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Antibody-Array-Based Proteomic Screening of Serum Markers in Systemic Lupus Erythematosus: A Discovery Study

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Abstract

A discovery study was carried out where serum samples from 22 systemic lupus erythematosus (SLE) patients and matched healthy controls were hybridized to antibody-coated glass slide arrays that interrogated the level of 274 human proteins. On the basis of these screens, 48 proteins were selected for ELISA-based validation in an independent cohort of 28 SLE patients. Whereas AXL, ferritin, and sTNFR2 were significantly elevated in patients with active lupus nephritis (LN) relative to SLE patients who were quiescent, other molecules such as OPN, sTNFR1, sTNFR2, IGFBP2, SIGLEC5, FAS, and MMP10 exhibited the capacity to distinguish SLE from healthy controls with ROC AUC exceeding 90%, all with $p < 0.001$ significance. These serum markers were next tested in a cohort of 45 LN patients, where serum was obtained at the time of renal biopsy. In these patients, sTNFR2 exhibited the strongest correlation with eGFR ($r = -0.50$, $p = 0.0014$) and serum creatinine ($r = 0.57$, $p = 0.0001$), although AXL, FAS, and IGFBP2 also correlated with these clinical measures of renal function. When concurrent renal biopsies from these patients were examined, serum FAS, IGFBP2, and TNFR2 showed significant positive

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ASSOCIATED CONTENT

Supporting Information

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AUTHOR INFORMATION

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correlations with renal pathology activity index, while sTNFRII displayed the highest correlation with concurrently scored renal pathology chronicity index ($r = 0.57$, $p = 0.001$). Finally, in a longitudinal cohort of seven SLE patients examined at ~3 month intervals, AXL, ICAM-1, IGFBP2, SIGLEC5, sTNFRII, and VCAM-1 demonstrated the ability to track with concurrent disease flare, with significant subject to subject variation. In summary, serum proteins have the capacity to identify patients with active nephritis, flares, and renal pathology activity or chronicity changes, although larger longitudinal cohort studies are warranted.

Graphical abstract

Keywords

AXL; FAS; IGFBP2; TNFRII; biomarkers; nephritis; pathology

INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease that affects multiple end organs including the kidneys, skin, joints, and heart. Indeed, renal disease is a leading cause of morbidity and mortality in SLE, affecting about 60% of these patients. About a quarter of all patients with lupus nephritis (LN) succumb to end-stage renal disease (ESRD). Given that early detection of renal involvement in SLE and prompt management of the disease can have a significant impact on disease outcome, accurate diagnosis of LN is absolutely critical.^{1–4} The current gold standard is to perform a renal biopsy to assess renal pathology. However, this procedure cannot be repeated serially and is associated with

untoward risks. Hence, there is an urgent need to identify biomarkers of LN that enable early detection and serial follow-up of the disease.

Most of the previous research efforts aimed at identifying serum biomarkers for LN have typically examined individual proteins preselected based on their known biology. Indeed, there has been a large number of publications in recent years identifying specific elevated serum proteins as potential biomarkers of SLE or specific clinical manifestations associated with SLE, including circulating levels of β 2-microglobulin, syndecan-1, BAFF, FABP4, ficolins, HMGB1, human neutrophil peptide 1–3, IGF1, IL-6, IL-23, milk fat globule epidermal growth factor 8, OxLDL, resistin, various oxidative stress markers, S100A8/A9, S100A12, thiols, soluble MER, urokinase plasminogen activator receptor, CSF1, RAGE, TLR2, E-selectin, and VCAM-1.^{5–27}

Multiplex and high-throughput array systems allow for the screening of large numbers of protein biomarker candidates. During the past decade, glass-slide-based protein array platforms have been designed and fabricated for the detection of specific antigens or antibodies, cancer-related proteins,^{29–33} and cytokine production in cell culture or body fluids³⁴. These technologies have been tailored by interrogating autoantigens to scan various autoantibodies in the sera from patients with autoimmune diseases such as rheumatoid arthritis (RA) and SLE.^{35–38} To profile the serum proteome in SLE and systemic sclerosis (SS), Wingren and colleagues developed a recombinant antibody microarray where 135 human recombinant single-chain fragment variable (scFv) antibody fragments directed against 60 different immunoregulation-related proteins were printed onto glass slides.³⁹ This array facilitated the interrogation of various immune-related proteins including complement proteins (C1q, C3, and C4) and cytokines such as IFN- γ , IL-1 β , IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p70, IL-13, and TNF- α in the sera of SLE and SS.³⁹ Along these lines, 52 different soluble mediators, including cytokines, chemokines, and soluble receptors, were examined by Munroe and colleagues using validated multiplex bead-based or enzyme-linked immunosorbent assays in plasma from SLE patients.⁴⁰ They reported that several soluble mediators were elevated preflare, including Th1-, Th2-, and Th17-type cytokines, sTNFR1, sTNFR2, FAS, FASL, and CD40L.

The present study constitutes perhaps the largest screen thus far for 274 potential biomarker proteins (including cytokines, chemokines, and other mediators) using serum from patients with SLE/LN and healthy controls. Given the high costs of planar arrays, this discovery study was focused on a limited number of patient samples, totaling 22. Molecules revealed to be significantly different in SLE serum using this screen were next validated using ELISA assays and an independent cohort of SLE patients. Serum proteins that were consistently elevated in patients with SLE or active LN were next examined in patients undergoing renal biopsy so that serum biomarker levels can be compared with clinical and pathological indices of LN. Finally, serial changes in the levels of selected serum proteins were also assessed in longitudinal blood samples obtained from a limited cohort of patients with renal or nonrenal SLE.

Collectively, this discovery study suggests that serum levels of AXL, FAS, ferritin, ICAM-1, IGFBP2, SIGLEC-5, and sTNFR2 are potential indicators of active LN or concurrent renal pathology indices or disease flares, worthy of further validation studies.

MATERIALS AND METHODS

Patients and Samples

For the initial exploratory/discovery biomarker discovery studies using protein arrays, the ELISA-based validation studies, and the renal biopsy-concurrent studies, patients with SLE, including those with active LN as well as matched controls, were recruited from the Parkland and St. Paul University Hospitals of the University of Texas Southwestern Medical Center at Dallas, and the collected samples were archived in an internal biobank (Tables 1–3). All human subject-related procedures were performed following institutionally approved IRB protocols. All patient informed consents were obtained prior to sample collection. The study protocols adopted are similar to our previous studies focusing on VCAM-1.^{41, 42} In brief, LN was diagnosed and classified based upon ISN/RPS 2003 classification. Inclusion criteria included LN patients with biopsy-proven LN. Exclusion criteria were patients with ESRD or other concurrent autoimmune diseases. Clinical data were gathered by chart review, and SLEDAI was calculated based on chart review.⁴³ For the longitudinal studies, patient samples were obtained from the rheumatology clinics at the University of Rochester and Johns Hopkins University Medical School (Table 3).

For the initial biomarker screening study, sera from 14 (active or inactive) LN patients was tested, with a mean age of 35.4 years, median SLEDAI of 8, and median renal-related SLE disease activity index (rSLEDAI) of 8, as summarized in Table 1. Eight healthy individuals with mean age of 35 years, matched for gender and ethnicity, served as controls for this array-based screening study. For the validation studies, serum samples from 28 LN patients were studied using an orthogonal method, ELISA. Of these patients 35.7% had inactive LN (rSLEDAI = 0) and 64.3% had active LN (rSLEDAI ≥ 4). There were no patients with intermediate SLEDAI values (0–3). Detailed information pertaining to these patients is provided in Table 1. The healthy controls ($N = 9$) for the validation study were matched for age, gender, and ethnicity. For the renal biopsy-concurrent samples, serum samples were obtained at the same time when the biopsy was done on the same patient. In total, 45 renal-biopsy concurrent samples were obtained; the demographics and clinical characteristics of these patients are summarized in Table 1.

Seven SLE patients in a longitudinal study with severe flare were identified based on the SLEDAI 2K composite,³¹ from patients followed up routinely in the Division of Rheumatology at University of Rochester, NY. The average time between visits in these patients was 2.9 months. Patient demographics and clinical characteristics at visits preceding, during and following the flare, are shown in Table 4. The presence or absence of proteinuria, low complements, and elevated anti-dsDNA was determined at time of the visits, while the presence or absence of ANA (as well as other autoantibodies such as RNP, Sm, Ro, and La) was derived from medical records.

All serum samples were procured and processed as previously described,⁴⁴ following standard operating procedures (<https://edrn.nci.nih.gov/resources/standard-operating-procedures/standard-operating-procedures/serum-sop.pdf>). In brief, whole blood was collected in BD Vacutainer Serum tubes (cat. no.: 367812). Tubes were incubated undisturbed at room temperature for 30 min and then centrifuged at 3000 rpm for 10 min at 4 °C. The supernatant (serum) was divided into 200 µL aliquots and frozen at –80 °C for storage. No additives, preservatives, or antiprotease cocktails were added. Hemolysed samples were not used. Each aliquot of serum was retrieved and thawed only once for the assays in this study.

Targeted Protein Array

Serum samples from LN patients ($n = 14$) and age, gender, and ethnicity-matched healthy controls ($n = 8$) were diluted 5-fold into sample buffer (1% BSA in PBS) and hybridized to glass slide arrays that interrogate the level of 274 different human proteins. The biomarker screening was conducted using the RayBio Human Cytokine Antibody Array G-Series 4000 (cat. no. AAH-CYT-G4000-8), which consists of eight subarrays in one slide and allows for the interrogation of one sample per subarray. Three such arrays (totally harboring $8 \times 3 = 24$ subarrays) were loaded with serum samples from LN patients or healthy controls. In brief, monoclonal antibodies against various cytokines (or other soluble mediators) were printed onto the slides as baits to capture the corresponding cytokines (or other mediators) in the applied body fluids (serum in this study), then incubated with a cocktail of prevalidated biotinylated secondary antibodies, and finally detected with Cy3-labeled streptavidin. Each analyte was assayed in duplicate. The slides were then scanned using a GenePix 4000B scanner (Molecular Devices). Signals were acquired and transformed to digits using Genepix software. In the array, Positive Control spots (POS1, POS2, POS3) composed of standardized amounts of biotinylated IgGs printed directly onto the array. All other variables being equal, the Positive Control intensities should be the same for each subarray. This allows for normalization of results from different subarrays (or samples). Also, included on the array were Negative Control (NEG) spots consisting of the assay buffer alone (used to dilute antibodies printed on the array). The presence of analytes was marked by signal intensities that exceeded two standard deviations above the mean background signal intensity. GenePix PMT was set at 80% and the gain setting was 550 for all scans in this study. The intra-array coefficient of variation between replicates was ascertained to be at least 90%; otherwise the data were excluded or repeated. One LN sample was used as an internal calibrator across all three arrays and used for normalization of the array data to adjust for interarray differences in array intensities.

ELISA Assay

Serum samples obtained from the renal clinics at Parkland and St. Paul Hospitals (Dallas, TX) were aliquoted prior to storage at –80 °C. Only one aliquot was retrieved for each assay to avoid multiple freeze/thaw cycles. All potential biomarker levels were measured using duoset ELISA kits from R&D systems (Minneapolis, MN) or precoated ELISA kits from Raybiotech (Norcross, GA). For each assay, serum was diluted 1:5–1:500 into sample diluent (R&D systems), and duplicate assay was performed for each sample.

Statistics

Data were plotted and analyzed using GraphPad Prism 5 (GraphPad, San Diego, CA) or Medcalc software (Mariakerke, Belgium). A *t* test was used where the normality test passed; otherwise, the nonparametric Mann–Whitney test was used to analyze the data. Likewise, the Pearson method or the nonparametric Spearman method was used for correlation analyses. The hypotheses being tested in various stages of the analysis include the following: (a) the biomarker levels are higher in patient samples, particularly those with active disease, and (b) serum biomarker levels correlate with specific clinical or histological features of disease.

The initial screening data were subjected to parametric tests and nonparametric tests (when the biomarker levels strongly deviated from normality). In addition, to correct for multiple testing, *q* values were calculated using the Benjamini and Hochberg's method. Differences between patients and controls were displayed using a Volcano plot showing the distribution of the biomarkers log fold-change versus negative log (base 10) *p* values from *t* tests on lupus and healthy groups. Biomarkers with absolute fold change 1.3 and *p* value 0.05 (in either the parametric or nonparametric tests) were considered promising in this screening stage. Although most of these markers did not attain a *q* value of 0.05, they were nevertheless selected for further validation so as not to miss an otherwise discriminatory biomarker from the screening stage.

The biomarker results from the ELISA-based validation assays were similarly analyzed. The Shapiro–Wilk test was used to check for normality of the data. If both comparison groups passed the test, a *t* test was used; this is reflected using nonitalicized entries in Tables 2 and 3. If either group did not pass the normality test, a Mann–Whitney U test was used, and this is reflected by the italicized entries in Tables 2 and 3.

For identifying groups of serum biomarkers that may be discriminatory, the biomarker values were log-transformed. We identified molecules that were different across the three groups (active LN, inactive LN, healthy controls) as well as active versus all others (*p* value <0.05).

For the biomarkers assayed at the time of renal biopsy, we first used univariate analyses to examine their association with clinical disease or pathological disease measured at the same time. Biomarkers that were significantly associated (*p* value <0.05) in the univariate analysis were entered into a multivariable linear regression model to assess whether they are independent predictors of clinical or pathological disease.

Results

For the initial discovery study, serum samples from LN patients (*n* = 14) and age-, gender-, ethnicity- matched healthy controls (*n* = 8) were hybridized to glass slide arrays that interrogate the level of 274 different human proteins, as detailed in the Materials and Methods. The median SLEDAI and renal-SLEDAI of these patients were 8 and 8, respectively (Table 1). Of the serum proteins that were significantly elevated in SLE patients compared with healthy controls, 14 proteins were elevated 2-fold or greater, while 19 proteins were elevated between 1.3- to 2-fold, as displayed in the Volcano plot in Figure 1A.

The most highly elevated serum proteins in SLE, as revealed by the planar arrays, are plotted in Figure 1B; this list includes angiopoietin-2, AXL, BLC, CD30, FAS, ferritin, GDF-15, growth hormone, ICAM-1, MMP3, sTNFR2, and VCAM-1. In contrast, a smaller number of serum proteins were observed to be significantly reduced in SLE (Figure 1A).

Because several of the serum proteins that were elevated in SLE sera compared with healthy control sera at $P < 0.05$ (by parametric or nonparametric tests) lost significance after multiple testing correction, we proceeded to experimentally interrogate promising candidates using independent patient samples and an orthogonal assay platform (referred to as “validation” in this report). Specifically, 48 proteins were selected for ELISA-based validation in an independent cohort of 28 SLE patients, including 18 with active lupus nephritis (“active” or “LN”). The median SLEDAI and renal-SLEDAI of these patients were 10 and 5, respectively (Table 1). As expected, most of the tested molecules were elevated in SLE sera relative to healthy control sera, as evidenced by the red-shaded cells in the heatmap displayed in Figure 2. Of these, 17 serum proteins were validated to be significantly elevated (2-fold or greater) in SLE at $p < 0.05$, as listed in Table 2. Of these molecules, a couple of serum proteins were also noted to be significantly elevated in patients with active LN (rSLEDAI = 4) relative to SLE patients with no active disease (rSLEDAI = 0), notably AXL, ferritin, and sTNFR2, as displayed in Figure 2 and Table 3. Although serum IGFBP2, BLC, MMP3, growth hormone, and activin A were elevated in patients with active LN, these elevations did not attain statistical significance (Table 3). Most of the other serum proteins that were elevated in SLE sera were elevated in both active as well as inactive disease (Figure 2E–M).

Next, ROC curves were generated for all 48 ELISA-tested markers to ascertain the capacity of each serum marker to distinguish SLE from healthy and active LN from inactive disease. Among these, seven serum markers had the capacity to distinguish SLE from healthy controls with ROC AUC exceeding 90%, all with $p < 0.001$ significance; these included sTNFR2, OPN, sTNFR1, IGFBP2, SIGLEC5, FAS, and MMP10 (Table 2). Most importantly, four serum proteins (AXL, Ferritin, Angiostatin, and sTNFR2) had the capacity to distinguish active LN from inactive disease with ROC AUC exceeding 80%, all with $p < 0.01$ significance (Table 3). A comparison of the initial array-based screening results and the subsequent ELISA-based validation assays for selected molecules that appeared promising is presented in Supplementary Figure S1.

A subset of these serum markers, AXL, FAS, IGFBP2, sTNFR2, ICAM1, and SIGLEC5, was further tested in a cohort of 45 LN patients where serum was obtained at the time of renal biopsy. The median SLEDAI and renal-SLEDAI of these patients were 16 and 8, respectively (Table 1). As shown in Figure 3 (row 1), AXL, FAS, IGFBP2, and sTNFR2 showed positive correlation with SLEDAI, with FAS ($r = 0.38$, $p = 0.005$) and IGFBP2 ($r = 0.44$, $p = 0.001$) being the best correlated. All four markers correlated negatively with eGFR, with sTNFR2 exhibiting the strongest correlation ($r = 0.50$, $p = 0.0014$) (Figure 3, row 2). Concurrently measured serum creatinine correlated significantly with serum IGFBP2 ($r = 0.52$, $p < 0.0007$) and sTNFR2 ($r = 0.57$, $p = 0.0001$) (data not plotted). All four serum proteins also correlated significantly with proteinuria, with r values ranging from 0.28 to 0.34 (data not plotted). Serum FAS, IGFBP2, and sTNFR2 showed significant positive

correlations with renal pathology activity index in concurrent biopsies (Figure 3, row 3). Finally, sTNFR2 displayed the highest correlation with concurrently scored renal pathology chronicity index ($r = 0.57$, $p = 0.001$) (Figure 3, row 4). Multivariate analysis also indicated that serum IGFBP2 was an independent predictor of renal pathology activity index and eGFR, while serum TNFR2 was an independent predictor of renal pathology chronicity, as marked by the P_m values in Figure 3. In contrast with the above markers, serum SIGLEC5 correlated significantly only with serum creatinine ($r = 0.35$, $p = 0.02$), while ICAM1 did not correlate significantly with the examined clinical or pathological parameters (data not shown).

Finally, the above panel of markers, as well as VCAM1, which we have previously validated to be a good serum marker of active LN,^{41, 42} were serially assessed, about 3 months apart, in a panel of seven SLE patients, three of whom exhibited renal flare during the follow-up, with the remaining four developing nonrenal flare during follow-up, as detailed in Table 4 and Figure 4. Interestingly, in different patients, different serum biomarker candidates performed optimally in tracking changes in SLEDAI. In some patients such as P5, almost all of the tested markers exhibited concordant changes with SLEDAI, while in other patients, only a subset of the markers varied concordantly with changes in SLEDAI (e.g., IGFBP2, SIGLEC5, and sTNFR2 in patient P1). Importantly, no single marker exhibited the capacity to track with disease flares in all patients.

DISCUSSION

Since ESRD is irreversible and can be fatal, it is imperative that LN be diagnosed as early as possible. Given the potential risk of complications associated with needle biopsy of the kidney, which is currently used for the diagnosis of LN, noninvasive biomarkers of LN are urgently needed. This is especially so given that early detection of renal involvement in SLE and prompt management can have a significant impact on disease outcome.^{1–4} Serum proteins constitute one promising category of potential biomarkers. Reports over the past 5 years have suggested that molecules such as MCP-1, TWEAK, NGAL, IP-10, and VCAM1 may have potential as early markers of LN, as reviewed.^{5, 45} A large number of publications in the past year have added to this fast growing list of potential LN biomarkers, including circulating levels of β 2-microglobulin, syndecan-1, BAFF, FABP4, ficolins, HMGB1, IGF1, IL-6, IL-23, milk fat globule epidermal growth factor 8, OxLDL, resistin, various oxidative stress markers, S100A8/A9, S100A12, thiols, soluble MER, urokinase plasminogen activator receptor, CSF1, RAGE, TLR2, E-selectin, and VCAM-1.^{5–27}

The present discovery study adds to this growing list of serum biomarkers in SLE, beginning with a relatively unbiased but targeted antibody-based protein screen. One of the most promising candidates to emerge from this study is serum sTNFR2, which is significantly elevated in patients with active LN and highly correlated with concurrently measured eGFR and serum creatinine as well as concurrent renal pathology activity and chronicity indices. Moreover, it tracks with renal or nonrenal flares in some of the serially monitored SLE patients. This molecule, also known as p75 and TNFRSF1B, is expressed on certain populations of lymphocytes, including T-regulatory cells (Tregs),^{46, 47} endothelial cells, microglia, neuron subtypes,^{48, 49} oligodendrocytes,^{50, 51} cardiac myocytes,⁵²

thymocytes,^{53, 54} islets of Langerhans, and human mesenchymal stem cells.⁵⁵ Not surprisingly, it has been studied and implicated as a potential biomarker under several other conditions. In cardiovascular disease, it was reported that circulating levels of sTNFRII were increased in heart failure with preserved ejection fraction relative to heart failure with reduced ejection fraction and significantly associated with increasing grade of diastolic dysfunction and severity of symptoms.⁵⁶ Serum sTNFRII levels were significantly increased in patients with primary progressive multiple sclerosis compared with other MS forms and healthy controls.⁵⁷ A recent study in lupus has shown that the baseline levels of soluble TNFRII are significantly elevated in preflare patients compared with nonflare patients and healthy controls.⁴⁰ In cancer, serum sTNFRII is associated with the outcome of patients with diffuse large B-cell lymphoma treated with the R-CHOP regimen.⁵⁸

Serum IGFBP2 was another serum protein that was found to be associated with active SLE, correlating well with concurrently measured SLEDAI, eGFR, serum creatinine, and renal pathology activity index. It also tracked with nonrenal flares in some of the serially monitored SLE patients. IGFBP2 and related family members are known to regulate the metabolic functions of insulin-like growth factors (IGFs) I and II, synthesized by a variety of cell types.⁵⁹ In vitro and in vivo models suggest that IGFBP2 has mainly inhibitory effects on IGF action.^{60, 61} Serum IGFBP2 has also been shown to have biomarker potential in metastatic prostate cancer, ovarian cancer, CNS tumors, and colorectal cancer.^{62–66} IGFBP2 has also been reported to identify insulin-resistant individuals at high cardiovascular risk as well as metabolic syndrome.⁶⁷ Most relevant to this report, high serum IGFBP2 at baseline was associated with a decreased eGFR over an 8-year period in type 2 diabetes.⁶⁸ Taken together, our findings suggest that increased circulating IGFBP2 might be a predictor of longitudinal deterioration of renal function in multiple nephropathies, including LN. Whether the increased IGFBP2 in SLE patients is a function of insulin resistance or metabolic syndrome warrants further study.

Serum AXL was another molecule found in this study to be elevated in patients with active LN. Although it did coincide with renal flares in some of the serially monitored SLE patients, it exhibited only modest correlations with concurrently recorded SLEDAI, eGFR, serum creatinine, as well as renal pathology indices. This molecule is a receptor tyrosine kinase whose extracellular domain can be cleaved off to release soluble AXL.⁶⁹ AXL is preferentially expressed on monocytes, stromal cells, and a fraction of CD34-positive progenitor cells.⁷⁰ AXL, DTK, and MER constitute a receptor tyrosine kinase subfamily that binds the vitamin K-dependent protein growth-arrest-specific gene 6 (Gas6) that is structurally related to the anticoagulation factor protein S. These receptors are suggested to be involved in apoptotic cell clearance, autoimmunity, cancer, inflammatory bowel disease, and colitis-associated cancer.^{71, 72} A previous report has documented that plasma concentrations of Gas6 and soluble AXL correlate with disease activity in SLE, in resonance with our findings.⁷³

Ferritin is a fourth molecule that was noted to be significantly elevated in patients with active LN. This molecule was not pursued further in this work, as we have previously studied this molecule.⁷⁴ Just like ferritin, other iron-binding proteins such as transferrin and hepcidin have also been previously investigated as potential biomarkers for LN.^{74, 75} FAS is

another molecule that was noted to be increased in some patients with active LN. Although serum FAS did not coincide with flares in the patients we studied serially, it did show modest correlation with concurrently recorded SLEDAI, eGFR, and renal pathology activity index. Recently, plasma FAS and FASL levels have been reported to be significantly elevated in preflare SLE patients who developed disease flare 6–12 weeks after a baseline assessment.⁴⁰

The strength of this study is that it began with a relatively unbiased screen of 274 proteins, and resulted in the validation of a handful of potential biomarkers, as discussed above. Ongoing developments in the field of targeted proteomics are continuously expanding the spectrum of protein markers that can be screened, which currently have surpassed 1000. Both antibody-based and aptamer-based approaches may one day allow researchers to interrogate a large fraction of the human proteome in an unbiased way, in the same manner that the genome and transcriptome can currently be screened. This is likely to have a transformational impact on novel biomarker identification.

This study does have some limitations. Although four independent sets of SLE patients were used for successive validation of candidate biomarkers, including patients interrogated at the time of renal biopsy as well as longitudinally followed patients, the numbers of subjects studied were relatively small and could be substantially increased. Although protein markers that were significant by parametric tests or nonparametric tests were identified in the screening stage, several of these failed multiple testing correction. This could have been rectified with significantly larger sample sizes for the screening arrays, although this would have dramatically multiplied the assay costs. Larger sample sizes would also allow us to ascertain the impact of any particular medications or clinical features on specific biomarker levels. Because identical SLEDAI measures could arise from different originating symptoms, future studies would also have to focus on studying these biomarker levels in relation to specific end-organ manifestations of the disease, for example, renal lupus. It would also be important to expand the spectrum of disease controls examined, including other systemic autoimmune diseases and renal diseases, to ascertain the degree of specificity of the examined biomarkers. Indeed, we have already initiated several large-scale validation studies with selected markers in different ethnic groups.^{64–67}

For the longitudinal studies it would also be important to procure blood or urine samples a couple of weeks preceding the flare as this might enhance the chance of uncovering potential “predictive” biomarkers that could forebode impending flares rather than peak concurrently with the flare. The current set of serial samples examined in this study may not be particularly useful for identifying predictive biomarkers as they were collected ~3 months apart on average. It is intriguing that different markers track differently with flares in individual patients (Figure 4). It is not clear if the particular molecule(s) that are most instructive in a given patient is/are influenced by the genetic background of the patient, the end-organs affected, or the molecular pathways underlying pathogenesis in different subjects. These uncertainties would need larger sample sizes to tease out.

In summary, serum proteins have the capacity to identify patients with active nephritis, flares, and renal pathology activity or chronicity changes, although larger longitudinal

cohort studies are clearly warranted. Some of the most promising serum markers to emerge from this discovery study include AXL, FAS, ferritin, IGFBP2, Siglec5, and sTNFR1I. Whether composite disease indices composed of some of these serum markers coupled with traditional disease measures could perform better in monitoring disease progression and treatment response in SLE/LN remains to be seen.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Antibody-array-based screening of SLE sera for circulating protein biomarkers. Serum samples from patients with SLE ($n = 14$) and healthy controls ($n = 8$) were applied to antibody-coated slide arrays that can interrogate the levels of 274 proteins and then developed with a cocktail of secondary antibodies specific to the same 274 molecules. (A) Volcano plot of the expression profiles of the 274 proteins expressed as a fold change (in SLE sera vs healthy control sera) and statistical significance of the difference, both being expressed in log scales. (B) Among the proteins that exhibited significant increase in SLE sera, the ones that exhibited the highest fold change in the screening arrays are plotted as bar charts, where the SLE patients ($n = 14$) and healthy controls ($n = 8$) are represented as black and white bars, respectively.

Figure 2.

ELISA-based validation of antibody array findings using an independent cohort of subjects. On the basis of the array screening results, 48 proteins were next validated using ELISA assays in an independent cohort of SLE patients, including those with active LN ($n = 18$) and inactive disease ($n = 10$) as well as healthy controls ($n = 9$). (A) Plotted in the heat-map are the relative expression profiles of these molecules in healthy controls and SLE patients. For each row of signals, red represents expression above the row median while green represents expression below the row median. (B–M) Plots of the serum levels of indicated molecules in the respective study groups, in pg/mL. Each dot represents an individual subject, and the horizontal lines represent group means. These serum molecules were selected for display because these molecules represent those that were most significantly elevated in active LN versus inactive LN at 2-fold or greater differences and ROC-AUC > 0.80 (IGFBP2, $p < 0.05$; sTNFRII, $p < 0.05$; AXL, $p < 0.001$) or were most significantly elevated in all SLE patients (i.e., with or without active LN) versus healthy controls at 2-fold or greater differences and ROC-AUC > 0.85 (FAS, $p < 0.01$, GDF15, $p < 0.01$; Acrp30, $p < 0.01$; MMP10, $p < 0.01$; OPN, $p < 0.0001$; sTNFR1, $p < 0.0001$; ICAM1, $p < 0.01$; Furin, p

< 0.01; SIGLEC5, $p < 0.0001$). Other molecules that were significantly elevated in SLE/LN are listed in Table 2.

Figure 3.

Correlation of serum protein biomarker levels with clinical and pathological indices of disease in paired biopsy/serum concurrent samples. In a set of 45 LN patients, independent from the above patients, serum samples for biomarker assays were obtained at the time of renal biopsy. Plotted are the correlation patterns of serum AXL, FAS, IGFBP2, and sTNFR2 in ng/mL against these patients' SLEDAI (row 1), eGFR (row 2), renal pathology activity index (row 3), or renal pathology chronicity index captured from concurrent renal biopsies. For all correlations where significance values were <0.08 , the correlation coefficient, r , and univariate statistical significance, P , are indicated. P_m refers to the multivariate p value following multivariate analysis. Correlation patterns with other disease parameters and other concurrently assayed markers are detailed in the Results.

Figure 4.

Longitudinal changes in serum biomarker levels and disease activity in patients with SLE. P1–P7 refer to the seven patients studied in this longitudinal study. Serum biomarker levels (plotted in blue; left Y axis) and SLEDAI (plotted in red; right Y axis) were serially monitored at three consecutive hospital visits that were spaced out an average of 2.9 months apart. The biomarker data have been normalized so that the levels recorded during flare have been set to 100%, and the biomarker levels at the other time points have been expressed as a percentage of the former.

Table 1**Demographics and Clinical Characteristics of Patients^a**

	protein array	validation	renal biopsy concurrent
total no. of subjects	14	28	45
female, no. (%)	14 (100%)	25 (89.3)	41 (91.1%)
age, mean $\pm$ SE, years	35.4 $\pm$ 3.4	37.3 $\pm$ 1.8	31.4 $\pm$ 1.4
ethnicity, african american/hispanic/caucasian, no	7/7/0	17/8/2	19/21/4/1
SLEDAI, median (interquartile)	8 (0–12)	10 (3–16)	16 (9–20)
renal SLEDAI, median (interquartile)	8 (0–8)	5 (0–8)	8 (8–12)
no. of patients with renal SLEDAI = 0 (%)	5 (36)	10 (35.7)	1 (2.2)
protein/creatinine ratio, mg/mg, mean $\pm$ SE	2.1 $\pm$ 0.6	2.0 $\pm$ 0.5	4.0 $\pm$ 0.5
serum Cr, mg/dL, mean $\pm$ SE	1.1 $\pm$ 0.2	1.3 $\pm$ 0.2	1.8 $\pm$ 0.2
comorbidities, no. (%)			
hypertension	11 (78.6)	20 (71.4)	30 (66.7)
dyslipidemia	3 (21.4)	12 (42.8)	11 (24.4)
cardiovascular disease	2 (14.3)	4 (14.3)	3 (6.7)
anemia	4 (28.6)	16 (57.1)	
antiphospholipid syndrome	1 (7.1)	3 (10.7)	9 (20.0)
venous thromboembolism	1 (7.1)	3 (10.7)	
diabetes mellitus		3 (10.7)	
hypothyroidism			4 (8.9)
others	3 (21.4)	14 (50%)	3 (21.4)
current medications, no. (%)			
prednisone	10 (71.4)	17 (60.7)	33 (73.3)
mycophenolic acid	2 (14.3)	7 (25)	9 (20.0)
cyclophosphamide	4 (28.6)	1 (3.6)	4 (8.8)
azathioprine/MTX	2 (14.3)	6 (21.4)	3 (6.7)
cyclosporine/tacrolimus		2 (7.1)	
hydroxychloroquine	7 (50.0)	12 (42.9)	22 (48.9)
angiotensin blocking agents	7 (50.0)	14 (50)	13 (28.9)

^aListed are the numbers of patients and the percentages in parentheses. Renal SLEDAI refers to the summed renal components of the SLEDAI.

Table 2

Validation Assays of Serum Proteins in SLE and Their Potential to Discriminate Lupus from Controls

protein	serum marker level (pg/mL), mean (median)		fold change	AUC of ROC curve
	healthy (n = 9)	lupus (n = 28)	lupus/healthy 1	lupus/healthy
ACrp30	5.3×10^7 (4.0×10^7)	1.9×10^8 (1.8×10^8)	3.5 ^{***}	0.86 ^{**}
Activin A	9.1×10^4 (1.0×10^5)	1.5×10^5 (1.0×10^5)	1.6	0.53
Angiogenin	1.7×10^5 (1.4×10^5)	4.9×10^5 (4.0×10^5)	2.9 ^{**}	0.85 ^{**}
Angiopoietin2	8.8×10^{-3} (1.0×10^{-3})	1.7×10^0 (4.6×10^{-2})	$\gg$ ^{**}	0.72
Angiostatin	1.2×10^4 (1.0×10^4)	1.2×10^4 (1.1×10^4)	1	0.51
AXL	3.1×10^5 (3.0×10^5)	4.9×10^5 (3.5×10^5)	1.6	0.58
BLC	0.0×10^0 (0.0×10^0)	2.6×10^2 (1.0×10^1)	$\gg$ [*]	0.79 ^{**}
CD30	1.7×10^3 (1.4×10^3)	4.3×10^3 (3.9×10^3)	2.5 ^{**}	0.79 ^{**}
CTACK	4.4×10^2 (4.3×10^2)	8.1×10^2 (8.8×10^2)	1.8 [*]	0.75 [*]
CXCL10	2.6×10^5 (2.3×10^5)	3.9×10^5 (1.6×10^5)	1.5	0.53
EGFR	1.5×10^5 (1.4×10^5)	9.9×10^4 (1.0×10^5)	0.7 ^{***}	0.87 ^{**}
FAS	1.5×10^3 (1.5×10^3)	3.2×10^3 (2.8×10^3)	2.2 ^{***}	0.91 ^{***}
FcyRIIB	1.8×10^5 (1.4×10^5)	2.5×10^5 (1.5×10^5)	1.3	0.65
Ferritin	1.2×10^3 (8.3×10^2)	1.5×10^3 (5.6×10^2)	1.3	0.53
FLRG	1.1×10^4 (0.0×10^0)	1.8×10^4 (5.4×10^3)	1.6	0.59
Follistatin	3.0×10^1 (1.4×10^1)	7.9×10^1 (7.5×10^1)	2.6 ^{**}	0.82 ^{**}
Furin	1.3×10^3 (0.0×10^0)	1.1×10^4 (7.5×10^3)	8.6 ^{***}	0.88 ^{***}
GDF15	9.0×10^2 (3.9×10^2)	4.2×10^3 (4.0×10^3)	4.7 ^{**}	0.87 ^{***}
GH	1.1×10^2 (0.0×10^0)	2.2×10^2 (8.3×10^1)	2	0.68
HGF	2.4×10^2 (1.3×10^2)	6.5×10^2 (5.1×10^2)	2.8 ^{**}	0.83 ^{**}
HVEM	9.3×10^3 (8.1×10^3)	1.8×10^4 (1.6×10^4)	2 ^{**}	0.79 ^{**}
ICAM-1	1.1×10^5 (1.1×10^5)	2.3×10^5 (2.1×10^5)	2 ^{***}	0.84 ^{**}
IGFBP2	1.2×10^4 (0.0×10^0)	4.4×10^5 (3.6×10^5)	37 ^{***}	0.97 ^{***}
IGFBP6	3.0×10^5 (1.7×10^5)	5.5×10^5 (3.5×10^5)	1.8	0.68
IL-13	0.0×10^0 (0.0×10^0)	8.8×10^1 (3.0×10^1)	$\gg$ [*]	0.79 ^{**}
INF-gamma	1.5×10^3 (0.0×10^0)	1.6×10^4 (3.3×10^3)	10.9	0.71
KLK3	2.6×10^1 (3.5×10^0)	1.9×10^0 (1.3×10^0)	0.1 [*]	0.73 [*]
Leptin	4.9×10^1 (5.5×10^1)	8.0×10^1 (7.8×10^1)	1.6 [*]	0.73 [*]
LIMP	5.4×10^3 (3.2×10^3)	7.4×10^3 (4.1×10^3)	1.4	0.58
LYVE	1.2×10^5 (1.2×10^5)	1.5×10^5 (1.5×10^5)	1.3	0.69
Marapsin	9.0×10^3 (7.3×10^1)	1.7×10^4 (1.8×10^4)	1.9 ^{**}	0.80 ^{**}
MIP3-beta	2.2×10^{-2} (7.0×10^{-3})	1.1×10^1 (1.8×10^{-1})	$\gg$ ^{**}	0.81 ^{**}
MMP10	6.1×10^1 (7.3×10^1)	2.2×10^2 (1.9×10^2)	3.6 ^{***}	0.91 ^{***}

protein	serum marker level (pg/mL), mean (median)		fold change	AUC of ROC curve
	healthy (<i>n</i> = 9)	lupus (<i>n</i> = 28)	lupus/healthy 1	lupus/healthy
MMP3	2.2×10^3 (1.5×10^2)	4.2×10^4 (2.2×10^4)	19.4 ^{**}	0.86 ^{**}
Nidogen	3.3×10^3 (2.8×10^3)	3.1×10^3 (2.9×10^3)	1	0.54
OPG	2.6×10^2 (5.0×10^0)	5.9×10^2 (5.3×10^2)	2.3	0.69
OPN	2.1×10^5 (1.9×10^5)	6.6×10^5 (6.3×10^5)	3.1 ^{***}	1.00 ^{***}
RAGE	8.9×10^2 (5.0×10^2)	9.6×10^2 (6.7×10^2)	1.1	0.52
Serpin E1	3.2×10^4 (3.1×10^4)	5.2×10^4 (4.2×10^4)	1.6	0.65
SGP130	2.7×10^5 (2.6×10^5)	1.9×10^5 (1.6×10^5)	0.7 [*]	0.73 [*]
Siglec5	3.9×10^{06} (3.9×10^6)	1.0×10^7 (1.1×10^7)	2.6 ^{***}	0.96 ^{***}
SSA	2.8×10^4 (2.8×10^4)	4.9×10^4 (5.1×10^4)	1.7 ^{**}	0.74 [*]
sTNFR1	2.8×10^2 (0.0×10^0)	7.2×10^3 (6.9×10^3)	25.4 ^{***}	0.99 ^{***}
sTNFR2	1.6×10^2 (1.5×10^2)	1.2×10^3 (7.2×10^2)	7.6 ^{***}	1.00 ^{***}
TIMP2	6.2×10^4 (5.0×10^4)	6.8×10^4 (6.1×10^4)	1.1	0.59
Trem-1	1.6×10^2 (0.0×10^0)	4.7×10^2 (2.7×10^2)	2.9	0.67
VEGFC	4.7×10^3 (0.0×10^0)	5.3×10^3 (4.6×10^3)	1.1	0.54
VEGFR3	3.2×10^4 (3.0×10^4)	5.7×10^4 (5.2×10^4)	1.8 ^{**}	0.81 ^{**}

^a Results shown pertain to Student's *t* test if Normality test passed (nonitalic); otherwise, a nonparametric test was done (italics). The last column depicts the AUC values for ROC curves generated for each molecule.

* $p < 0.05$;

** $p < 0.01$;

*** $p < 0.001$.

Table 3

Validation Assays of Serum Proteins and Their Potential to Discriminate Active from Inactive Lupus

protein	serum marker level (pg/mL), mean (median)		fold change	AUC of ROC curve
	inactive lupus	active lupus	active/inactive 1	active/inactive
ACrp30	2.4×10^8 (2.3×10^8)	1.6×10^8 (1.6×10^8)	0.7 *	0.75 *
Activin A	8.7×10^4 (1.0×10^5)	1.8×10^5 (1.1×10^5)	2.1	0.58
Angiogenin	5.8×10^5 (6.3×10^5)	4.4×10^5 (2.8×10^5)	0.8	0.65
Angiopoietin2	2.8×10^{-1} (1.6×10^{-2})	2.5×10^0 (2.7×10^{-1})	9.0	0.69
Angiostatin	1.4×10^4 (1.4×10^4)	1.0×10^4 (1.0×10^4)	0.7 ***	0.83 **
AXL	2.0×10^5 (2.2×10^5)	6.6×10^5 (6.6×10^5)	3.3 **	0.87 ***
BLC	8.2×10^1 (0.0×10^0)	3.4×10^2 (6.4×10^1)	4.2	0.68
CD30	3.8×10^3 (2.9×10^3)	4.5×10^3 (4.1×10^3)	1.2	0.61
CTACK	9.1×10^2 (1.0×10^3)	7.6×10^2 (7.5×10^2)	0.8	0.61
CXCL10	7.5×10^5 (2.6×10^5)	2.3×10^5 (1.4×10^5)	0.3	0.63
EGFR	7.7×10^4 (7.4×10^4)	1.1×10^5 (1.2×10^5)	1.4 *	0.78 *
FAS	2.7×10^3 (2.5×10^3)	3.5×10^3 (3.0×10^3)	1.3	0.63
FcyRIIB	2.5×10^5 (1.5×10^5)	2.4×10^5 (1.6×10^5)	1.0	0.5
Ferritin	3.7×10^2 (3.2×10^2)	2.1×10^3 (1.1×10^3)	5.5 **	0.84 **
FLRG	2.9×10^4 (5.6×10^3)	1.3×10^4 (5.4×10^3)	0.5	0.53
Follistatin	8.3×10^1 (8.3×10^1)	7.6×10^1 (6.3×10^1)	0.9	0.59
Furin	1.2×10^4 (8.8×10^3)	1.1×10^4 (7.5×10^3)	0.9	0.57
GDF15	4.0×10^3 (4.1×10^3)	4.3×10^3 (3.9×10^3)	1.1	0.5
GH	1.0×10^2 (0.0×10^0)	2.7×10^2 (9.9×10^1)	2.6	0.7
HGF	7.2×10^2 (5.0×10^2)	6.1×10^2 (5.3×10^2)	0.9	0.52
HVEM	1.5×10^4 (1.4×10^4)	2.1×10^4 (1.7×10^4)	1.4	0.61
ICAM-1	1.8×10^5 (1.4×10^5)	2.4×10^5 (2.5×10^5)	1.3	0.69
IGFBP2	2.4×10^5 (1.9×10^5)	5.3×10^5 (4.4×10^5)	2.2	0.72
IGFBP6	4.8×10^5 (3.2×10^5)	5.8×10^5 (4.3×10^5)	1.2	0.55
IL-13	9.1×10^1 (1.2×10^2)	8.7×10^1 (5.1×10^0)	1.0	0.6
INF- γ	2.5×10^4 (6.5×10^3)	1.2×10^4 (3.0×10^3)	0.5	0.58
KLK3	2.2×10^0 (2.8×10^0)	1.7×10^0 (8.4×10^{-1})	0.8	0.64
Leptin	9.2×10^1 (8.3×10^1)	7.3×10^1 (6.5×10^1)	0.8	0.7
LIMP	6.8×10^3 (4.9×10^3)	7.8×10^3 (4.0×10^3)	1.1	0.58
LYVE	1.5×10^5 (1.5×10^5)	1.5×10^5 (1.5×10^5)	1.0	0.51
Marapsin	1.9×10^4 (2.0×10^4)	1.7×10^4 (1.6×10^4)	0.9	0.58
MIP3-beta	3.2×10^{-1} (6.4×10^{-2})	1.7×10^1 (3.2×10^{-1})	>>	0.68
MMP10	2.0×10^2 (1.6×10^2)	2.4×10^2 (2.0×10^2)	1.2	0.61
MMP3	2.1×10^4 (1.5×10^4)	5.1×10^4 (2.5×10^4)	2.4	0.64
Nidogen	2.9×10^3 (2.4×10^3)	3.2×10^3 (3.2×10^3)	1.1	0.58

protein	serum marker level (pg/mL), mean (median)		fold change	AUC of ROC curve
	inactive lupus	active lupus	active/inactive 1	active/inactive
OPG	6.2×10^2 (4.0×10^2)	5.8×10^2 (5.3×10^2)	0.9	0.5
OPN	5.8×10^5 (6.3×10^5)	6.9×10^5 (6.3×10^5)	1.2	0.63
RAGE	1.0×10^3 (6.0×10^2)	9.4×10^2 (6.7×10^2)	0.9	0.51
Serpin E1	7.8×10^4 (5.5×10^4)	4.1×10^4 (3.7×10^4)	0.5	0.71
SGP130	2.7×10^5 (1.8×10^5)	1.5×10^5 (1.5×10^5)	0.6	0.64
Siglec5	9.4×10^6 (1.0×10^7)	1.1×10^7 (1.1×10^7)	1.2	0.7
SSA	5.0×10^4 (5.6×10^4)	4.9×10^4 (4.1×10^4)	1.0	0.55
sTNFR1	7.3×10^3 (8.0×10^3)	7.2×10^3 (5.3×10^3)	1.0	0.51
sTNFR2	5.2×10^2 (3.5×10^2)	1.5×10^3 (9.2×10^2)	2.9*	0.81**
TIMP2	6.0×10^4 (6.0×10^4)	7.3×10^4 (6.1×10^4)	1.2	0.57
Trem-1	5.0×10^2 (3.4×10^2)	4.6×10^2 (9.5×10^1)	0.9	0.58
VEGFC	5.5×10^3 (3.2×10^3)	5.2×10^3 (4.6×10^3)	0.9	0.51
VEGFR3	5.1×10^4 (5.0×10^4)	6.0×10^4 (5.4×10^4)	1.2	0.63

^aResults shown pertain to Student's *t* test if the Normality test passed (nonitalic); otherwise a nonparametric test was done (italics). The last column depicts the AUC values for ROC curves generated for each molecule.

* $p < 0.05$;

** $p < 0.01$;

*** $p < 0.001$.

Demographics and Clinical Characteristics of Patients Used for the Longitudinal Analyses^a

Table 4

subject ID	race/gender	age	ANA	visit	PGA	total SLEDAI	renal SLEDAI	proteinuria	low C _r	elevated anti-dsDNA
P1	W/F	42	+	pre- flare	0.5 2	2 10	0 0	- -	+	-
P2	B/F	63	+	post- pre- flare	0.5 1 2	0 0 13	0 0 12	- - +	- - -	-
P3	B/F	44	+	post- pre- flare	2 1.5 1.5	3 10 16	0 0 0	- - -	+	-
P4	B/F	28	+	post- pre- flare	2 2.5 2.8	10 12 16	0 8 12	- - +	- + +	-
P5	W/F	69	+	post- pre- flare	2.5 0 1.5	16 0 8	12 0 0	+	-	-
P6	B/F	31	+	post- pre- flare	1 2 2	2 3 10	0 0 0	- - -	-	-
P7	W/F	48	+	post- pre- flare	2 0.5 2	10 4 24	0 0 12	- - +	- + +	-
				post-	1.5	16	12	+	-	+

^aP1–P7 refer to the seven patients studied in this longitudinal study. SELENA-SLEDAI and flares were defined as reported elsewhere.³¹ The average time interval between the visits was 2.9 months.