Biomarker Discovery in Human SLE Nephritis

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Abstract

The treatment of systemic lupus erythematosus (SLE) nephritis, while effective, is associated with significant morbidity and mortality. These side effects can be mitigated if the onset, severity, and response of renal flare can be predicted, and therapy modified accordingly. In this review, an approach to derive prediction equations of SLE nephritis flare is discussed. Integral to generating such prediction equations is the identification of biomarkers of lupus nephritis that can serve as input variables for modeling flare. The use of urine as a source of SLE nephritis biomarkers is described, and the results of urine biomarker discovery studies using candidate and proteomic approaches are presented.

Kidney involvement in patients with systemic lupus erythematosus (SLE) is a common and serious complication that is often associated with a poor long-term prognosis. Aggressive immunosuppression is effective in controlling renal lupus flares and has improved disease outcomes, but is associated with significant morbidity (infection, malignancy, metabolic disturbances, infertility) and mortality. Until more specific and less toxic therapies are developed, currently available immunosuppressive drugs could be used more effectively and with fewer side effects if clinicians could accurately predict who will flare, when flare will occur, flare severity, and response to treatment. This would allow the treating physician to: (1) start preemptive therapy early, with the goal of achieving remission quickly and decreasing duration of treatment; (2) identify patients who will have less severe flares and will thus require less aggressive treatment; (3) shorten the duration of treatment in patients destined to respond quickly and sustain a durable remission; or (4) lengthen and intensify treatment in patients who will not respond quickly. Tailoring therapy based on these predictors should provide tangible benefits to patients. For example, both early treatment and rapid response translate to improved kidney survival in SLE nephritis.1,2

This approach represents a fundamental change in the way SLE nephritis is treated, and will require the development of predictive models of renal flare. Such models are critically dependent on identifying biomarkers that monitor renal flare activity. Despite considerable investigation there are currently no validated biomarkers that accurately reflect flare status. Biomarker discovery in lupus nephritis will thus address a significant unmet clinical need.

Status of Traditional Clinical Lupus Biomarkers

The most frequently ordered serologic tests for the evaluation of lupus renal flare are the third and fourth components of complement (C3, C4), and anti-double stranded DNA antibodies (ADNA). We examined the reliability of these markers to detect renal flare in the Ohio SLE Study (OSS) cohort. The OSS1,4 included 71 SLE patients with biopsy-proven immune complex glomerulonephritis, and 35 SLE patients with no evidence of kidney involvement. Both groups were followed prospectively every two months (median follow up currently 33 months) for renal and nonrenal flare. The diagnosis and severity of renal flare in the OSS population was based on prespecified changes in urine sediment, serum creatinine, and proteinuria (Table 1), and did not take into
consideration complement or ADNA levels. Despite conventional clinical wisdom, a low C3, C4, or the presence of ADNA were poor markers of concurrent renal flare in the OSS. For this analysis, the reference ranges of C3 and C4 from The Ohio State University Clinical Laboratory were used to identify the lower limits of normal, and the presence or absence of ADNA was determined by a Crithidia luciliae assay. Using data from 70 renal flares and over 800 patient follow-ups, the false negative rates for C3, C4, and ADNA were 30%, 51%, and 47%, respectively, and the false positive rates were 27%, 26%, and 29%, respectively. The sensitivity, specificity, and positive predictive values (PPV) for C3 were 70%, 73%, and 22%; for C4, they were 49%, 74%, and 17%; and for ADNA, they were 53%, 71%, and 14%. Furthermore, the change in C3 or C4 from two months pre-flare to flare did not forecast impending renal flare (PPV of 7.4% and 5.5%, respectively).

The literature on complement and ADNA as biomarkers of concurrent or future SLE flares is inconsistent at best. In 98 patients who experienced 146 flares, Petri and colleagues showed that hypocomplementemia and ADNA antibodies accompanied SLE relapse in only 54% and 27% of patients, respectively, and the data specifically for renal flares were not provided. Other studies that looked at SLE nephritis showed decreased C3 or C4 levels or both in 55% to 95% of patients, and ADNA antibodies in 61% to 100% of patients at renal flare. In a prospective investigation of 202 patients who had 27 renal flares, the sensitivity and specificity for predicting renal exacerbation was 56% and 74% for a preceding fall in C3, 53% and 65% for a preceding fall in C4, and 53% and 69% for a preceding increase in ADNA antibodies. Similarly, Coremans and coworkers found that only 64% of patients had a significant increase in ADNA before renal flare, and Ho and associates showed that previous decreases in C3 and C4 and previous increases in ADNA did not predict impending renal relapse. In contrast, other investigators have reported more encouraging results, especially for ADNA. An increasing level of ADNA was a predictor of renal exacerbation with a sensitivity and specificity between 90% and 100% in two prospective studies that included 21 and 13 renal flares in 143 and 72 patients, respectively. A preceding decline in complement was a less sensitive forecaster than ADNA, with 80% of patients showing reduced C4 levels and 50% of patients showing reduced C3 levels 4 months before renal flare. Potential reasons for discrepancies in the results of these studies include the lack of a uniform definition of flare, the

### Table 1 Lupus Nephritis Flare Criteria Used in the OSS

<table>
<thead>
<tr>
<th>Mild Renal Flare</th>
<th>Moderate Renal Flare</th>
<th>Severe Renal Flare</th>
</tr>
</thead>
<tbody>
<tr>
<td>↑ in glomerular hematuria from</td>
<td>If baseline creatinine is:</td>
<td>If baseline creatinine is:</td>
</tr>
<tr>
<td>&lt; 5 to &gt;15 RBC/hpf,</td>
<td>&lt; 2.0 mg/dl, an ↑ of 0.20-1.0 mg/dl</td>
<td>&lt; 2.0 mg/dl, an ↑ of &gt; 1.0 mg/dl</td>
</tr>
<tr>
<td>with ≥ 2 acanthocytes/hpf</td>
<td>≥ 2.0 mg/dl, an ↑ of 0.40-1.5 mg/dl</td>
<td>≥ 2.0 mg/dl, an ↑ of &gt; 1.5 mg/dl</td>
</tr>
<tr>
<td>and/or recurrence of ≥ 1 RBC cast, WBC</td>
<td>and/or</td>
<td>and/or</td>
</tr>
<tr>
<td>cast (no infection), or both</td>
<td>If baseline Pr/Cr is:</td>
<td>an absolute ↑ Pr/Cr &gt; 5.0</td>
</tr>
<tr>
<td></td>
<td>&lt; 0.5, an ↑ to ≥ 1.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5-1.0, an ↑ to ≥ 2.0, but &lt; absolute ↑ of 5.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt; 1.0, an ↑ of ≥ 2-fold with absolute Pr/Cr &lt; 5.0</td>
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**Figure 1** Statistical approach to modeling SLE nephritis flare. This algorithm summarizes the procedure for analyzing traditional and novel biomarkers as input variables to model lupus renal flare. See text for details.
use of the test being evaluated to define flare, different assay techniques, and different sampling frequencies.

Although used less frequently in clinical practice than other markers, the level of antibodies to complement component C1q (anti-C1q) has been reported to be a lupus nephritis biomarker.\textsuperscript{6,15-19} From these investigations, the prevalence, sensitivity, specificity, and positive and negative predictive values of anti-C1q were 50% to 71%, 71% to 100%, 58% to 92%, 27% to 87%, and 81% to 100%, respectively. These results are comparable, in general, to C3, C4, and ADNA. Interestingly, three studies showed a 97% to 100% negative predictive value, suggesting that active lupus nephritis may not occur in the absence of anti-C1q. A prospective examination of anti-C1q antibodies in lupus found that levels started increasing 4 months before renal flare and were significantly different than baseline 2.3 months prior to flare.\textsuperscript{7} A positive predictive value of 71% for impending activation of SLE nephritis was calculated.

The main reason for these generally unimpressive results is that individual tests are probably not sufficient to describe a condition as complex as an SLE nephritis flare. Rather, an approach that simultaneously considers multiple dynamic phenotypes, genotypes, and interactions among variables may be considerably more informative. We have recently used a multivariate approach that incorporates stepwise logistic regression within a generalized estimating equation (GEE) framework to test the relationships between phenotypes and genotypes as markers and forecasters of renal flare (Fig. 1). A critical component of the GEE framework is the measurement of multiple input variables (i.e., biomarkers) prospectively at fixed intervals in a lupus nephritis cohort over several years and several flare cycles, leading to a large number of repeated measures over time. For each input biomarker, univariate logistic regressions are then fit to specific response models. For example, to develop a forecaster model of impending renal flare the response will be flare status (flare/no flare) at the next scheduled visit (Fig. 1). Biomarkers that survive univariate analysis at a $p < 0.25$ are identified as ‘informative.’ Informative biomarkers are tested together as covariates in a stepwise multivariate analysis in the GEE framework, to fit multiple logistic regressions with each response. Several rounds of successive multivariate analyses that include interaction terms are run with increasingly stringent survivor cutoffs, until no biomarkers are eliminated at a $p \leq 0.05$. These remaining biomarkers are designated ‘significant effectors’ of SLE renal flare and can be used to derive a prediction equation for each response model. The strength of this approach is illustrated by our analysis of the relationship of stress markers to SLE flare.\textsuperscript{4} Fluctuation of self-perceived stress in SLE patients was found to be a predictor of SLE nephritis flare, but only in the presence of a specific serotonin receptor gene allele that reduces serotonergic neurotransmission, and has been associated with depression and anxiety.\textsuperscript{4} Thus, through the GEE technique, an interaction between a dynamic phenotype (stress variability) and a genotype (serotonin receptor) was identified as the key pro-flare effect.

More relevant to the traditional clinical measures of SLE activity discussed above, we used this approach with biomarkers such as C3, C4, erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP) as input variables. The analysis returned a highly significant ($p < 0.001$) forecaster equation that included C4, ESR, and patient age as the significant effectors for impending renal flare in the next 60 days. However, the forecaster model based on these biomarkers has a relatively small impact on flare prediction. The model was tested with data from 26 renal flares in which the mean increase in ESR was 2.3 mm/hour and the mean fall in C4 was 1.4 mg/dL from baseline to 2 months pre-flare. The forecaster equation calculated the increased risk of flare to be only 10.6% 2 months later. This suggests that in addition to traditional clinical markers, novel biomarkers will be needed as inputs in the GEE framework to develop more robust prediction models.

Urine is a potential source of novel biomarker discovery in SLE nephritis. The advantages of urine for this purpose are its accessibility and the fact that urine components often directly reflect pathological events within the kidneys. Although it may sometimes be difficult to distinguish whether a urine component has been filtered or locally produced, filtered factors can adversely affect the kidneys, and if validated as biomarkers, their origin is less important. To interpret urine biomarkers correctly, urine concentration must be taken into account and there must be awareness that contamination from the lower urinary tract could confound results.

The Candidate Biomarker Approach

A common approach to identifying novel disease biomarkers is to study factors that are known or thought to be involved in the pathogenesis of organ injury. With respect to inflammatory diseases of the kidney, leukocyte infiltration is mediated by proinflammatory chemokines,\textsuperscript{19-23} and the chemokine monocyte chemoattractant protein-1 (MCP-1) has been shown to be pathogenic for kidney injury in murine lupus nephritis, and significantly associated with human SLE nephritis.\textsuperscript{24-34}

We thus studied urine MCP-1 (uMCP-1) as a candidate biomarker for SLE renal flare. MCP-1 was measured by ELISA (enzyme-linked immunosorbent assay) in the urine of patients from the OSS cohort at the time of renal flare, and, when available, in samples from before and after flare.\textsuperscript{7} The mean uMCP-1 at renal flare was significantly greater than that of healthy control subjects, patients with stable renal lupus (disease controls), and patients with active or inactive nonrenal SLE. Urine MCP-1 was a sensitive indicator of renal flare, with 73% of the flare values above the 95th percentile of disease controls. Additionally, uMCP-1 was not confounded by systemic, nonrenal SLE activity, suggesting specificity for renal flare. Urine MCP-1 levels were higher in patients with impaired kidney function, patients undergoing severe flare (as defined in Table 1), and patients...
with class III or IV nephritis (versus class V), consistent with a role for MCP-1 in flare pathogenesis. Most patients were receiving chronic immunosuppressive therapy, but there was no relationship between the cumulative amount of therapy received during the 30 days preceding flare and uMCP-1 levels, suggesting that uMCP-1 is a robust marker of SLE renal activity that is valid in patients on maintenance therapy.

In longitudinal studies of a small (N = 12) number of patients, uMCP-1 appeared to increase as early as 2 to 4 months before flare, consistent with the possibility that serial monitoring of uMCP-1 could be used to predict flare onset. After appropriate treatment for renal flare, uMCP-1 remained high for at least 4 months and displayed three patterns of response. In clinically improved patients (50%), uMCP-1 fell to control levels, while in patients who showed no improvement (25%), uMCP-1 remained elevated. In the remaining 25%, uMCP-1 was persistently elevated despite clinical improvement, raising the possibility that uMCP-1 may indicate ongoing, subclinical inflammation in this group.

Other investigations also support uMCP-1 as a biomarker for SLE nephritis. For example, the urine sediment of patients with SLE nephritis showed higher levels of MCP-1 mRNA during active nephritis. Additionally, the protein TWEAK (TNF-like weak inducer of apoptosis), a regulator of renal MCP-1 production, was recently found in the urine of patients with active SLE nephritis, and showed a trend toward increased expression at flare in longitudinal samples. These data along with our findings support testing uMCP-1 as an input variable in developing prediction models of SLE nephritis.

A number of urine protein biomarker candidates, including other chemokines, cytokines, growth and proinflammatory factors, have been examined as SLE biomarkers, but none have been independently validated to date. A disadvantage of the candidate approach is that not all cytokines having a pathogenic role in SLE nephritis will be disease biomarkers. A good example is interleukin-8. Like MCP-1, this chemokine is thought to play a role in human kidney injury in SLE, but we found no evidence that urine IL-8 expression reflected renal flare cycle activity. Additionally, the candidate approach is labor-intensive and not high through-put. Thus, other approaches to lupus biomarker discovery are currently being examined.

The Protein Profiling Approach to Biomarker Discovery

Urine protein profiling using proteomic techniques is high through-put; has the potential of identifying novel, unexpected biomarkers; and does not rely on preconceived notions of disease pathogenesis. Using an immobilized antibody array containing antibodies for 70 cytokines, we found that adiponectin, an adipocyte-derived cytokine, was present in high amounts in the urine of patients with SLE nephritis.

This finding was not anticipated, but turned out to be potentially interesting, because, in addition to its metabolic effects on obesity and glucose regulation, adiponectin can modulate inflammation. Interestingly, adiponectin can also induce MCP-1 production. We, thus, measured urine adiponectin (uAdip) in the OSS cohort by ELISA, and found it to be significantly higher during SLE renal flare, compared to healthy and renal disease controls, and patients with active or inactive nonrenal SLE, verifying the antibody array data. Time course studies showed that uAdip increased acutely at renal flare and fell quickly after treatment, in contrast to the patterns observed with uMCP-1. Like uMCP-1, uAdip is a novel input variable that can be tested in building predictive models of SLE nephritis.

Another proteomic technique that is being studied as a tool for biomarker development is urine mass spectrometry. We have used surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) to profile the differential expression of proteins in the low-molecular-weight (LWM) urine proteome during class flare. Figure 2 SELDI-TOF-MS of a WHO class IV SLE nephritis flare cycle. Urine samples from a period of disease quiescence (baseline), 4 and 2 months pre-flare, flare, and 2 and 4 months post-flare were analyzed by SELDI-TOF-MS after albumin and other high-molecular-weight proteins were removed. The raw SELDI spectrum is shown, and the peaks represent individual, LWM protein ions. The values along the y-axis represent peak intensity, a measure of protein ion abundance, and the values along the x-axis represent mass to charge ratios from 2000 to 10,000 daltons.
III and IV SLE nephritis flares. The LMW proteome is especially relevant, because it contains cytokines and growth factors that are likely to be important in the pathogenesis of SLE nephritis flare. A representative SELDI-TOF MS profile of a lupus flare cycle is shown in Figure 2 and illustrates the dynamic changes in LMW proteins over the course of a flare. Applying this technique to 25 class III/IV flare cycles, 24 protein ions were found to have significantly different expression at one or more points of the flare cycle. The next step in this analysis is to identify the proteins represented by these ion peaks, so they can be validated in the OSS cohort, preferably using an immunodetection platform. Validated proteins will then be used as input variables for predictive modeling.

Summary

The urine of patients with SLE nephritis is a potentially rich source of biomarkers that may reflect various aspects of the renal flare cycle. Identification of urine biomarkers has traditionally been approached by evaluating candidate proteins chosen because of a relationship to the pathogenesis of SLE nephritis. The candidate approach is thus limited by the level of understanding of disease mechanisms. Non-biased, high throughput proteomic approaches offer the promise of simultaneously evaluating a large number of proteins and identifying novel biomarkers that would be overlooked by the candidate approach. In this way, proteomics can also provide new insights into disease pathogenesis. A caveat to proteomic approaches is that these novel biomarkers must be validated in a disease cohort using a different technique. We envision that a combination of both techniques will lead to the discovery of lupus nephritis biomarkers that can be incorporated into multivariate prediction models. These models will allow a more rational and individualized approach to the therapy of SLE renal flare, and should result in improved renal prognosis with less morbidity.

Disclosure Statements

None of the authors have a financial or proprietary interest in the subject matter or materials discussed in the manuscript, including, but not limited to, employment, consultancies, stock ownership, honoraria, and paid expert testimony.

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References


