Glomerular Autoimmune Multicomponents of Human Lupus Nephritis In Vivo: α-Enolase and Annexin AI

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ABSTRACT
Renal targets of autoimmunity in human lupus nephritis (LN) are unknown. We sought to identify autoantibodies and glomerular target antigens in renal biopsy samples from patients with LN and determine whether the same autoantibodies can be detected in circulation. Glomeruli were microdissected from biopsy samples of 20 patients with LN and characterized by proteomic techniques. Serum samples from large cohorts of patients with systemic lupus erythematosus (SLE) with and without LN and other glomerulonephritides were tested. Glomerular IgGs recognized 11 podocyte antigens, with reactivity varying by LN pathology. Notably, IgG2 autoantibodies against α-enolase and annexin AI were detected in 11 and 10 of the biopsy samples, respectively, and predominated over other autoantibodies. Immunohistochemistry revealed colocalization of α-enolase or annexin AI with IgG2 in glomeruli. High levels of serum anti–α-enolase (>15 mg/L) IgG2 and/or anti-annexin AI (>2.7 mg/L) IgG2 were detected in most patients with LN but not patients with other glomerulonephritides, and they identified two cohorts: patients with high anti–α-enolase/low anti-annexin AI IgG2 and patients with low anti–α-enolase/high anti-annexin AI IgG2. Serum levels of both autoantibodies decreased significantly after 12 months of therapy for LN. Anti–α-enolase IgG2 recognized specific epitopes of α-enolase and did not cross-react with dsDNA. Furthermore, nephritogenic monoclonal IgG2 (clone H147) derived from lupus-prone MRL-1pr/lpr mice recognized human α-enolase, suggesting homology between animal models and human LN. These data show a multiantibody composition in LN, where IgG2 autoantibodies against α-enolase and annexin AI predominate in the glomerulus and can be detected in serum.


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Lupus nephritis (LN) is the most serious organ manifestation of systemic lupus erythematosus (SLE).\(^1\) It occurs in a relevant proportion of SLE patients and if not timely recognized and treated, may lead to renal failure and death. There is a general consensus that LN is mediated by antibody deposition in glomerular tuft,\(^2\)–\(^5\) but the mechanisms leading to the formation of immune deposits and the development of renal lesions are not completely clarified. The glomerular targets of pathogenic antibodies, in particular, are not well defined. Consequently, no surrogate biomarker is available that could allow early identification of candidate patients to LN and/or drive the therapeutic approach.

Several theories have been developed over the years.\(^6\)–\(^12\) They consider a pre-eminent role of circulating anti–double stranded DNA (dsDNA) antibodies that they can interact, for mimicry, with glomerular antigens expressed at the cell surface of podocytes and mesangial cells\(^7,13–15\) or in the glomerular basement membrane.\(^12,16–20\) Chromatin and nucleosomes deriving from ineffective fragmentation may interact with negatively charged constituents of the basement membrane\(^7,16\) and become the planted antigen for anti-dsDNA (and autoantibodies of other specificities).\(^6,7,9,16\)

Other potentially nephritogenic antibodies different from anti-dsDNA have been proposed\(^10,11,21–26\); overall, it has been estimated that anti-DNA deposition in LN accounts for not more than 10%–20% of eluted IgG overall,\(^27\) implying that IgG not recognizing DNA represents the vast majority of antibodies in glomeruli. Renal targets of autoimmunity in human LN are, however, unknown.

An \textit{in vivo} approach based on antibody microelution from glomeruli dissected from renal biopsies of LN patients and their characterization with proteomics seems potentially conclusive to elucidate main features of human LN. Studies published so far on direct analysis of antibodies eluted from glomeruli in patients with LN have been done only on autopic samples of kidney tissue and focused only on renal targets of eluted anti-dsDNA.\(^27,28\) Ultraprecise techniques of dissection, such as laser capture, allow repetitive analysis of minute amounts of renal tissue and can be used routinely \textit{in vivo},\(^29\) which has already been done in membranous nephropathy.\(^30,31\)

The aim of the present study was to evaluate the target antigens of antibodies eluted from glomeruli of LN patients to characterize their isotype and determine their serum levels in different cohorts of patients with SLE (with and without nephritis). Antigen targets in murine models of LN were characterized as well.

**RESULTS**

**Anti-Podocyte Antibodies in Glomerular Eluates and Serum**

For the analysis of glomerular eluates, we used renal biopsies obtained from 20 SLE patients with proteinuria who underwent a renal biopsy for diagnostic purposes (Table 1). Glomeruli were dissected from frozen kidneys by laser capture, and eluted Igs were analyzed by Western blot using podocyte extracts as fixed

### Table 1. Clinical data and pathology characteristics relative to 20 patients with SLE who underwent a renal biopsy at the time of enrolment and had their renal tissue processed with laser capture for antibody characterization

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age (yr)</th>
<th>Biopsy Year</th>
<th>LN Class</th>
<th>SCreat (mg/dl)</th>
<th>UProt (g/24 h)</th>
<th>C3 (mg/dl)</th>
<th>C4 (mg/dl)</th>
<th>ANA</th>
<th>Anti-DNA</th>
<th>Steroid</th>
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SCreat, serum creatinine; UProt, proteinuria; ANA, antinuclear antibody; Cycloph, cyclophosphamide; Cyclosp, cyclosporine; MMF, mycophenolate mofetil; M, man; Pos, positive; W, woman; nd, not determined.
antigens. An example showing laser capture efficiency and precision is shown in Supplemental Figure 1.

Pooled samples from different pathologic classes of LN (classes III–V) were first analyzed; a separate set of analysis of eluates from each renal biopsy was then carried out with dot blot after antigens had been characterized (see below for information on specific antibodies). As shown in Figure 1, glomerular IgGs recognized 11 proteins (Figure 1, A–D) that were characterized by matrix-assisted laser desorption ionization (MALDI)–mass spectrometry (MS) and liquid chromatography (LC)-MS. The predicted sequence for each recognized protein is reported in Table 2. The presence of specific autoantibodies in glomerular eluates versus this panel of proteins varied in different patients according to the pathology classes of LN, with only anti–α-enolase and anti-annexin AI being present in all eluates (Table 3). Five autoantibodies were detected in at least two pathology classes (anti–α-tubulin, anti–glutathione synthetase, anti–heat shock cognate 71 kDa, anti–Xaa dipeptidase, and anti–lactate dehydrogenase), and four autoantibodies were found in just one class (i.e., anti–ezrin/moesin, anti–transketolase, anti–vimentin, and heat shock protein/peroxiredoxin).

Serum IgGs from the same LN patients recognized most of the antigens above (Figure 1, E and F) with the following exceptions: anti–Xaa Pro dipeptidase (spot D), transketolase (spot F), and anti–heat shock protein peroxiredoxin 6 (spot J) were not detected in serum, whereas anticytosolic dipeptidase antibodies were found in serum but not glomeruli. All other components were confirmed in both.

Based on the presence of anti–α-enolase and anti–annexin AI antibodies in all the LN pathology classes and serum, focused studies addressed isotypes of these antibodies in glomeruli and serum, single biopsy expression, antigen–antibody colocalization, and specificity.

Renal Anti–α-Enolase/Anti–Annexin AI Antibodies: Isotype, Glomerular Expression, and Colocalization

Isotype analysis of anti–α-enolase in glomerular eluates showed IgG2 and a minimal amount of IgG1 (Figure 2A); anti–α-enolase IgG3 and IgG4 were negative. Overall, specific anti–α-enolase IgG2 was detected in 11 of 20 renal biopsy samples processed with microdissection (Figure 2A).

![Figure 1](https://www.jasn.org/)

**Figure 1.** Characterization of autoantibodies eluted from glomeruli of patients with LN and in corresponding serum. (A and E) Representative two-dimensional electrophoresis of podocyte cell extracts stained by colloidal Coomassie. The same cell extract was incubated with antibodies eluted from microdissected glomeruli obtained from patients with different classes of LN: (B) class III, (C) class IV, and (D) class V. Microeluted antibodies from normal kidneys did not react with any protein. Several spots were recognized by LN eluates and identified as 11 different proteins (A–K). One was characterized by LC-MS (spot I; α-enolase), and the remaining spots were characterized by MALDI-MS (Table 2). Their identity as predicted by MS is reported in Table 2. The same podocyte cell extract separated by two-dimensional electrophoresis was incubated with normal sera (not shown) and (F) pooled LN sera (classes III–V) and then developed with anti-IgG (total) antibodies. Several proteins were detected, and most corresponded to proteins recognized in glomerular eluates (A–C and G–I); three proteins were recognized only in serum or a few glomerular eluates (K and L). All spots were characterized by LC-MS or MALDI-MS as above (Table 2, Supplemental Table 1). Their identity is reported in Table 2.
single biopsy for antibodies versus implanted antigens anti-C1q, anti-DNA, and anti-histones H2A, H3, and H4, which are typical findings in LN. With this type of approach, the intensity relative to all autoantibodies can be evaluated simultaneously in all biopsies. The results confirmed predominance of antibodies versus endogenous glomerular antigens followed by anti-DNA and anti-C1q.

Other experiments showed colocalization of α-enolase and annexin AI with IgG2 in glomeruli of patients with LN. Figure 3 shows two examples of patients with LN classes III and V presenting with diffuse α-enolase expression and widespread IgG2 deposition. Vast areas of colocalization with IgG2 could be shown for both α-enolase and annexin AI that were observed also after DNase/RNase treatment (Supplemental Figure 2), implying that the target antigen is not a planted DNA or nucleosome structure.

Serum Anti-α-Enolase/Anti-Annexin AI: Isotype, Circulating Levels, and Correlations

The isotype of serum anti-α-enolase and anti-annexin AI was less selective than in glomeruli; even IgG2 was the major component. Minor amounts of anti-annexin AI IgG1 and IgG3 (Figures 4A and 5A) were observed.

Circulating levels of anti-α-enolase IgG2 (Figure 4, B and C) and anti-annexin AI IgG2 (Figure 5B) were determined with dot blot analysis and ELISA in several SLE and non-SLE patients; antibodies of the other isotypes were evaluated as well (Figure 4B, Supplemental Figure 4). The following patient categories were considered (Table 4, Supplemental Table 1): 104 LN patients recruited at the time of renal biopsy, 112 SLE patients without LN, 50 patients following patient categories were considered

Table 2. MALDI-MS/LC-MS spectra analyses of protein spots (A–L) from two-dimensional electrophoresis

<table>
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<tr>
<th>Spot</th>
<th>Gene Name</th>
<th>Protein Name</th>
<th>Technique</th>
<th>Score</th>
<th>Coverage</th>
</tr>
</thead>
<tbody>
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<td>Tubulin α-18 chain</td>
<td>MALDI-MS</td>
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<td>GSHB</td>
<td>Glutathione synthetase</td>
<td>MALDI-MS</td>
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<td>HSP7C</td>
<td>Heat shock cognate 71 kD protein</td>
<td>MALDI-MS</td>
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Proteins identified by two-dimensional electrophoresis from podocyte extracts were characterized by MALDI-MS in most cases (spots A–H and J–L) and LC-MS in one case (spot I). Structure prediction features (score and coverage) are reported.

Table 3. Positivity of antibodies versus different podocyte antigens in serum and glomerular eluates of patients with different classes of LN as evaluated by two-dimensional electrophoresis/Western blot

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<td>Heat shock cognate 71 kD protein</td>
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<td>Heat shock protein β-1</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

Isotype analysis of glomerular anti-annexin AI showed IgG2 as a unique category (Figure 2B). Analysis in every separate renal biopsy sample showed the presence of anti-annexin AI IgG2 in 10 of 20 samples (Figure 2B); in other renal samples, the expression was scanty. In six cases, a double positivity for anti-α-enolase and anti-annexin AI IgG2 was observed. Multiple positivity for separate renal biopsy samples was analyzed with cluster analysis (heat map), and results are shown as intensity variation from black (maximum) to white (minimum) in Figure 2C. Other than results on anti-α-enolase and anti-annexin AI antibodies, in Figure 2C, it is also reported, for comparison, positivity of patients with isolated glomerulonephritis.

Serum levels of anti-α-enolase IgG2 were higher than the 95% limit of normal levels in 82% of the LN patients (Figure 4, B and C). Anti-α-enolase IgG1 and IgG3 serum levels were undetectable in all patient categories (Figure 4B); anti-α-enolase IgG4 was negative in LN and SLE patients but high, which is known, in patients with membranous nephropathy.31 (Supplemental Figure 4B). Fewer SLE patients and no patients with rheumatoid arthritis or primary GN presented anti-α-enolase IgG2 levels exceeding normal values (Figure 4C). The median of serum levels of anti-α-enolase IgG2 was statistically higher in LN
(45.37 mg/L) versus SLE (17.44 mg/L) patients and much more versus the other patient cohorts (Table 5). The area under the receiver operating characteristic curves for anti–α-enolase IgG2 was significantly greater in LN patients compared with SLE patients, rheumatoid arthritis patients, and patients with other nephropathies (Figure 4, E–L). The results relative to anti–α-enolase IgG2 were confirmed by a self-made ELISA (Supplemental Figure 3). Some of the LN patients who had been evaluated at the beginning of disease were re-evaluated after 6 and 12 months, during which time they had received specific treatments.

Figure 2. Glomerular α-enolase and annexin Al: isotypes and levels in single biopsies. The following studies were done to better characterized (A) anti–α-enolase and (B) anti-annexin Al antibodies: study 1, definition of isotype with dot blot analysis; study 2, single biopsy analysis; and study 3, competition experiment using the same glomerular elutes as above and increasing amounts of α-enolase/annexin Al from 5 to 15 ng to saturate antibodies. Results show complete inhibition and confirm the presence of anti–α-enolase/anti-annexin Al IgG2 in glomerular eluates. (C) Hierarchical cluster analysis heat map for a single antibody in each renal biopsy; antibody intensities (black, high; gray, medium; white, low) are reported in lines and refer to single patient biopsies that are reported at the bottom of the figure. Results are given for 20 LN patients of our study cohort and four normal kidneys.
(steroids and cyclophosphamide, mycophenolate mofetil, or tacrolimus): a significant decrease of anti-α-enolase IgG2 was reached at T12 (Figure 4D).

Circulating anti-annexin A1 IgG2 levels were determined with a self-made ELISA in the same patient cohorts. Results reported in Figure 5B and Table 5 indicate median levels one order of magnitude lower than anti-α-enolase IgG2 levels. Also, in this case, LN patients had higher levels than SLE patients (median of 4.250 versus 2.249 mg/L), and the difference was much more evident in other patient cohorts (Table 5). Twelve months after the start of therapy, LN patients presented a significant decrease of anti-annexin A1 levels (2.251 mg/L).

Patients with double positivity for circulating anti-α-enolase and anti-annexin A1 IgG2 were identified with heat map analysis (Figure 5D). A clear demarcation was observed among patients with LN regarding serum levels of the two antibodies, with one population presenting high anti-α-enolase in concomitance with low anti-annexin A1 IgG2 (red indicates high levels and green indicates low levels in Figure 5D) and vice versa. Intermediate levels (Figure 5D, black) could be detected in a few patients. This result identifies two distinct patient cohorts with potentially different clinical characteristics and possibly, different mechanisms. Before definitive clinical considerations can be done, we need to have an overall picture of all antibodies present in glomeruli and serum of patients with LN, which also includes implanted antigens (G.M. Ghiggeri, unpublished data).

**Anti–α-Enolase Antibodies Antigen Binding Epitopes and Specificity**

To better characterize anti–α-enolase antibodies, we analyzed the epitopes recognized by these antibodies: α-enolase cyanogen bromide (CNBr) digests were probed with IgG2 purified from glomeruli and serum of patients with LN and SLE or IgG4 eluted from glomeruli of patients with membranous nephropathy (a category of patients that has been recently recognized as having high levels of anti–α-enolase IgG4 in glomeruli and serum (Figure 6, A–C).30,31 IgG2 and IgG4 recognized different peptides derived from CNBr fragmentation with 1.3 kD (ILPVGAAANFREAME) and 6.8 kD (DGTENKSFGANAILGVSLLAVCKAGAVEKGVPLYRHYILDAGNSVILPVPFPAFMNVINGSHAGNKLAM), respectively (arrows in Figure 6A). Epitope mapping for the IgG2 and IgG4 recognition sites in the α-enolase model is reported in Figure 6A.

A set of experiments was done to exclude interaction of anti-DNA antibodies with α-enolase. Anti-dsDNA antibodies were isolated from both serum and renal biopsies of several LN patients.32 On repeated experiments, purified anti-dsDNA recognized several histones (only H1 and H2b were negative) but did not recognize α-enolase, even when the amount of the protein in the dot blot assay was significantly increased (Figure 6, D–F). At variance, in the same dot blot essay using the same α-enolase amount, anti-α-enolase antibodies recognized the protein at low concentration (5 μg). Taken together with the results of colocalization after DNase/RNase treatment (Supplemental Figure 2), these findings exclude an interaction between anti–α-enolase/anti-annexin A1 IgG2 with DNA and an interaction of anti-DNA antibodies with α-enolase.

**Lupus-Prone Mice**

LN is reproduced in mice by infusion of monoclonal anti-DNA IgG2 produced by cells derived from lupus-prone MRL-lpr/lpr mice.13 The prototype produced by clone H147 (an IgG2 encoded by 7183/81X VH gene)33 induces the formation of glomerular or tubular basement membrane, mesangial immune deposits, and proliferative GN after passive transfer to normal mice.12 The target antigen of these antibodies was recognized as a protein of 46 kD, which is the same molecular mass of α-enolase in the work by D’Andrea and colleagues,12 but it has not been characterized until now. We attempted to fill this gap by characterizing the target
protein of the mouse IgG2 above (a gift from M.M.) and found that these antibodies at various dilutions recognize α-enolase at dot blot (Figure 7A). This finding shows important similarities in LN in humans and mice and strongly supports the implication of anti-α-enolase antibodies in murine LN.

Figure 4. Serum anti-α-enolase isotype and levels. (A) Characterization of serum anti-α-enolase isotype with dot blot analysis. (B) The same technique was used to determine serum anti-α-enolase IgG1-IgG2-IgG3-IgG4 in patients with lupus erythematosus with (LN; n=104) and without (SLE; n=112) nephritis and several other control populations, including rheumatoid arthritis (RA; n=50), membranous nephropathy (MN; n=186), IgA nephropathy (IgA; n=60), FSGS (n=32), and normal controls (n=135). (C) Levels of anti-α-enolase IgG2 were very high in most patients with LN, whereas (D) other isotypes (IgG1, IgG3, and IgG4) were undetectable. (D) Patients with LN were evaluated at T0, and after 6 and 12 months of therapy, results showed a decrease of antibody levels after therapy. Dot blot analysis using recombinant α-enolase linked to nitrocellulose as antigen (Supplemental Figure 4); results (evaluated as the signal intensity of chemiluminescence detected by VersaDoc and computed with QuanTyOne software; Bio-Rad) were transformed (milligrams per liter) using a standard curve of chemiluminescent IgG2. The horizontal line is set at the 95th percentile of levels titrated in normal controls. Receiver operating characteristic curves for anti-α-enolase IgG2 were significantly greater in LN patients compared with other patient series: (E) normal subjects, (F) SLE, (G) RA, and (H–J) other nephropathies.
Infusion of Anti-α-Enolase Antibodies in Mice

For anti-α-enolase antibodies, 22 BALB/c mice were injected intraperitoneally with hybridomas producing IgG anti-α-enolase antibodies or IgM anti-dsDNA antibodies as a negative control. Proteinuria (100–300 mg%) was detected in 25% of injected animals after 10 days from injection (Figure 7B); proteinuria in control mice was constantly less than 10 mg%. Renal histology showed diffuse glomerular proliferative lesions in 5 of 22 mice injected with anti-α-enolase monoclonals (Figure 7D). In two mice, proliferative lesions coexisted with basement membrane thickening, and in one case, proliferative lesions coexisted with FSGS. Few tubular infiltrates were observed. In mice infused with anti-α-enolase antibodies, mesangial deposits of nonmuscle myosin were seen in glomeruli and small vessels (where nonmuscle myosin is physiologic), whereas no mesangial staining could be found in mice injected with anti-DNA IgM (Figure 7E). Mice injected with monoclonal IgM anti-DNA developed only focal glomerular infiltrates.

Four of six SCID mice similarly injected with the same hybridomas developed proliferative GN with crescents and tubulointerstitial lesions (Figure 7F). Proteinuria was, in these cases, minimal, which is in agreement with the basic finding of cell proliferation within Bowman cells and interstitial infiltration.

DISCUSSION

This study is the first study attempting to define the identity of glomerular targets of autoantibodies in human LN in vivo. New technologies based on laser capture microdissection and proteomics made this evolution possible. The crucial point of the program was to use human biopsies from a relevant number of patients with LN and cross-match the presence of microeluted antibodies versus renal and implanted antigens with the homolog sera. The strength of our study is the in vivo approach based on renal biopsies and the reproducibility of results. The novelty of our findings is the characterization of an autoantibody panel in kidneys of human LN, which defines IgG2 as a major isotype. The potential benefit is linked to the definition of mechanisms and the characterization of specific surrogate biomarkers of LN.

The characterization in vivo of specific autoantibodies versus podocyte antigens in glomeruli of LN patients was a main objective. Considering concomitance of autoantibodies in glomeruli and serum as a criterion to define importance, anti-α-enolase and anti-annexin AI emerged among a list of 11 proteins and were further evaluated. Actually, antibodies versus these two proteins were detected in more than 50% of
Figure 5. Serum anti-annexin Al isotype and levels. (A) Characterization of serum anti-annexin Al isotype with dot blot analysis. (B) Serum levels of anti-annexin Al IgG2 were determined with a self-made ELISA in the same cohort of patients described in Figure 4; the technique is described in Supplemental Material. (C) Patients with LN were evaluated at T0, and after 6 and 12 months of therapy, results showed a decrease of antibody levels after therapy. (D) Double positivity for both anti-α-enolase and anti-annexin Al IgG2 in the
biopsies that were relevant, because other autoantibodies, including antibodies versus implanted antigens, were less expressed. It is also worth noting that we could exclude any interaction of α-enolase with anti-DNA antibodies purified from serum and renal biopsies. Finally, characterization of a single autoantibody in glomeruli indicated IgG2 as the unique deposited component; this crucial finding influenced the search for circulating biomarkers, posing the basis for the definition of specific antibodies. The implication of anti-α-enolase IgG2 has been, in particular, addressed on the basis of homology with experimental models of LN and will be discussed below. Anti-annexin A1 antibodies in SLE are not new,34–36 because they have been proposed as markers of discolid lupus erythematosus,34 and more generally, high serum levels have been detected in small series of patients with systemic autoimmune diseases,14 particularly anti-phospholipid syndrome.35 The demonstration of specific anti-annexin A1 IgG2 in glomeruli emerges as a major finding of this work and strengthens their implication in LN. Other than anti-α-enolase and anti-annexin A1, the panel of antibody versus podocyte antigens included proteins of the cytoskeleton, such as vimentin and ezrin/moesin, or cytosol enzymes, such as glutathione synthetase, lactate dehydrogenase, and transketolase. It is of note that other antibodies previously proposed as markers of disease, such as anti-α-actinin and antilaminin antibodies,37–39 could not be detected in glomeruli with our approach. We cannot exclude that sensitivity problems of our methods caused the failure to detect these antibodies in glomeruli of LN patients, because α-actinin and laminin are expressed by cell lysates at very low levels. However, both anti-α-actinin and antilaminin antibodies have been never be documented in the kidney of human LN and data of the literature indicate that both proteins cross-react with anti-DNA antibodies.38,40 Therefore, additional studies must address the role of anti-α-actinin and antilaminin antibodies before concluding on this aspect.

A special focus of our study was α-enolase, because its serum levels are very high (in the range of 40–50 mg/L, with peaks up to 300 mg). Indeed, anti-α-enolase IgG has been detected in clinical conditions involving the kidney other than LN, and with the methodology limitations that characterized the preproteomics era, it has been generically associated with different autoimmune and inflammatory pathologies.25,26,41–43 Previous works on anti-α-enolase did not characterize the isotype of antibodies and reported variable results, overall suggesting a limited specificity for various clinical conditions, such as SLE (data on LN not reported), mixed cryoglobulinemia, systemic sclerosis, and ANCA vasculitis.41 Recently, we eluted anti-α-enolase IgG4 from glomeruli of patients with primary membranous nephropathy31 and detected specific antibodies of the same isotype in their serum.44 It is noteworthy that IgG2 was constantly absent in both glomerular eluates and circulating enolase antibodies of patients with membranous nephropathy.31,44 In our hand, patients with rheumatoid arthritis and other GNS were frankly negative for circulating anti-α-enolase IgG2.

It is of interest that anti-α-enolase IgG2 and IgG4 (purified from patients with membranous nephropathy) recognize different fragments deriving from the α-enolase CNBr digestion, suggesting that different parts of the protein become immunogenic in different clinical conditions. This basic finding opens up consideration of different mechanisms with determinant structural basis for autoantibody formation.

Analysis of target renal antigens in spontaneous animal models of LN strengthens the results from the human study. Actually, there are many factors influencing the development (variable genetics and environment) or progression (diet, lifestyle, sensitivity to therapies, etc.) of LN in humans that are controlled in animal models, suggesting that any comparison should be considered with circumspection. However, data on α-enolase in lupus-prone mice and anti-α-enolase in BALB/c mice go in the direction of a direct mechanism linked to anti-α-enolase and anti-annexin A1 antibodies. In fact, it is shown
Table 5. Serum levels of anti-α-enolase and anti-annexin AI IgG2 in LN and SLE patients and different cohorts of patients who were enrolled in the study compared with other cohorts presenting primary GN or rheumatoid arthritis

<table>
<thead>
<tr>
<th>Pathology Group</th>
<th>Number</th>
<th>Median (mg/L)</th>
<th>Interquartile Range</th>
<th>Probability Versus LN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-α-enolase IgG2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LN T0</td>
<td>104</td>
<td>45.37</td>
<td>73.36</td>
<td>0.09</td>
</tr>
<tr>
<td>T6</td>
<td>74</td>
<td>41.95</td>
<td>52.52</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td>T12</td>
<td>52</td>
<td>22.21</td>
<td>31.45</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SLE</td>
<td>112</td>
<td>17.44</td>
<td>32.13</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>RA</td>
<td>50</td>
<td>3.67</td>
<td>8.42</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IgAN</td>
<td>60</td>
<td>1.21</td>
<td>10.71</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MN</td>
<td>186</td>
<td>6.25</td>
<td>2.16</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FSGS</td>
<td>32</td>
<td>1.51</td>
<td>3.54</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Controls</td>
<td>135</td>
<td>5.24</td>
<td>9.09 (95°=15.62)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Anti-annexin AI IgG2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LN T0</td>
<td>104</td>
<td>4.077</td>
<td>4.250</td>
<td></td>
</tr>
<tr>
<td>T6</td>
<td>74</td>
<td>4.394</td>
<td>4.448</td>
<td></td>
</tr>
<tr>
<td>T12</td>
<td>52</td>
<td>2.633</td>
<td>2.251</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SLE</td>
<td>112</td>
<td>3.422</td>
<td>2.549</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>RA</td>
<td>50</td>
<td>2.075</td>
<td>0.904</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IgA</td>
<td>60</td>
<td>1.342</td>
<td>1.270</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MN</td>
<td>184</td>
<td>1.416</td>
<td>0.944</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FSGS</td>
<td>32</td>
<td>1.105</td>
<td>0.086</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Controls</td>
<td>116</td>
<td>1.320</td>
<td>0.616 (95°=2.686)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Results are given as milligrams per liter. Non-parametric Wilcoxon test for unpaired sample was used for comparison in different patient cohorts. Results are given as medians and interquartile ranges. RA, rheumatoid arthritis; IgAN, GN with mesangial IgA deposits; MN, membranous nephropathy.

here that α-enolase is recognized by nephritogenic mAbs derived from lupus-prone MRL-lpr/lpr mice that produce renal lesions reminiscent of human LN in mice. Data in other animal models are weaker because of difficulty to develop unbiased controls (i.e., antibodies anti-DNA IgM). Overall, data obtained in BALB/c mice suggested, however, that anti-α-enolase antibodies produce diffuse renal proliferation, highly reminiscent of LN, in only a percentage of mice. Once again, the limited nephrotoxic potential of anti-α-enolase antibodies in mice is in agreement with the finding reported above in human LN showing 50% positivity of anti-α-enolase antibodies in renal biopsies.

Overall, these data implicate a multicomposition of renal autoantibodies in LN with a significant presence of anti-α-enolase and anti-annexin AI antibodies. This concept has been proposed over the years and is strengthened here by the finding of a panel of autoantibodies versus endogenous and implanted antigens, such as anti-DNA, anti-histones, and anti-C1q. It is of interest that positivity for a single antibody did not exceed 50% of biopsies (see heat map analysis) for any case, implying a potential multiorigin of renal lesions. Confirmatory data and new results should constitute a sound start to approach pathogenesis of LN. It seems, in particular, that the key aspect is related to an implication of anti-dsDNA/histone antibodies as a starter of LN, because these antibodies could not be detected in a few biopsies. Our results, excluding an interaction of α-enolase with anti-dsDNA antibodies, suggest that other mechanisms and in vivo studies in the near future may modify the prevailing view of anti-dsDNA as a main player in LN.

A final point of interest is to define whether and how the present findings would modify the clinical approach to SLE patients. Indeed, treating LN is a possible task given that this condition is rapidly recognized and possibly, anticipated. A number of tests have been already been proposed as surrogate biomarkers of disease activity in LN. The list includes anti-dsDNA, antihistones, and antibodies versus endogenous antigens, such as antibi-actinin. Actually, definition of specific renal and serum levels of the above antibodies are in progress and will be reported very soon (G.M. Ghiggeri, unpublished data). To know the renal isotypes of these implanted components, it is crucial to look at the serum levels. Until definite data are available, it is difficult to conclude about sensitivity and correlation among different components, in which anti-α-enolase and anti-annexin AI IgG2 should be included in the list of candidates.

In conclusion, we describe here the presence of several specific antibodies versus endogenous glomerular antigens in glomeruli and sera of a relevant proportion of patients with LN. Anti-α-enolase and anti-annexin AI IgG2 seem to be the prevalent autoantibody components in glomeruli in vivo. Anti-α-enolase IgG2 seems to also be implicated in experimental LN. A multiantibody panel should be developed as a surrogate biomarker of LN.

CONCISE METHODS

Patients
Overall, 216 SLE patients were included in the study (Table 2), and their sera were used for studies on circulating autoantibodies; 103 patients with LN were recruited when they underwent a renal biopsy for diagnostic purposes. Sera were obtained from all patients at the time of diagnosis and after months of therapy (T6 and T12) from a significant portion of patients (63 and 68 patients, respectively, after 6 and 12 months). All patients had a diagnosis of LN based on typical renal lesions. Fresh frozen renal samples were available for 20 patients and used for the proteomic approach (Table 1). For histologic evaluation of kidney disease, Dubosq-Bresil solution-fixed tissues were embedded in paraffin, sectioned, and stained with hematoxylin/eosin, Masson’s trichrome, silver methenamine, and periodic-acid Schiff. Routine immunofluorescence studies on frozen sections were performed using anti-human IgG, IgA, IgM, C1q, C3, and fibrinogen antibody.
Normal Kidneys

Noncarcinomatous portions of kidneys removed for renal cancer were obtained from four patients and processed as negative tissue controls with laser capture.

Other Diseases

Serum was obtained from 50 patients with rheumatoid arthritis and 278 patients with isolated nephritis (186 patients with membranous nephropathy, 32 patients with FSGS, and 60 patients with IgA nephritis). One hundred thirty-five sera from normal people were also obtained and analyzed.

Ethical Committee

Permission for the study was given on June 10, 2010, by the Ethical Committee of San Carlo Borromeo Hospital in Milan, Italy. Informed consent to the study was obtained from all participants.

Antibodies

Antibody sources are reported as Supplemental Material.

Laser Capture Microdissection and Elution of Antibodies from Renal Biopsy Tissue

Laser capture microdissection and elution of antibodies were done as already described29,31; details are given in Supplemental Material.

Two-Dimensional Electrophoresis

Two-dimensional electrophoresis of podocyte extracts (a gift from M.A. Saleem) was performed in soft gels as described.55 A detailed description is given in Supplemental Material.

Monodimensional Electrophoresis

Gradient PAGE was done according to the work by Laemmli.56

Gel/Membrane Staining and Image Analysis

After separation in SDS-PAGE gels, proteins were visualized by a double staining procedure. The methyltrichloroacetate negative staining was followed by the blue silver colloidal Coomassie57 staining for preparative MS analysis. Images of stained gels were digitized using a GS800 photometer, and Western blots were acquired using a Versa DOC 400. All images were analyzed with PD Quest software (Bio-Rad, Hercules, CA).

Western Blot

Western blot with glomerular eluates and sera was done with podocyte cell line whole extracts separated by either mono- or two-dimensional electrophoresis. Equipment and technique of analysis are described in Supplemental Material.

Tryptic Digestion and Protein Identification by MALDI-MS and LC-MS

For the identification of proteins from two-dimensional electrophoresis, we used MALDI-MS and LC-MS as described in Supplemental Material. For MALDI-MS, we used a Voyager-DE PRO mass spectrometer (Applied Biosystems, Framingham, MA). For LC-MS, we used an Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA) coupled to an HPLC Surveyor (Thermo Fisher Scientific) and equipped with a Jupiter C18 column of 250×1 mm (Phenomenex). Details of the analysis procedure are given in Supplemental Material.
Classic Immunofluorescence and Colocalization
Renal biopsy specimens were embedded in optimal cutting temperature (Tissue Tek; Miles Inc., Elkhart, IN) and stored in liquid nitrogen. Three-micrometer sections were cut by a cryostat (Leica CM1850; Leica Microsystems) and placed on poly-L-lysine–coated glass slides for indirect immunostaining. Details of staining are given in Supplemental Material.

Characterization of Autoantibody Isotype and Levels in Single Glomerular Eluates and Serum
Autoantibody isotype characterization and single biopsy levels were evaluated with dot blot using a Bio-Dot apparatus (Bio-Rad) as described in Supplemental Material. The same technique was used for the determination of anti-α-enolase antibodies, mesangial deposits of αSMA were seen in glomeruli and small vessels (where αSMA is physiologic), whereas no mesangial staining could be seen in mice injected with anti-DNA IgM (×200). (F) Four of six SCID mice similarly injected with the same hybridomas developed proliferative GN with crescents and tubulointerstitial lesions.

Anti-α-Enolase ELISA
A method for determining serum α-enolase levels in serum was developed. Main characteristics and steps of the procedure are illustrated in Supplemental Material.

Anti-Annexin A1 ELISA
For anti-annexin A1, we used a self-made ELISA (Supplemental Material).

CNBr Digestion of α-Enolase and Analysis of Fragmentation Binding
For digestion with CNBr, α-enolase (10 μg) methods are illustrated in Supplemental Material.

Isolation of Serum Anti-DNA Antibodies
Isolation of anti-dsDNA antibodies from sera of patients with SLE was done according to the work by Chan and colleagues. Details are given in Supplemental Material.

Nepritogenic Monoclonal Anti-DNA IgG2 Clone (H147) Derived from Lupus-Prone MRL-lpr/lpr Mice
Nepritogenic monoclonal anti-DNA IgG2 clone (H147) derived from lupus-prone MRL-lpr/lpr mice was furnished by M.M. Techniques for developing hybridomas have been reported in detail in previous works. mAbs were purified from hybridoma supernatants by affinity chromatography.

Anti-α-Enolase IgG in BALB/c Mice
Twenty-two BALB/c and six SCID mice were injected intraperitoneally with 1 × 10^6 hybridoma cells producing anti-α-enolase mAbs; proteinuria and renal pathology were tested at weekly intervals (Supplemental Material).

Statistical Analyses
Serum levels of antibodies were expressed as median and interquartile range. A nonparametric Wilcoxon test for unpaired samples was used for comparison of anti-α-enolase IgG2 or anti-annexin A1 IgG2 serum titer in different patient cohorts, whereas a nonparametric Wilcoxon test for paired samples was used for comparison of anti-α-enolase IgG2 or anti-annexin A1 IgG2 LN serum titer at different times. Statistical analysis was performed using the R software.
Differences were considered statistically significant at two-tailed $P$ values $<0.05$.

**ACKNOWLEDGMENTS**

This study was supported by funds from Cinque per mille of Personal Income Tax (IRPEF)-Finanziamento della ricerca sanitaria and the Italian Ministry of Health Ricerca Corrente contributo per la ricerca intramuraria. The Institute Giannina Gaslini provided financial and logistic support to the trial. This work was also supported by the Renal Child Foundation and Fondazione La Nuova Speranza (Progetto integrato per la definizione dei meccanismi implicati nella glomerulosclerose focale).

The present study was investigator-initiated and -driven. All members of the study steering committee are listed as authors of the present report, had access to the study data, and vouch for the accuracy and completeness of the data reported. A patent on the potential use of

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**Figure 7.** Continued.
anti-α-enolase IgG2 antibodies as surrogate biomarkers of lupus nephritis is pending.

DISCLOSURES
None.

REFERENCES

This article contains supplemental material online at http://jasn.asnjournals.orglookup/suppl/doi:10.1681/ASN.2013090987/-/DCSupplemental.
SUPPLEMENT METHODS

Cell culture. Human conditionally immortalized podocyte cell lines were a gentle gift from Dr Saleem (University of Bristol, UK). They cultured in RPMI 1640 supplemented with 10% inactivated fetal calf serum (FCS), insulin transferrin selenium, 100 U/ml penicillin, and 100 mg/ml streptomycin. Cells were expanded at 33°C. For immunofluorescence, cells were plated in 6 cm Petri dishes at a density of 3.5x10^3 cells/cm² and differentiated for 15 days at 37°C in 5% CO2 - 95% air. Human leukemic monocyte lymphoma cell lines (U937) engineered for expressing high levels of membrane αenolase were utilized as positive control.


Secondary antibodies - Affinity-purified fluorescein isothiocyanate (FITC) F(ab’)2 donkey anti-rabbit IgG (affinity-purified Texas Red conjugated donkey anti-mouse IgG) were all purchased from Jackson Immunoresearch (West Grove, PA, USA).

Recombinant proteins. αenolase: Recombinant, Abnova Corporation (Taipei, Taiwan); AnnexinA1: Recombinant, Creative BioMart, (Shirley, NY, USA); Histones: Recombinant, New
England BioLabs inc. (Whitby, Canada); \textit{C1q}: purified protein, Calbiochem-Merck KG, (Darmstad, Deutschland); \textit{DNA}: plasmide purified, Invitrogen, (Carlsbad, CA, USA).

\subsection*{Laser Capture Microdissection (LCM) and Elution of antibodies from renal biopsy tissue.}

Laser Capture microdissection and elution of antibodies were done as already described \textsuperscript{2,3}. Cryostatic sections (5 µm) of kidney tissue specimens were placed on metal frame slides with thermoplastic membrane (Molecular Machines & Industries AG; Glattburg, Zurich, Switzerland), stained, and dehydrated using an Arcturus HistoGene, LCM Frozen Section Staining Kit (Arcturus Bioscience, Mountain View, CA) according to the manufacturer's instructions. Air-dried sections were then viewed with the NIKON ECLIPSE-TE 2000 inverted microscope (Nikon-Instruments, Sesto Fiorentino, Italy). Glomeruli were identified and isolated with the Molecular Machines & Industries Cellcut LMD system by focal melting of the membrane through laser activation. The Molecular Machines & Industries Cellcut Laser Capture Microdissection system is equipped with a solid-state ultraviolet laser that guarantees precise cutting without damaging the tissue. High precision xy-stage and CCD camera allow identification, documentation, and dissection of multiple regions of interest from the same tissue specimen. For each specimen, a total of 25 to 30 glomeruli were microdissected and removed sequentially in separate isolation cap (Nikon Instruments) with special adhesive material in the lid.

After visual control of the completeness of dissection, captured tissue was immersed in denaturation buffer and used for proteomic analysis. Sections of human kidney derived from non-carcinomatous portions of kidneys removed for renal carcinoma were used as negative control.

IgGs were recovered from glomeruli by means of acid elution as described previously. Briefly, after washings with PBS (0.01 M, pH 7.2) groups of 20 glomeruli were incubated with 0.15 M
NaCl and 0.1 M glycine buffer (pH 2.5) at 4°C. After 30 minute 10 µl of 1.875 M Tris-HCl buffer was added to achieve a pH of 7.2.

**Two-dimensional electrophoresis** Two dimensional electrophoresis was performed in soft gels as described 4. Sample delipidation was achieved using a solution consisting of tri-n-butyl-phosphate: acetone: methanol (1:12:1), cooled in ice. Fourteen milliliters of this mixture were added to each sample to reach a final acetone concentration of 80% (v/v) and it was incubated at 4°C for 90 min. The precipitate was pelleted by centrifugation at 2800 g for 20 min at 4°C. After washing with the same de-lipidizing solution, it was centrifuged again and then air-dried. Finally, samples were dissolved in the focusing solution, i.e. 7 M urea, 2 M thiourea, 4% (w/v) 3-[3-(cholamidopropyl)-dimethylammonium]-1-propanesulphonate (CHAPS), 5 mM tributyl-phosphine (TBP), 20 mM iodoacetamide (IAA), 40 mM Tris, 0.1 mM ethylene-diamine tetra-acetic acid (EDTA) pH 8.5 and a 1% (v/v) carrier ampholyte cocktail, containing 60% of the pH 3.5-10 and 40% of the pH 4-8 intervals. Prior to isoelectric-focusing (IEF), samples were incubated in this solution for 3h, to allow proper reduction and alkylation. To prevent overalkylation during the IEF step, excess IAA was destroyed by adding a molar amount of dithiothreitol (DTT). The first dimension strips used for 2D maps were 18-cm long, soft home made immobilized-pH-gradient (IPG) gels. In the second dimension, proteins were separated based on their size in 8-16%T gradient polyacrylamide gel slabs having the following dimensions: 180 x 160 x 1.5 mm.

**Western blot.** Western blot with glomerular eluates and sera was done with podocyte cell line whole extracts separated by either mono- or bi-dimensional electrophoresis. After separation, protein extracts were trans-blotted to nitrocellulose membranes Protean BA (Schleicher & Schuell, Dassel Germany) with a Novablot semidry system utilizing a
continuous buffer system with 2-amino 2-iodoxymethyl 1,3-propanediol tris 38 mM, glycine 39 mM, sodium dodecyl sulphate (SDS) 0.035% w/v, and methanol 20% v/v. The transfer was performed at 1.55 mA/cm² for 1.5 h. Two-hundreds µl of serum (diluted in 20 ml TBS) were incubated overnight at room temperature with membranes, rinsed with TBS-T 0.05% v/v and incubated with HRP conjugated anti-human IgG (Invitrogen Corporation, Camarillo, CA - 2h, 1:5000) for immunodetection.

**MALDI-MS.** Spots excised from two-dimensional gel electrophoresis were rinsed with 50% (v/v) acetonitrile (ACN) in 5 mM ammonium bicarbonate pH 8.9 until full decolouration, rinsed twice in 100% (v/v) ACN, and briefly rinsed in 1 mM CaCl₂ and 100 mM ammonium bicarbonate pH 8.9. Enzymatic digestions were performed using trypsin in 100 mM ammonium bicarbonate buffer pH 7.8 overnight at 37°C. After incubation, the reaction was quenched by the addition of formic acid to pH 2. Digest samples were removed and subjected to a desalting/concentration step on μZipTipC18 column (Millipore, Bedford, MA, U.S.A.) using acetonitrile as eluent before MALDI (matrix-assisted laser-desorption ionization) - MS analysis. Peptide mixtures were loaded on the MALDI target, using the dried-droplet technique and α-cyano-4-hydroxycinnamic acid as matrix, and analysed using a Voyager-DE PRO mass spectrometer (Applied Biosystems, Framingham, MA, U.S.A.). Internal mass calibration was performed with peptides derived from enzyme autoproteolysis. PROWL software package was used to identify spots unambiguously from National Center for Biotechnology Information and SwissProt non-redundant sequence databases.

**LC-MS.** LC-ESI MS-MS/MS was utilized for characterizing enolase isoforms deriving from two-dimensional electrophoresis. Protein spots were treated as above. Enzymatic digestions were performed using trypsin in 100mM ammonium bicarbonate buffer pH 7.8 overnight at 37 °C.
After incubation, the reaction was quenched by the addition of formic acid to pH 2. Mass spectrometric measurements were performed using a Orbitrap mass spectrometer (Thermo Electron, San Jose, USA) coupled to a HPLC Surveyor (Thermo Electron) and equipped with a Jupiter C18 column 250mm × 1mm (Phenomenex). Peptides were eluted from the column using an acetonitrile gradient, 5% B for 6’ followed by 5 to 90% B within 109’ (eluent A: 0.1% formic acid in water; eluent B: 0.1% formic acid in acetonitrile) at flow-rate of 50µl/min. The column effluent was directed into the electro spray source. The spray voltage was 5.0kV. The ion trap capillary was kept at 200 °C and the voltage at 2.85V. Spectra were obtained in automated MS/MS mode: each full MS scan (m/z 400–1800) was followed by five MS/MS of the most abundant ions. The ions analyzed this way, were automatically excluded for 30”.

The raw data acquired by the mass spectrometer were converted in a peaklist file for database search using Extract_msn in Bioworks 3.3.1.

Protein identification was performed using SEQUEST software 3.3.1 from Thermo Electron, operating on a 10 processor computer cluster (AETHIA, (Torino, Italia) Turin, Italy). Peptide MS/MS assignments were filtered following very high stringent criteria: Xcorr ≥ 1.9 for the singly charged ions, Xcorr ≥ 2.2 for doubly charged ions, and Xcorr ≥ 3.7 for triply charged ions, peptide probability ≤ 0.01, Delta Cn ≥ 0.1 and Rsp ≤ 4 according to the HUPO criteria. The mass tolerance for precursor ions was set to 2 amu and the mass tolerance for fragment ions was 1 amu. To identify the largest panel of peptides, the option no enzyme was used for the in silica digestion of human databases, so every combination of human peptides was evaluated. Moreover the search for parameters did not included variable or fix modifications.

*Immunofluorescence staining for αenolase and annexin AI.* Renal biopsy specimens were embedded in OCT (Tissue Tek, Miles Inc., Elkhart, IN, USA) and stored in liquid nitrogen. Samples were cut to 3 µm sections by a cryostat and placed on poly-L-lysine coated glass
slides for indirect immunostaining. Cryosections were fixed in modified Carnoy solution for 10’ at 4°C and subsequently washed in phosphate buffer solution (PBS-pH 7.2). Non specific binding was blocked by incubation in bovine serum albumin (BSA) 3% w/v in PBS for 20’ at RT. Sections were then incubated for 2h at RT with purified polyclonal rabbit anti-αenolase (AbD Serotec MorphoSys, UK) diluted 1:100 in PBS and with monoclonal (clone 251/3 and 276/3) antibodies diluted 1:25 in PBS. Affinity-purified fluorescein isothiocyanate conjugate (FITC) affinity-purified donkey anti-mouse IgG (Jackson Immunoresearch, PA) was used as secondary antibody diluted 1:20 in PBS.

Negative controls were processed in parallel using PBS or an equivalent concentration of non immune rabbit or mouse serum as primary antibody.

**Co-localization of αenolase and annexin A1 with IgG2 and confocal microscopy analysis.**

Renal biopsy specimens were embedded in OCT (Tissue Tek, Miles Inc., Elkhart, IN, USA) and stored in liquid nitrogen. Cryosections (3 µM) were fixed in modified Carnoy solution for 10’ at 4°C and washed in PBS pH7.2. Non specific binding was blocked by incubation in bovine serum albumin (BSA) 3% w/v in PBS for 30’ at RT. Sections were then incubated in succession with purified polyclonal rabbit anti-αenolase antibody (AbD Serotec MorphoSys Ltd, Endeavour House, Kidlington Oxford, UK) diluted 1:100 with PBS for 2h at RT. Following additional PBS washes, slides were exposed to Texas Red-conjugated donkey anti-rabbit IgG F (ab’)2 diluted (1:20) for 1h at RT. Subsequently, monoclonal mouse antibody anti-human IgG2 (Invitrogen, CA) diluted 1:10 in PBS was applied for 1h at RT. IgG2 deposits were characterized utilizing FITC-conjugated purified donkey anti-mouse IgG (Jackson Immunoresearch, PA) diluted 1:100 for 1h at RT.

Sample were observed using a confocal system (LSM 510 Meta scan head integrated with the Axiovert 200 M inverted microscope Carl Zeiss, Jena Germany) with a 43x/1.30 oil objective.
Image acquisition was carried out in multitrack mode, namely through consecutive and independent optical pathways.

**Characterization of auto-antibody isotype and levels in single biopsy eluate.** Auto-antibody isotypes for both endogenous and implanted antigens and single biopsy levels were evaluated with dot-blot utilizing a Bio-Dot apparatus (Bio-Rad, Hercules, CA, USA): the nitrocellulose membrane was pre-wetted in TBS and mounted on the apparatus. Constant amounts of recombinant protein (100 ng) in TBS were placed on the nitrocellulose membrane for 4 hours at room temperature and then at 4°C overnight; a vacuum was then applied for a few minutes. The nitrocellulose was then gently removed and saturated with 5% albumin in TBS. Sera diluted 1:50 in TBS-T (TBS-T 0.05% v/v, 1% w/v albumin) were placed on the membrane then left for six hours at room temperature and then at 4°C overnight; at the end the membrane was washed three times in TBS-T. Incubation with HPR–anti human IgGs (IgG1, IgG2, IgG3, IgG4 diluted in 1% w/v albumin in TBS-T) was performed for 4 hours at room temperature. The membrane was then washed three times, 15 min each, with TBS-T prior to developing the immune reaction with SuperSignal West Pico Chemiluminescent substrate (Thermo scientific, Rockford, USA).

**Anti-αenolase IgG2 dot-blot analysis.** For the determination of anti-αenolase IgG2 autoantibody levels in serum, we utilized the same dot-blot method as above and utilizing anti-human IgG2 labeled with HPR (Clone: HP6014-InVitrogen Corporation, Camarillo, CA). A standard curve with the same IgG2 at different dilution is prepared to test the linearity limits of the assay.
**Anti- αenolase IgG2 ELISA.** 100ng of recombinant αenolase was put in MaxiPrep plate 96 wells, in PBS buffer and incubate at room temperature for 5 hours and then at 4°C overnight. Aliquots (200μl) of blocking solution (PBS, 5% w/v BSA and 0.05% v/v Tween20) were put in each well. Serum samples (100μl) diluted 1:50 in PBST (PBS – Tween20 0.05% v/v – BSA 1% w/v) was added and incubated for 4 hours at room temperature and then at 4°C overnight. After three washes in PBST, HRP-conjugated rabbit anti human IgG2 (Clone: HP6014-InVitrogen Corporation, Camarillo, CA) diluted 1:3,000 in PBST and 1% w/v BSA were incubated at room temperature for 4 hours and after 3 washes in PBST, 100 μl of substrate TMB/H$_2$O$_2$ (10:1) was added and incubated up to 30 minutes. The reaction was stopped by adding 100 μl of 0.45 M of H$_2$SO$_4$ at any wells before reading absorbance at 450 nm. A standard curve was prepared utilizing HRP-IgG2 at different dilutions

**Anti- Annexin AI ELISA.** The same procedure as above was utilized for determining serum levels of anti-annexin AI levels with the unique difference that less amount of recombinant annexin AI was put in the Maxiprep wells (5 ng).

**CNBr digestion of αenolase and analysis of fragmentation binding.** For digestion with cyanogen bromide (CNBr), αenolase (10 μg) in 0.4 M ammonium bicarbonate was incubated with 1% v/v 2-mercaptoethanol at room temperature for 1 h in a dark box. Then, the sample was dried in speed vacuum and resuspended in 5 μL of deionized water, 15 μL of trifluoroacetic acid (TFA) and 5 μL of 5 M CNBr in acetonitrile (ACN). The tube was wrapped in aluminum foil and left overnight at 4 °C. The reaction was terminated by drying down under vacuum. Finally the sample was resuspended in Tris-HCl 62,5 mM pH 6.8, 2% w/v SDS and 10% glycerol. Electrophoresis for the analysis of fragmentation products was carried out with gradient polyacrylamide gel electrophoresis was done according to Judd RC $^6$. 
Isolation of serum anti-DNA antibodies. Isolation of anti-dsDNA antibodies from sera of patients with SLE was done according to Chan and col 7. This method utilizes affinity chromatography on native-DNA-cellulose columns (Amersham Pharmacia Biotech.) equilibrated with 25 mM tris-buffer pH 8.0. Proteins without affinity for DNA were discarded with the same buffer whereas anti-DNA antibodies were eluted with a linear gradient NaCl gradient7.

Nepritogenic monoclonal anti-DNA IgG2 clone (H147) derived from lupus prone MRL-lpr/lpr mice. Nepritogenic monoclonal anti-DNA IgG2 clone (H147) derived from lupus prone MRL-lpr/lpr mice was furnished by Dr Madaio (see authorship). Techniques for developing hybridomas have been reported in details in previous papers 8,9; mAb were purified from hybridoma supernatants by affinity chromatography10.

Anti-αenolase IgG in BALB/c. Twenty-two BALB/c and six SCID mice were injected intraperitoneum with 1x10^6 hybridoma cells producing monoclonal anti-αenolase antibodies; hybridoma cells producing IgM anti-DNA antibodies were injected in 3 BALB/c and 2 SCID mice as control. Ten days after intraperitoneal treatment, all the mice injected with the hybridoma producing anti-αenolase antibodies developed ascites and anti-αenolase activity was detectable in sera. Proteinuria was detected by urinary dipstick. Mice were sacrificed after 3-4 weeks and kidneys were processed for light microscopy. Histology was evaluated by one of the authors (M. Madaio). Experiments in mice followed NIH Guide for the Care and Use of Laboratory Animals.
REFERENCES


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**Supplement Table 1. Clinical characteristics of subjects enrolled in the study.** Clinical data were registered simultaneously with serum samples collection. Proteinuria in normal controls was tested by urine dipstick. Anti-DNA antibodies were detected by different assays; in order to unify and analyze data, the “anti-DNA ratio” was conceived (see Methods section). Briefly, an “anti-DNA ratio” of 1 indicates the lowest positive value of each method. Values smaller than 1 indicate a negative test. Anti-DNA in diseases different than SLE were always negative. All data are presented as median and range because of their non-normal distribution. Only age is presented as mean and SD.
Supplement Fig 1. Laser capture. Micro-dissection of a renal sample of biopsy from a patient with LN. Laser capture resulted in high precision separation of a renal glomeruli from the surrounding tissue: a) renal frustule prior laser capture; b) resultant of micro-dissection.
Supplement Fig 2. Co-localization of annexin A1 (red) and IgG2 (green) in renal biopsies after (a-c) DNase/RNase treatment. Double IF staining was evaluated for αenolase (in red) and IgG2 (in green). Merged images (yellow) Scale bar: 20 μm. Original magnification, x630.
Supplement Figure 3. Correlation between serum levels of αenolase evaluated with Dot-blot and ELISA (b). Both methods are described in dedicated sections of Supplements.

Heat map of αenolase IgG2 levels comparing dot-blot and ELISA according to the data above. (a).

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**Supplement Figure 3.** Correlation between serum levels of αenolase evaluated with Dot-blot and ELISA (b). Both methods are described in dedicated sections of Supplements.

Heat map of αenolase IgG2 levels comparing dot-blot and ELISA according to the data above. (a).
Supplement Fig 4.

(a) Examples of dot blot analysis for serum anti-α-enolase IgG2 in all classes of patients recruited in this study. (b) Examples of dot blot for serum anti-α-enolase IgG1, IgG3 and IgG4 in different classes of patients recruited in this study. For details on the methodology see supplement Methods.
Supplement Fig 4.

(a) Examples of dot blot analysis for serum anti-α-enolase IgG2 in all classes of patients recruited in this study. (b) Examples of dot blot for serum anti-α-enolase IgG1, IgG3 and IgG4 in different classes of patients recruited in this study. For details on the methodology see supplement Methods.