GLOMERULAR AUTOIMMUNE MULTI-COMPONENTS IN HUMAN LUPUS NEPHRITIS (1): ALPHA-ENOLASE AND ANNEXIN AI

***by Bruschi* et *al*.**

SUPPLEMENT METHODS

Cell culture. Human conditionally immortalized podocyte cell lines [1] 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/cm^2 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.

Antibodies. *Alpha enolase-1:* Rabbit anti Human Non-Neuronal Enolase (NNE) (alpha-alpha), AbD Serotec MorphoSys Ltd. (Endeavour House, Kidlington Oxford , UK). *Anti-AnnexinA1:* Rabbit anti-human, Millipore Corp. (Billerica, MA, USA.). *Anti-Histones2A,3,4.*: Rabbit anti-human , Novus (Biologicals , Cambrige, UK). *Anti-C1q.* mouse anti-human (Abcam , Canbrige, UK. *Anti-IgG1-IgG2-IgG3-IgG4 :* Purified mouse monoclonal antibodies to human IgG1-4 (Clones: HP6070, HP6014, HP6047 and HP6023 respectively for IgG1, IgG2, IgG3 and IgG4) were purchased from InVitrogen Corporation, (Camarillo, CA).

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. *œnolase*: Recombinant, Abnova Corporation (Taipei, Taiwan); *AnnexinA1*: Recombinant, Creative BioMart, (Shirley, NY, USA)*;* *Histones*: Recombinant, New

England BioLabs inc. (Whitby, Canada); *C1q*: purified protein, Calbiochem-Merck KG, (Darmstad, Deutschland); *DNA*: plasmide purified, Invitrogen, (Carlsbad, CA, USA).

Laser Capture Microdissection (LCM) and Elution of antibodies from renal biopsy tissue.

Laser Capture microdissection and elution of antibodies were done as already described [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-idroxymethyl 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/cm^2 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 $CaCl_2$ 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 [5]. 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 AI 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) diluited 1:100 with PBS foe 2h at RT. Following additional PBS washes, slides were exposed to Texas Red-conjugated donkey anti-rabbit IgG F (ab')2 diluited (1:20) for 1h at RT.

Subsequently, monoclonal mouse antibody anti-human IgG2 (Invitrogen, CA) diluited 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) diluited 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 4C° 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 4C° 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_2O_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_2SO_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 gradient [7].

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 chromatography [10]

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 inta peritoneal 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.

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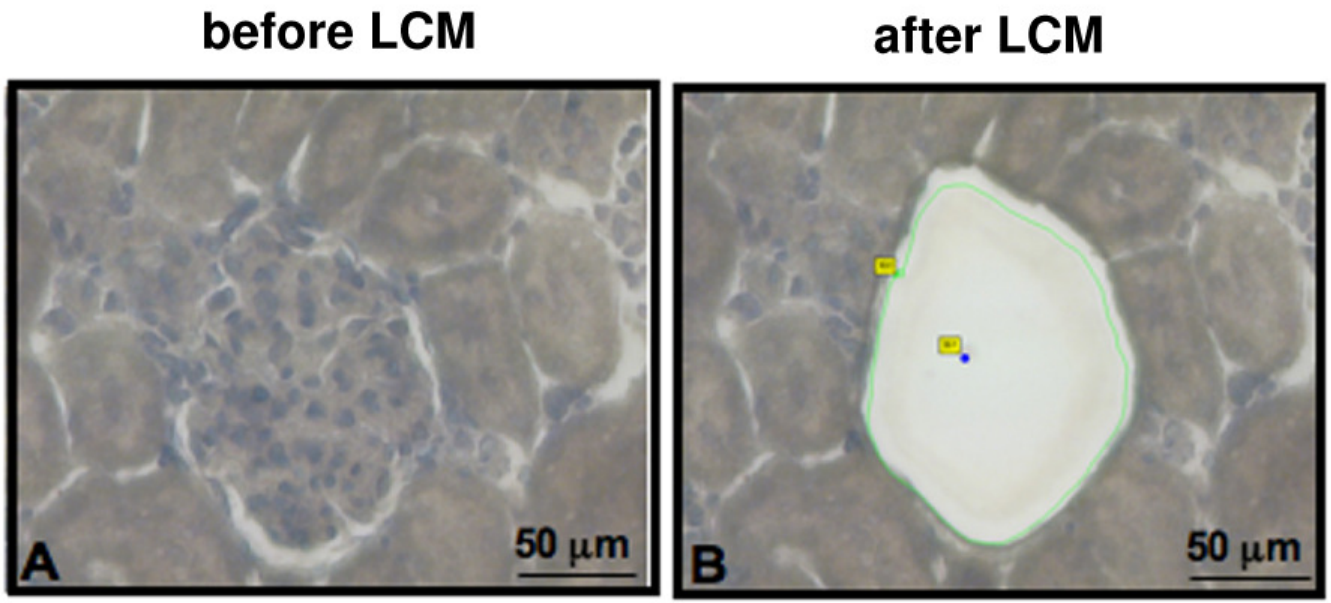
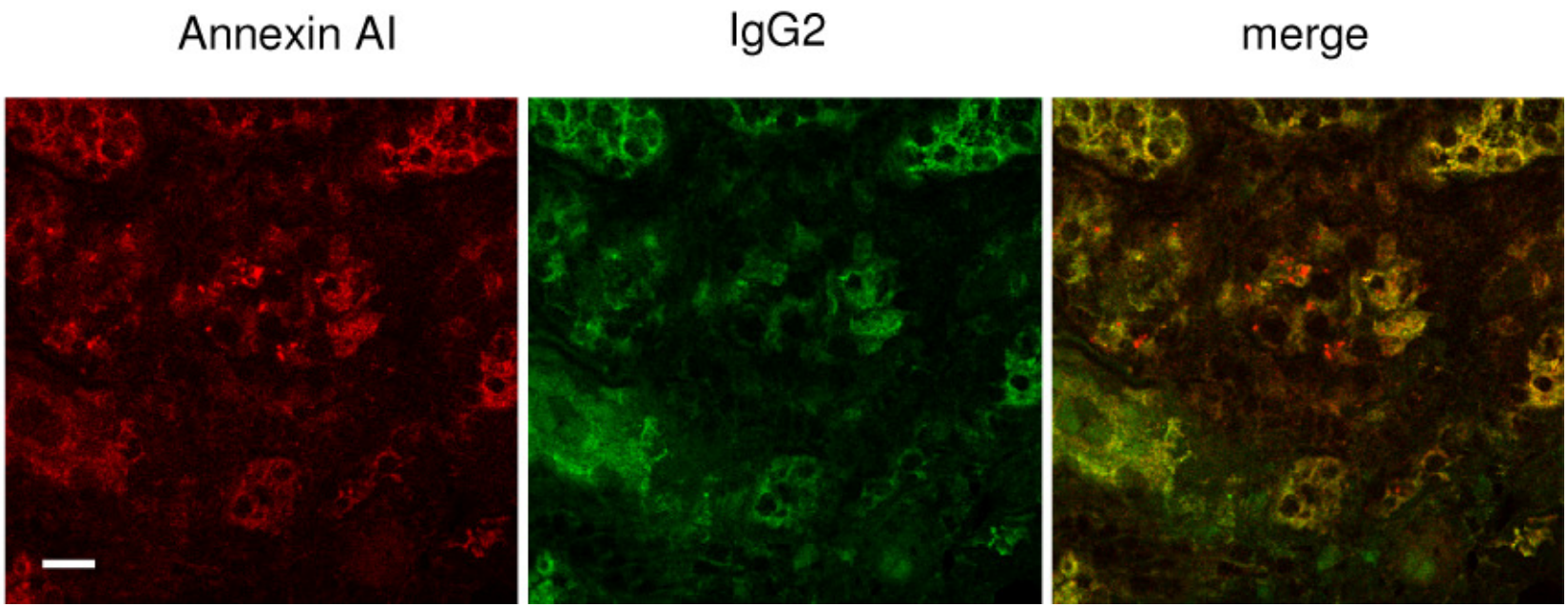
	RA (n = 50)	MN (n = 186)	FSGS (n = 32)	IgAN (n = 60)	Normal (n = 96)
Male sex	9 (18%)	121 (65%)	19 (59%)	38 (63%)	56 (58%)
Age (years)	58±22	59±16	18±3	40±4	49±10
Serum creatinine (mg/dl)	0.8 (0.6 – 1.1)	1.1 (0.3 – 6)	0.6 (0.3 – 1)	0.9 (0.5 – 1.3)	0.9 (0.6 – 1.2)
Proteinuria (g/day)	0.1 (0 – 0.3)	5.8 (0.3 – 28)	6.0 (3.6 – 12.4)	1.55 (0.9 – 3.4)	0
Anti-DNA ratio	0	0	0	0	-

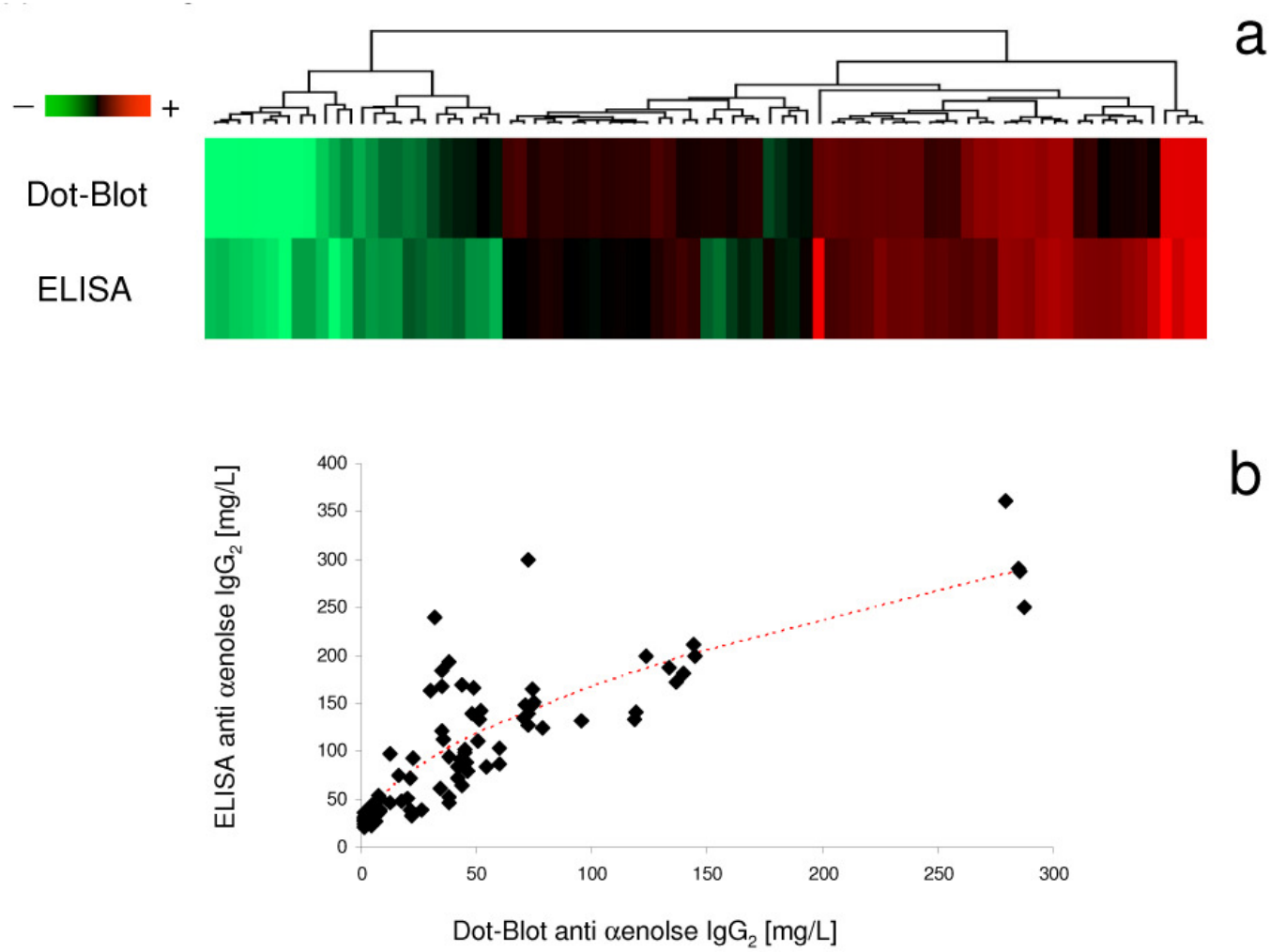
Supplement Table 1. Clinical characteristics of subjects enrolled in the study. Clinical data were registered simultaneously with serum samples collection. Proteinuria in normal controls was tested by urine dipstick. Anti-DNA antibodies were detected by different assays; in order to unify and analyze 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) resultants of micro-dissection.

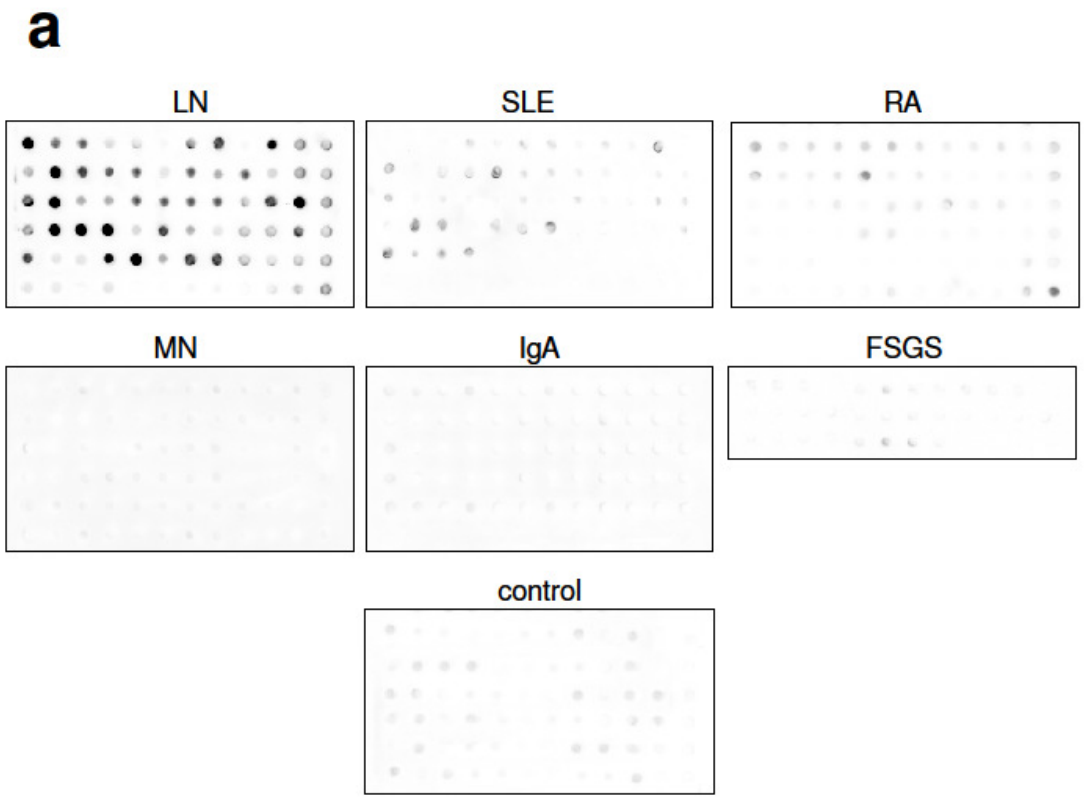


Supplement Fig 2. Co-localization of annexin AI (red) and IgG2 (green) in renal biopsies after (a-c) DNasa/RNase treatment. Double IF staining was evaluated for αenolase (in red) and IgG2 (in green). Merged images (yellow) Scale bar: 20 μm. Original magnification, x630.



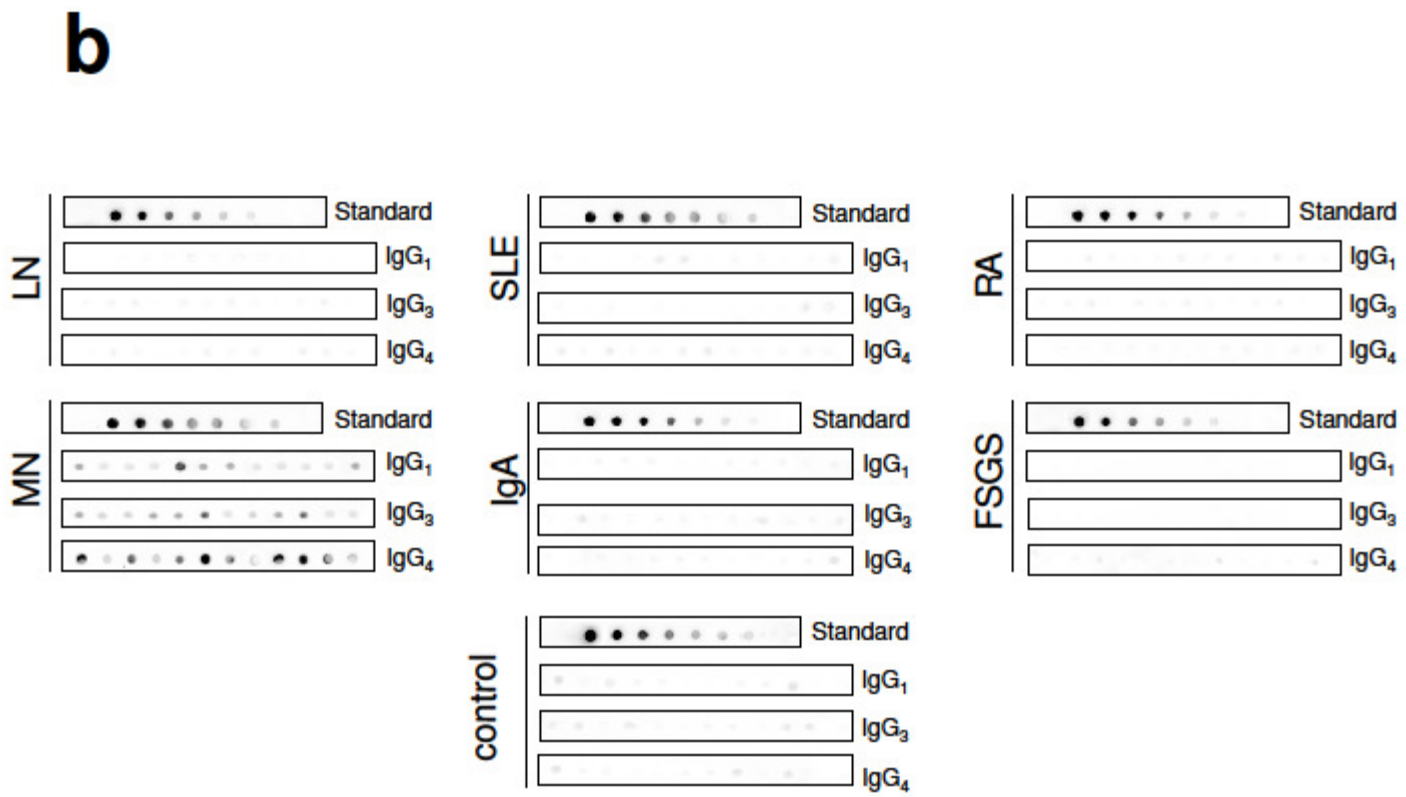
Supplement Figure 3. Correlation between serum levels of αenolase evaluated with Dot-blot and ELISA (b). Both methods are described in dedicated sections of Supplements.

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



Supplement Fig 4.

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



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