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GLOMERULAR AUTOIMMUNE MULTICOMPONENTS OF HUMAN LUPUS NEPHRITIS *IN VIVO* (2): IMPLANTED ANTIGENS

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 were 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 for dot-blot and ELISA

Anti-a 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).

Secondary antibodies for dot-blot. Purified mouse monoclonal HRP conjugated 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).

Antibodies for Immunofluorescence

Anti-a enolase-1 Rabbit anti-human non-neuronal enolase (NNE) (alpha-alpha), AbD Serotec MorphoSys Ltd, (Endeavour House, Kidlington Oxford, UK).

Anti-AnnexinA1: Anti-Histones2A,3,4: Mouse monoclonal anti-Annexin A1 antibody [3A8], rabbit polyclonal anti-Histone H2A , rabbit polyclonal anti-Histone H3 and mouse monoclonal anti-Histone H4, Abcam (UK)

Anti-DNA: Mouse monoclonal anti-DNA antibody, Merck KgaA (Darmstadt, Germany)

Anti-C1q. : Fluorescin (FITC)-conjiugated rabbit polyclonal anti-human C1q complement antibody, Dako (Glostrup, Denmark).

Anti-immunoglobulin isotypes antibodies for immunofluorescence: Anti-IgG1, anti-IgG2 and anti IgG4: mouse monoclonal antibodies, Life Technologies (Grand Island, NY); anti-IgG3: mouse monoclonal antibody, Southern Biotech (Birmingham, USA)

Secondary antibodies for immunofluorecsence : Fluorescin (FITC)-conjiugated Affinity purified Donkey Anti-Mouse IgG (H+L) and Anti-Rabbit IgG (H+L) were purchased from Jackson Immunoresearch (West Grove, PA, USA). Alexa Fluor 546 Donkey Anti-Mouse IgG and Alexa Fluor 546 Donkey Anti-Rabbit IgG were purchased from Life Technologies (Grand Island, NY)

Recombinant proteins. *αenolase*: 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 0.15% v/v and incubated with HRP conjugated anti-human IgG (Invitrogen Corporation, Camarillo, CA - 2h, 1:5000) for immunodetection.

Immunofluorescence. 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 mouse monoclonal anti-human IgG1 antibody diluted 1:10 in PBS or with mouse monoclonal anti-IgG3 antibody diluted 1:10 in PBS. Fluorescein isothiocyanate conjugate (FITC) affinity-purified donkey anti-mouse IgG (Jackson Immunoresearch, PA) was used as secondary antibody diluted 1:50 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 H2A, H3 **and** ***H4 istones with IgG2, C1q with IgG2 and IgG4, DNA with IgG2 and IgG3 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 rabbit polyclonal anti-H2A or rabbit polyclonal anti-Histone H3 or mouse monoclonal anti-Histone H4 or mouse monoclonal anti-DNA antibodies diluited 1:100 with PBS for 2h at RT. Following additional PBS washes, slides were exposed to Alexa Fluor 546 donkey anti-rabbit or anti-mouse IgG diluited 1:200 for 1h at RT. Subsequently, monoclonal mouse anti-human IgG2 or anti-human IgG3 antibodies diluted 1:10 in PBS was applied for 1h at RT. IgG2 and IgG3 deposits were

characterized utilizing FITC-conjugated purified donkey anti-mouse IgG diluited 1:100 for 1h at RT.

For anti-C1q IgG2 and anti-C1q-IgG4 co-localization, cryosections were processed in the same way as previously indicated but the method was a direct immunofluorescence by using fluorescin (FITC)-conjiugated rabbit polyclonal anti-human C1q antibody. This antibody was diluted 1:10 with PBS for 2h at RT. Subsequently, monoclonal mouse antibody anti-human IgG2 or anti-human IgG4 diluted 1:10 in PBS was applied for 1h at RT. IgG2 deposits were characterized utilizing Alexa Fluor 546 Donkey anti-mouse IgG diluted 1:200 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 63x/1.30 oil objective. Image acquisition was carried out in multitrack mode, namely through consecutive and independent optical pathways.

Traditional assays for SLE. For the clinical setting the following essays for anti-DNA and anti-C1q were utilized. Anti-DNA antibodies were detected by two different commercial quantitative enzyme-linked immunosorbent assays (Varelisa dsDNA Antibodies, Phadia GmbH, Freiburg, Germany; anti-dsDNA, Diamedix, Miami, Florida, USA); both assays utilize plasmid DNA and the results are reported in IU/ml (international units), traceable to the first international standard (Wo/80). Nevertheless, since the results were not strictly comparable in terms of IU [5], in order to unify and analyze data, an "anti-DNA ratio" was conceived. 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. Anti-C1q antibodies were detected using a home-made enzyme-linked immunosorbent assay as described by Sinico *et al* [6].

SUPPLEMENT FIGURES

Supplement Figure 1. Anti-C1q IgG4/IgG2 potential interaction. To rule out the possibility that high anti-C1q IgG4 and IgG2 staining was done by aggregation of the two antibodies, we repeated the same staining presented in Figure 1e and utilizing a positive sample (pt4) in a gradient of NaCl between 0.15 (the same of serum) and 1.5 M. Albumin and anti-albumin were run in parallel to see if NaCl could reduce the binding of primary and/or secondary antibodies to the antigen. It is here shown that for concentration of NaCl higher than 0.15 M, the interaction between albumin (as fixed antigen) and anti-abumin Abs is reduced. The same was seen for anti-C1q IgG4; anti-C1q IgG2 was always negative. Altogether, this experiment shows that the binding of a dot-blot membrane fixed antibody is maintained for NaCl concentration up to 0.15M. At this NaCl concentration anti-C1q IgG4 bound the fixed antigen while IgG2 did not. This results exclude the presence of aggregates between anti-C1q IgG4 and IgG2.

Supplement Figure 2. **Correlations among different variables**. We utilized Spearman analysis and presented results in the correlogram above in which the grade of correlation is indicated with colours from red (Max) to blue (Min). In bold are remarked those correlations characterized by a R>0.7 that means very high correlation probability. A tree dendrogram performed on row and column places similar variables near each other according to the results of cluster analysis.

Supplement Fig 3. Renal expression of IgG1 and IgG3 in LN biopsies. Renal IgG1 (a-h) and IgG3 (k-p) deposition was investigated by indirect immunofluorescence (IMF) on residual cryostatic tissue sections from renal biopsies of patients affected by class IV or class V lupus nephritis. Both immunoglobulins were variable expressed in glomerular tuft showing strong

/medium positivity in class IV (c,d,K,i) and while in a few class V (g,h,o,p) they were substantially absent. In positive cases IgG3 were markedly expressed in the capillary walls and in class IV in the mesangium as well. The signal was not detectable at the tubular and vascular level, indicating the specificity of the glomerular antibody binding..

Supplement Figure 4. Standards for determination of antibody levels in micro-dissected glomeruli. For dot blot analysis of concentration of each antibody a specific standard curve was utilized that included specific IgG versus all isotype categories. For dot-blot conditions see Methods.

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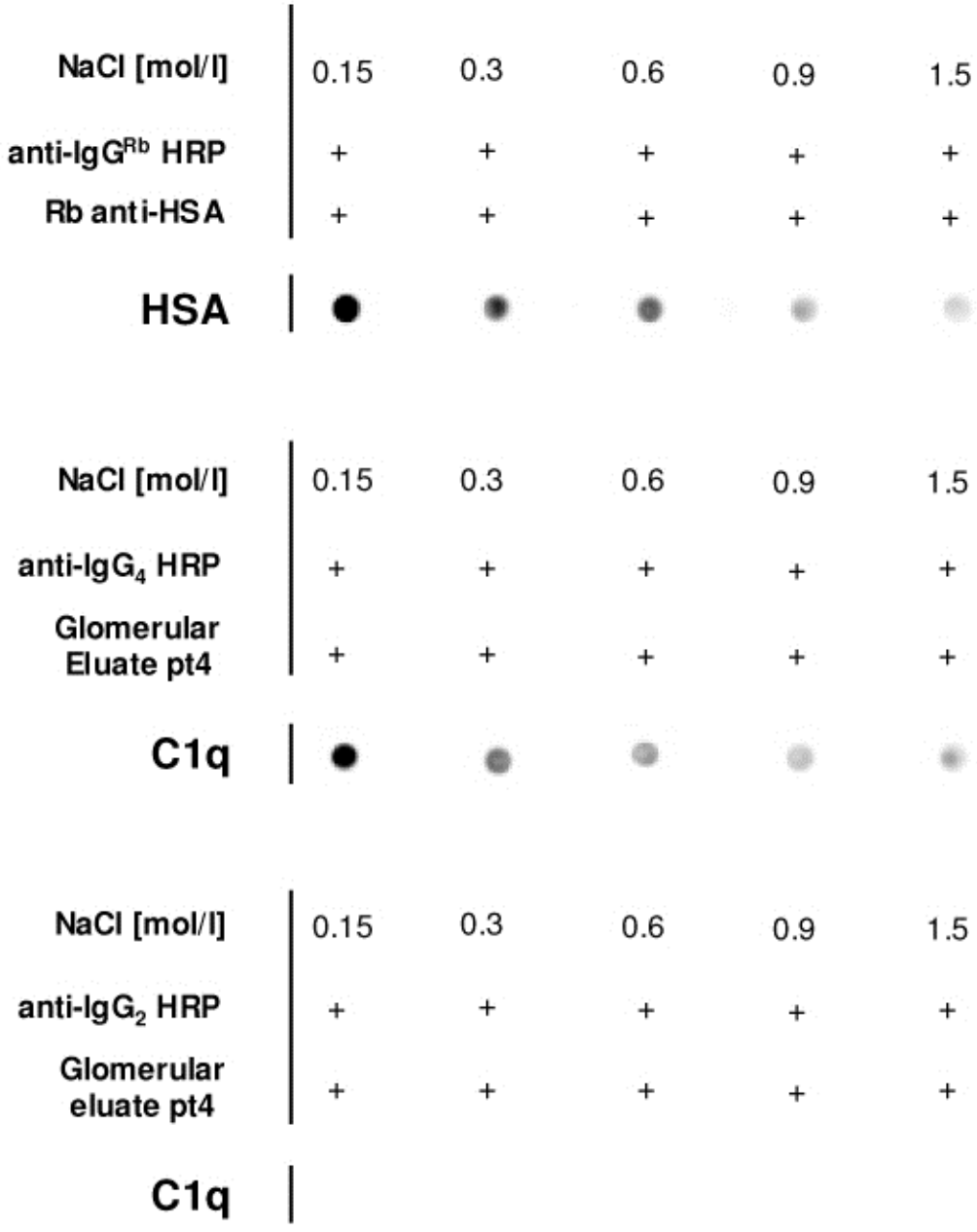
Supplement Figure 1



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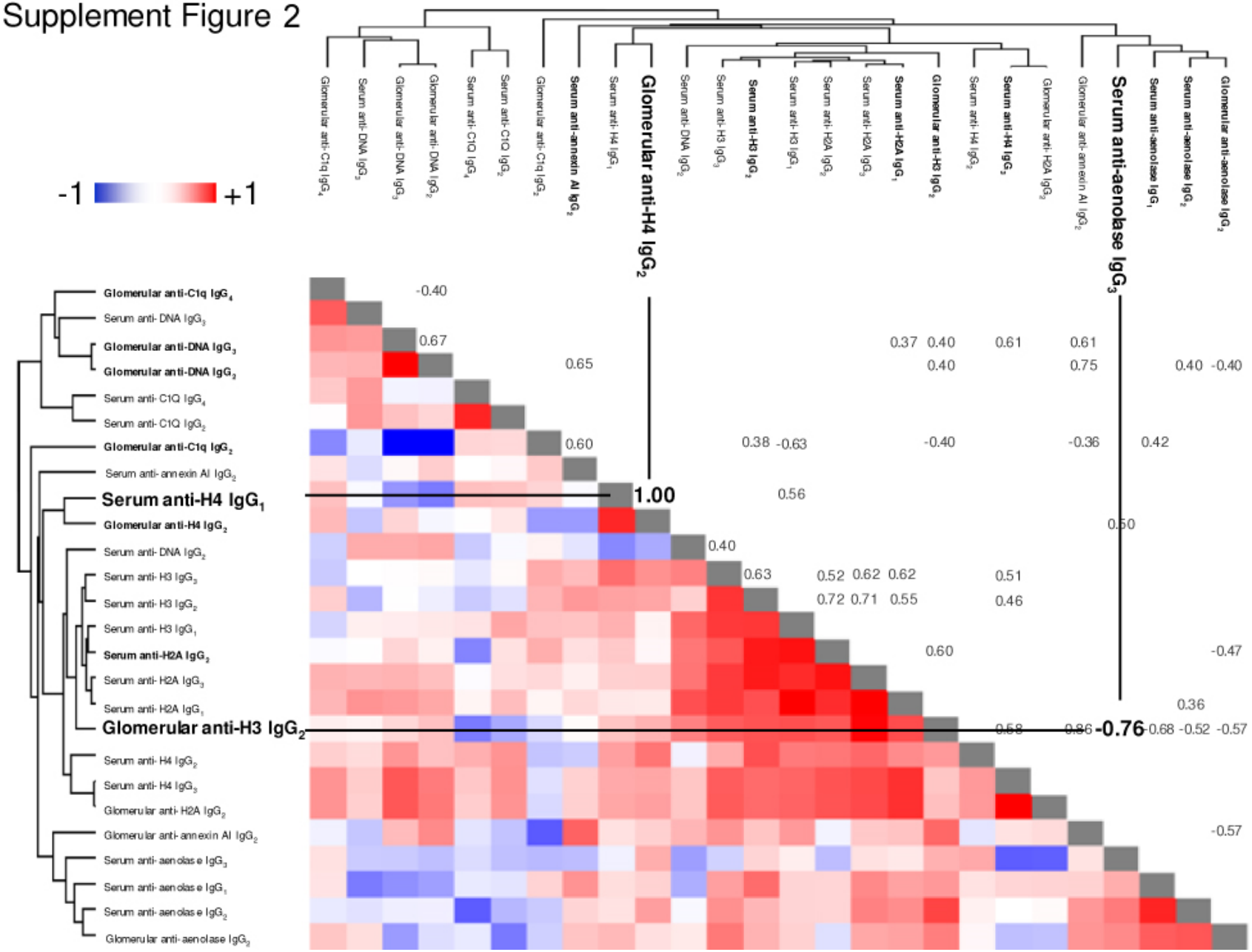

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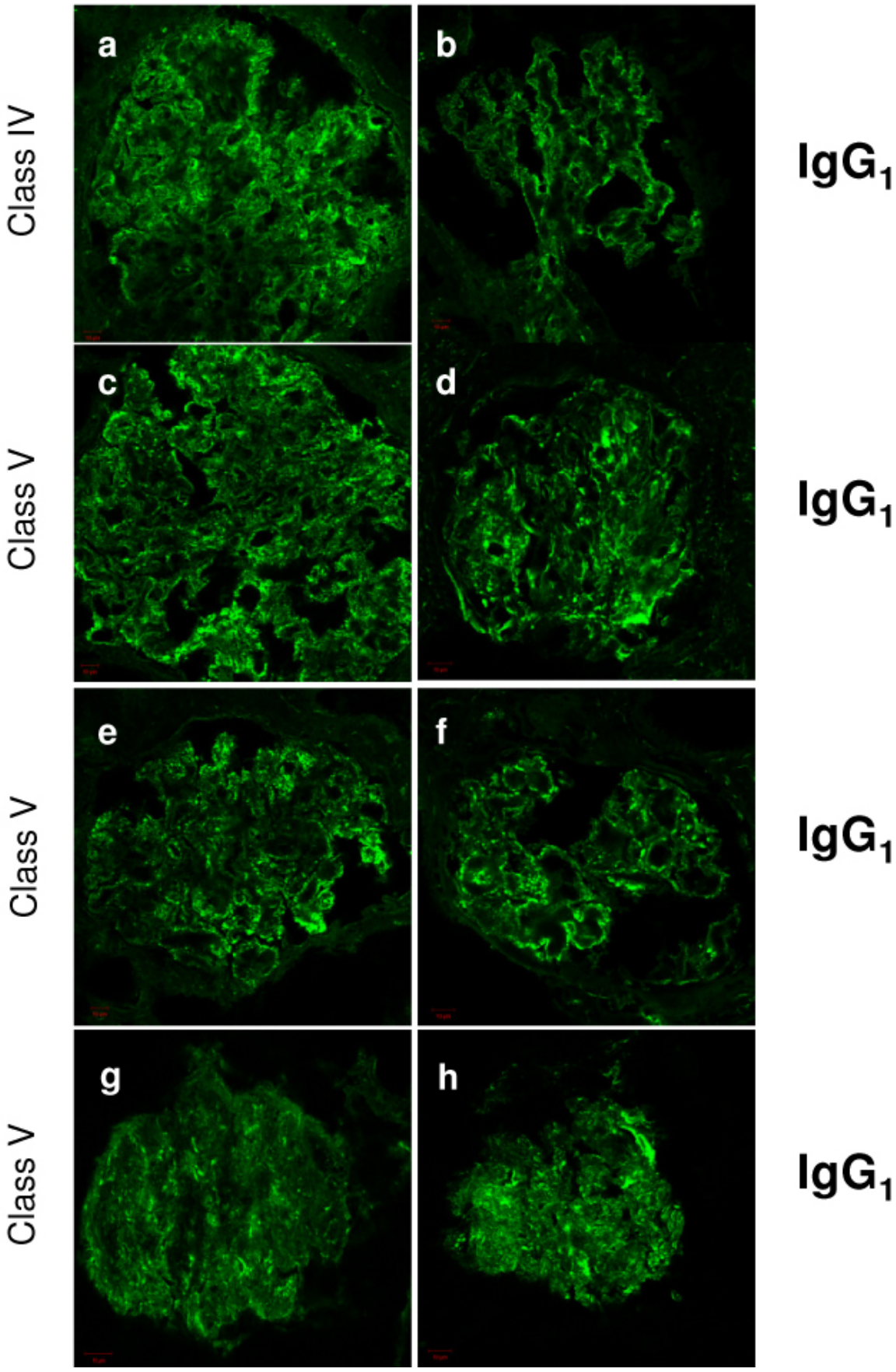

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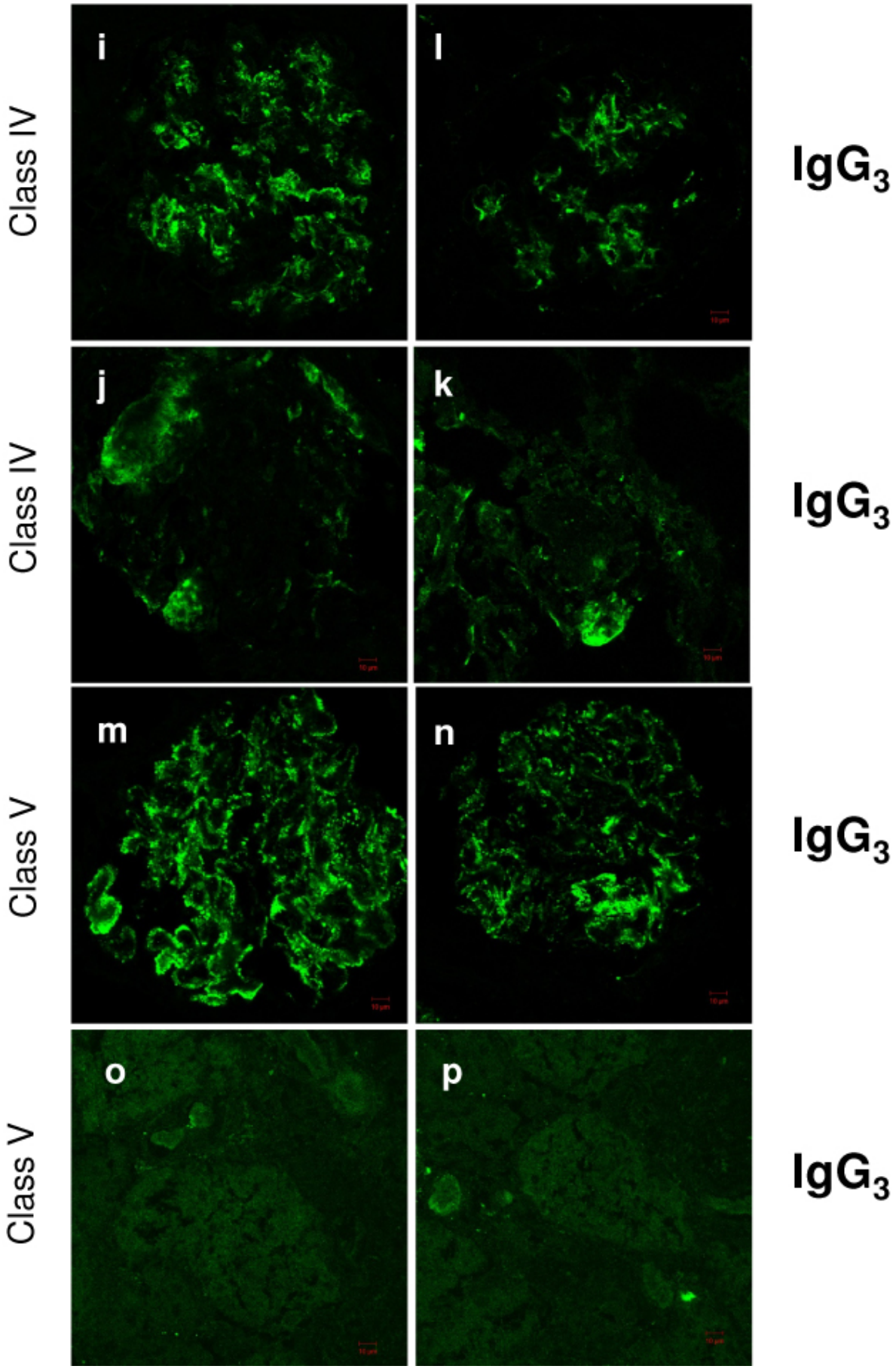

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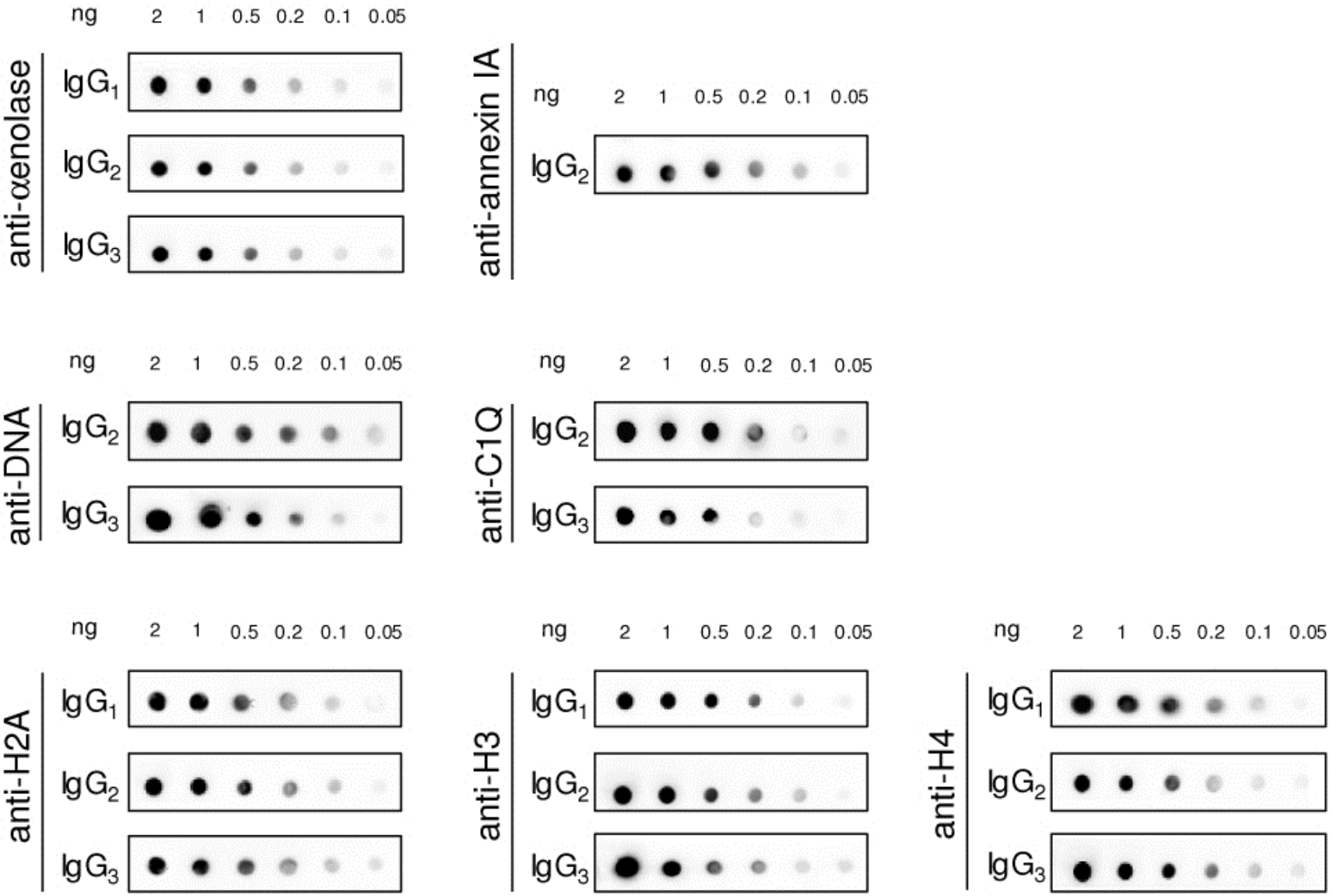

Pt.n	Sex	Age	Biopsy Year	LN Class	SCreat (mg/dL)	UProt (g/24h)	C3 (mg/dL)	C4 (mg/dL)	ANA	Anti-DNA	STEROID	CYCLOPH	CYCLOSP	MMF	PLAQUENIL
1-	M	16	2010	IV	0.5	2.2	103	16	pos	pos	yes	no	no	no	no
2-	M	22	2005	III	0.6	0.9	44	8	pos	pos	yes	no	no	no	no
3-	M	19	2010	V	2.4	0.9	78	12	pos	pos	yes	no	no	no	no
4-	F	18	2009	IV	0.4	1.4	65	10	pos	pos	yes	no	no	no	no
5-	F	25	2005	V	0.5	1.1	47	19	pos	pos	yes	no	no	no	yes
6-	F	28	2005	nd	2.1	0.2	102	20	pos	pos	yes	no	yes	yes	no
7-	F	20	2007	II	0.4	0.6	60	10	pos	pos	yes	no	no	no	no
8-	F	17	2009	IV-V	0.7	0.8	47	<3	pos	pos	yes	no	no	no	yes
9-	F	25	2004	IV	0.9	1.8	5	20	pos	pos	yes	no	no	no	yes
10-	F	43	2010	IV	0.8	1.4	53	6	pos	pos	yes	no	no	yes	yes
11-	F	44	2010	IV+V	0.7	0.9	46	4.8	pos	pos	yes	no	no	yes	yes
12-	F	53	2010	III	0.6	2.8	141	18	pos	pos	yes	no	no	yes	no
13-	F	34	2009	IV	0.8	2.8	53	11.5	pos	pos	yes	yes	no	no	yes
14-	F	40	2009	III	0.5	1.4	71	10.7	pos	pos	yes	no	no	yes	yes
15-	F	25	2009	V	0.6	3.3	63	12.1	pos	pos	yes	no	no	yes	yes
16-	F	38	2009	IV	0.6	4.5	41	4.1	pos	pos	yes	yes	no	no	yes
17-	F	50	2009	IV	1.0	1.9	75	10	pos	pos	yes	no	no	yes	yes
18-	F	35	2010	IV	0.7	2.1	47	2.5	pos	pos	yes	yes	no	no	yes
19-	F	31	2011	IV	0.8	5.7	23	2.4	pos	pos	yes	no	no	yes	no
20-	F	27	2011	nd	1.0	1.0	50	5	pos	pos	yes	no	no	no	no

Supplement Table 1. Clinical data and pathology characteristics relative to 20 patients with LN who underwent a renal biopsy at the time of enrolment and had their renal tissue processed with laser capture for antibody characterization. The same biopsies were studied in a first approach limited to anti-podocyte antibodies (i.e. anti-αenolase and anti-annexin AI IgGs); the table is here presented for simplifying the reading of this part of the study.

Abbreviations: LN , Lupus nephritis; **SCreat** , serum creatinine; **UProt** , proteinuria; **ANA** , antinuclear antibodies; CYCLOPH, cyclophosphimide, CYCLOSP. cyclosporine

	LN (n 104)	SLE (n 84)	RA (n50)	Contr. (n96)
Male sex	11 (12%)	17 (15%)	9 (18%)	56 (58%)
Age (years)	34 (14–77)	47 (16– 79)	58 (13-77)	49 (35-60)
Age at SLE onset (yrs)	26 (12–77)	37 (7 - 79)	-	-
Disease duration (yrs)	3 (0.1–24)	7 (0 – 34)	-	-
Serum creatinine (mg/dl)	0.9 (0.3–6.4)	0.7 (0.5 – 1.7)	0.8 (0.6-1.1)	0.9 (0.6 -1.2)
C3 (mg/dl)	63 (24 -147)	87 (42 – 154)	-	-
Anti-DNA ratio	2.9 (0– 26.7)	0.8 (0 – 11.8)	no	-
Anti-C1q (U/ml)	166 (10-600)	21 (10 – 257)		
Proteinuria (g/day)	2.5 (0.1– 20)	0.1 (0 – 0.3)	0.1 (0-0.3)	-
LN class				
Low grade	9 (8%)			
Proliferative (III, IV)	57 (55%)			
Membranous (V)	12 (12%)			
Mixed	26 (25%)			
Therapy				
None	14 (13%)	13 (15%)		
Steroids only	29 (28%)	17 (20%)		
MMF/Csa/Plaq	8 (8%)	9 (11%)		
Multiple therapies	53 (51%)	45 (54%)		

Supplement Table 2. Clinical data of LN and SLE patients. Data were collected from 188 SLE patients. One-hundred and four had overt LN in which case, serum collection coincided with the time of renal biopsy. The remaining 84 had SLE with normal urine and renal function. Fifty patients with Rheumatoid Arthritis **(RA)** were enrolled to compare positivity to the auto-antibody panel. For the clinical setting, anti-DNA antibodies were detected by two different commercial quantitative enzyme-linked immunosorbent assays [5], and data were expressed as "anti-DNA ratio where 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. Anti-C1q antibodies were detected using a home-made enzyme-linked immunosorbent assay as described by Sinico *et al.* [6]