S, Bollée G. Renal biopsy practice: what is the gold standard? World J Nephrol 2014;3(4):287–294.
22. Noris M, Remuzzi G. Overview of complement activation and regulation. Semin Nephrol 2013;33(6):479–492.
23. Morgan BP, Harris CL. Complement, a target for therapy in inflammatory and degenerative diseases. Nat Rev Drug Discov 2015;14(12): 857–877.
24. Walport MJ, Davies KA. Complement and immune complexes. Res Immunol 1996;147(2):103–109.
25. Antunes Andrade F, Goeldner Eibofner I, Pieczarka C, et al. Impact of VSIG4 gene polymorphisms on susceptibility and functional status of rheumatoid arthritis. Int J Immunogenet 2021;48(3):260–265.
26. Zhao H, Zheng D. Revealing common differential mRNAs, signaling pathways, and immune cells in blood, glomeruli, and tubulointerstitium of lupus nephritis patients based on transcriptomic data. Ren Fail 2023;45(1):2215344.
27. Shi Y, Yao W, Sun L, et al. The new complement inhibitor CRIg/FH ameliorates lupus nephritis in lupus-prone MRL/lpr mice. BMC Nephrol 2019;20(1):424.
28. Vogt L, Schmitz N, Kurrer MO, et al. VSIG4, a B7 family-related protein, is a negative regulator of T cell activation. J Clin Invest 2006; 116(10):2817–2826.
29. Stachura I, Ellis D, Kelly RH. Urinary immune complexes in patients with glomerular diseases. Their possible diagnostic implications. Arch Pathol Lab Med 1983;107(6):315–318.
30. Ward F, Bargman JM. Membranous lupus nephritis: the same, but different. Am J Kidney Dis 2016;68(6):954–966.
31. Li H, Lin S, Yang S, et al. Diagnostic value of serum complement C3 and C4 levels in Chinese patients with systemic lupus erythematosus. Clin Rheumatol 2015;34(3):471–477.
32. Birmingham DJ, Irshaid F, Nagaraja HN, et al. The complex nature of serum C3 and C4 as biomarkers of lupus renal flare. Lupus 2010; 19(11):1272–1280.
33. Li W, Li H, Song W, et al. Differential diagnosis of systemic lupus erythematosus and rheumatoid arthritis with complements C3 and C4 and C-reactive protein. Exp Ther Med 2013;6(5):1271–1276.