COEXISTENCE OF DIFFERENT CIRCULATING ANTI-PODOCYTE ANTIBODIES IN MEMBRANOUS NEPHROPATHY

By Corrado Murtas and coll.

SUPPLEMENTAL MATERIALS

SUPPLEMENT METHODS

Recombinant proteins

Recombinant proteins utilized for dot blot were obtained from the companies outlined below.

r AR: full-length recombinant protein with GST tag, Abnova Corporation (Taipei, Taiwan).

r SOD2: full-length recombinant protein with GST tag, Abnova Corporation (Taipei, Taiwan).

r αENO: full-length recombinant protein with GST tag, Abnova Corporation (Taipei, Taiwan).

r NEP: extracellular domain protein with 6-His tag, R&D Systems (Minneapolis, MN).

Antibodies

Antibodies for specific proteins were utilized for the calibration curve. They were obtained from the companies outlined below:

Anti-AR: Abnova Corporation, (Taipei,Taiwan).

Anti-SOD2: Abnova Corporation, (Taipei, Taiwan).

Anti-αENO: Rabbit anti Human Non-Neuronal Enolase (NNE) (alpha-alpha) from AbD Serotec MorphoSys Ltd, (Endeavour House, Kidlington Oxford , UK).

Anti-NEP: Abnova Corporation, (Taipei, Taiwan).

Anti-PLA2r: Abcam, (Cambrige, UK).

Antibodies HRP conjugated utilized for dot blot and western blot were obtained from the companies outlined below.

Purified mouse monoclonal antibody to human IgG_4 (Clone: HP6025) and purified mouse monoclonal antibody to human IgG_3 (Clone: HP6050): Southern Biotech, (Birmingham, AL, USA).

Purified mouse monoclonal antibody to human IgG_1: Invitrogen (Carlsbad, CA, USA).

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.

Dot-blot for anti-AR, anti-SOD2, anti-αENO and anti-NEP

For anti-AR, anti-SOD2, anti-α enolase and anti-NEP determination we utilized dot-blot and recombinant proteins as fixed antigen. The essay was done with a Bio-Dot apparatus (Bio-Rad, Hercules, CA, USA) following the instruction manual with minor modifications. All samples and the calibrator serum were diluted with Tris buffered saline (TBS) pH 7.4 (1:100). A calibration curve was prepared by keeping constant the amount of recombinant protein and increasing dilution of specific antibody from 1:500 to 1:32,000. Sera were diluted in the same buffer (1:100) to achieve the desired range of levels, obtained after testing several conditions.

Accordingly, the nitrocellulose membrane was pre-wetted in TBS and placed on a sheet of Whatman 3 mm filter paper embedded with the same buffer. Constant amounts of protein (300 ng) were placed in the 80 μl of TBS. After removal of the air bubbles between the two sheets by gentle pressure, the sample template was placed on the nitrocellulose membrane and a vacuum was applied for a few minutes, to fill up the 96 sample wells with 50 mL using a multi-channel pipette. The vacuum was applied until all the samples were adsorbed. The same operation was repeated five times with 150 uL of buffer each, to wash out the non-adsorbed sample. The nitrocellulose was then gently removed and saturated with 5% w/v bovine serum albumin (BSA) in TBS. Sera were then left for six hours at room temperature; at the end the membrane was washed

six times in 0.15% v/v Tween-TBS. Incubation with HPR–anti human IgG_{1-3-4} 0.5 μg/mL in 1% w/v BSA in TBS was performed for two hours room temperature. The membrane was then washed four times, 15 min each, with TBS-T prior to developing the immuno reaction with SuperSignal West Pico Chemiluminescent substrate (Thermo scientific, Rockford, IL, USA). Chemiluminescence was detected by VersaDoc and computed with QuantyOne software (Bio-Rad) and given as relative optical density [O.D. unit] that corresponds to one unit of signal intensity.

Western blot/Membrane Staining and Image Analysis for PLA2r

Western-blot after mono-dimensional electrophoresis of podocyte extracts was utilized for determining anti PLA2r circulating antibodies. Podocyte protein separation in gradient mono-dimensional electrophoresis. Cells were first lysated in ice-cold non reducing Laemmli buffer (2) (62.5 mM Tris-HCl pH 6.8, 2% w/v SDS, 1 mM EDTA and protease inhibitors); proteins were then separated in 8-16 T% gradient gels and blotted onto nitrocellulose membrane (30 μg for line). After saturation, the membrane was incubated overnight at 4°C with MN or control serum (1:100) in 3% w/v BSA in TBS-T, rinsed in TBS-T and then incubated with anti-human IgG_4 HRP-conjugated antibody (Southern Biotech). To identify PLA2r, the same test was performed using anti PLA2r polyclonal antibody (Abcam). Chemioluminescence protein bands were visualized by using a VersaDoc 4000 (Bio-Rad, Cambridge, MA, USA) apparatus and QuantityOne software (Bio-Rad) was used to quantify optical density. In Supplement Figure 2 it is shown a test sample reporting a series of MN sera with variable positivity is shown in the same figure. Intra-essay variation was less than 5%. In the standard run (consisting in podocyte extracts with the same amount of the other test lines) PLA2r was identified with anti-PLA2r antibodies.

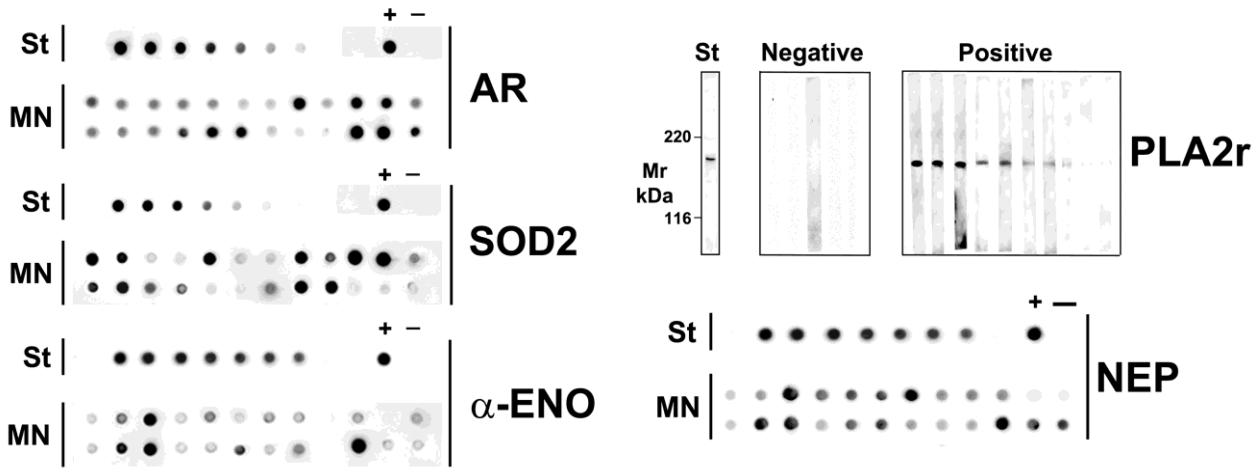
Immunofluorescence test for circulating anti-PLA2r

To validate the results obtained by western blot analysis a random portion of MN sera (73) were evaluated by indirect immunofluorescence with a commercially available test, according to manufacturer's instruction (Euroimmun, Lubeck, Germany). Briefly, anti-PLA2r total IgG titers were measured with indirect immunofluorescence on HEK 293 cells, previously transfected with full-length complementary DNA encoding PLA2r. Antibody positivity was defined as positive staining at serum dilutions of 1/10 or higher. There is a strong correlation between indirect immunofluorescence test and western blot analysis (Spearman analysis, r 0.91, $P<0.001$).

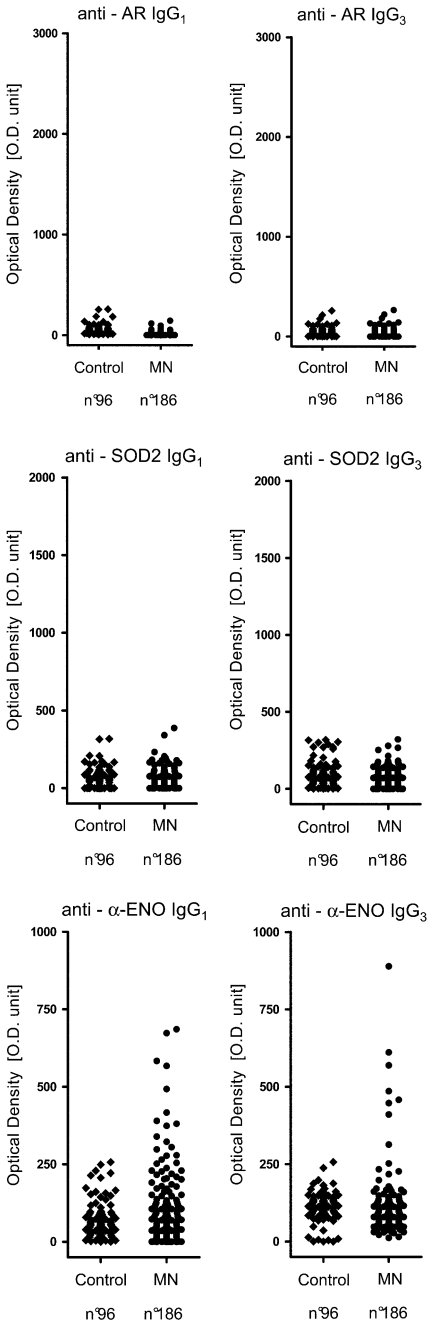
REFERENCES

1. Saleem, MA, O'Hare, MJ, Reiser, J, Coward, RJ, Inward, CD, Farren, T, Xing, CY, Ni, L, Mathieson, PW, Mundel, P: A conditionally immortalized human podocyte cell line demonstrating nephrin and podocin expression. *J Am Soc Nephrol* 13: 630-638, 2002
2. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685, 1970

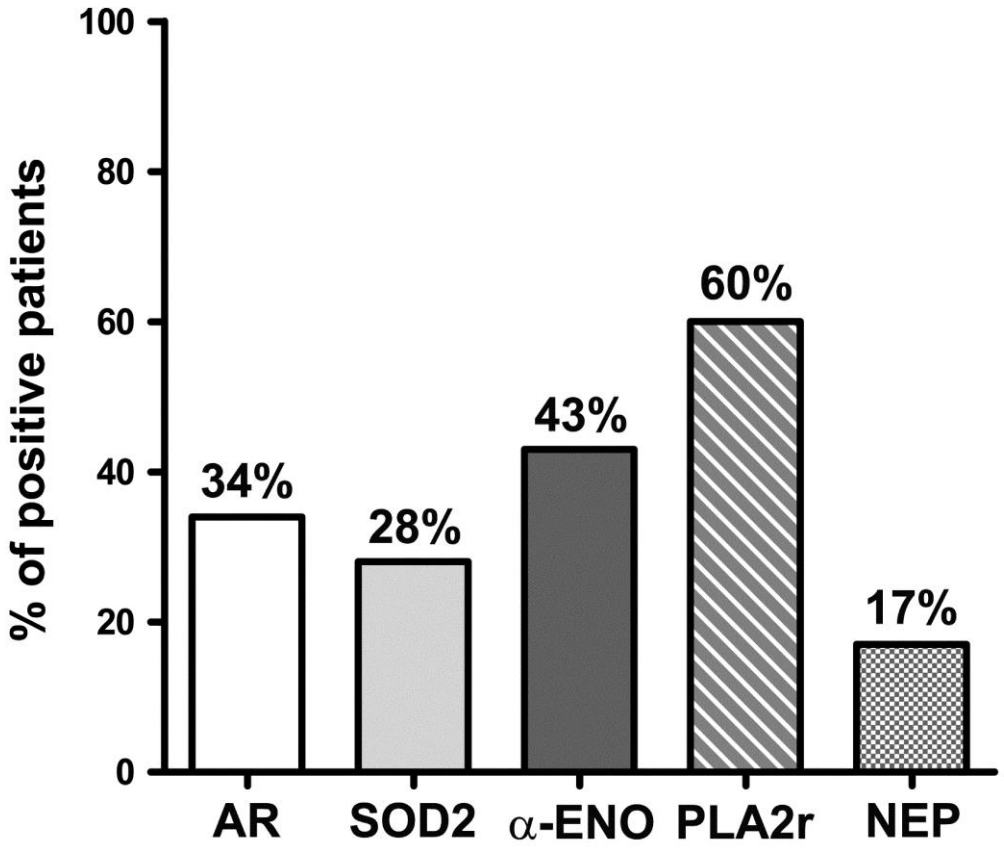
SUPPLEMENT FIGURES



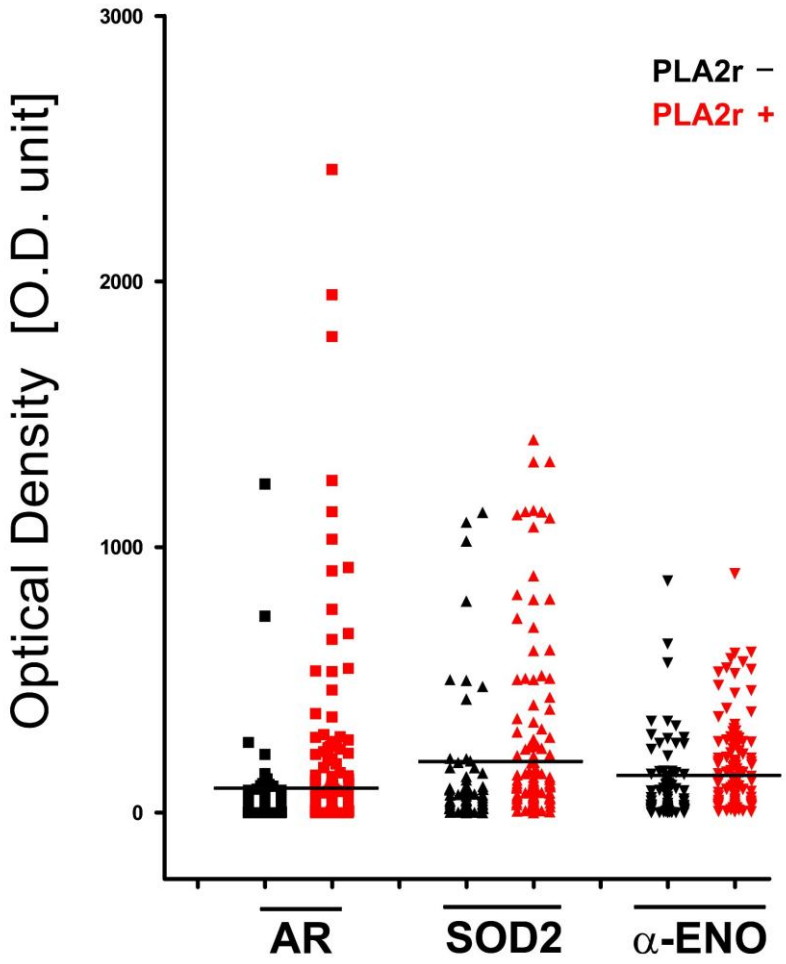
Supplement Figure 1. Dot blot for the determination of anti-AR IgG_4, anti-SOD2 IgG_4, anti- αENO IgG_4 and anti-NEP IgG_4. Western blot analysis of anti-PLA2r IgG_4. In dot blot analysis for each antibody titration, a calibration curve was prepared by keeping constant the amount of recombinant protein and increasing dilutions of specific antibody from 1:500 to 1:32,000. Sera were diluted in the same buffer (1:100). Positive and negative internal standards are indicated with + and –. Only patients with MN are presented in the figure. In western blot analysis St indicates a line run with podocyte extracts and analysed with anti-PLA2r antibodies.



Supplement Figure 2. Serum levels of circulating auto-antibodies (IgG_{1-3}) versus AR(a), SOD2(b) and αENO(c) in MN and normal populations. In all cases it was utilized the technique based on dot-blot analysis with recombinant protein linked to nitrocellulose as antigen (as detailed in Supplement Figure 1). Results are given as chemioluminescence optical density arbitrary units**.**



Supplement figure 3. Percent of MN patients positive for different anti-podocyte antibodies at diagnosis. In the case of anti-AR, anti-SOD2, anti-αENO and anti-NEP positivity was defined as a serum level exceeding the 95th percentile of levels titrated in normal controls. Anti-PLA2r were negative in normal sera and in other glomerular diseases.



Supplement Figure 4. 186 MN patients have been subdivided depending on anti-PLA2r status (negatives in black and positives in red) and serum levels of anti-AR, anti-SOD2 and anti- αENO IgG_4 have been reported. 111 patients were anti-PLA2r positive, 75 negative. In the case of anti-PLA2r negative 38 patients (51%) were positive for at least one other antibody. No histological or clinical characteristic distinguished anti-PLA2r positive and negative patients. The black horizontal line represents, for each antibody, the positivity limit.

SUPPLEMENT TABLES

	Whole group of patients (n = 186)	**Patients with 1 year follow-up** (n = 120)
Male sex (n - %)	121 – 65%	80 – 67%
Age (years)	59±16	59±15
Histological stage (n - %) I II III IV	 52 - 28% 74 - 40% 43 - 23% 17 - 9%	 40 - 33% 50 - 42% 19 - 16% 11 - 9%
Diabetes (n - %)	24 – 13%	10 – 8%
Serum creatinine (mg/dl)	1.1 (0.3 – 6)	1.1 (0.5 – 6)
Proteinuria (g/day) at diagnosis after 12 months	 5.8 (0.3 – 28) -	 5.7 (0.7 – 28) 1.1 (0 – 16.1)
Serum albumin (g/dl) at diagnosis after 12 months	 2.6 (1.0 – 4.3) -	 2.6 (1.0 – 4.3) 3.6 (1.5 – 4.8)
ACE-i / ARB therapy (n - %)	160 - 86%	105 - 87%
Immunosuppressant therapy (n - %) Ponticelli schedule Steroids only Cyclosporine A Synthetic ACTH Rituximab None	 113 - 61% 17 - 9% 5 - 3% 6 - 3% 1 - <1% 44 - 24%	 79 - 66% 20 - 17% 5 - 4% 2 - 2% 1 - <1% 13 - 11%

Supplement table 1. Clinical characteristics of MN patients enrolled in the study. Serum samples for antibodies titration were obtained at the time of renal biopsy in the whole group of patients. Clinical data were collected at the time of diagnosis. Only 120 patients completed a 1 year clinical

follow-up. Therapy protocol data were collected at the last available follow-up visit. Data are presented as median and range because of their non-normal distribution. In few case data are presented as mean and SD.

Indirect immunofluorescence antibody titer	n	Western blot serum level
0	17	0 (0 – 0)
1/10	1	18.39
1/32	6	10.97 (8.59 – 25.37)
1/100	9	29.79 (13.78 – 54.13)
1/320	24	87.34 (70.01 – 96.43)
1/1000	8	288.79 (192.23 – 388.42)
>1/1000	8	609.61 (449.83 – 673.50)

Supplement table 2: Titration of circulating anti-PLA2r in MN patients: comparison between WB and indirect IF. In a part of the MN cohort (73 patients) both test were performed. There is a strong correlation between the two techniques (Spearman analysis, r 0.91, P<0.001). A part of the small differences can be explained by the different serum antibody revealed: IgG_4 in WB and total IgG in IF. WB data are expressed in optical density arbitrary unit and presented as median and interquartile range.

Outcome		*Beta (β)*	*95% CI*	*P value*	*Exp[β]*	*95% CI*
Proteinuria	**log_2 AR**	0.11	0.04, 0.18	0.003	1.12	1.03, 1.19
	log_2 αENO	0.10	-0.01, 0.21	0.063	1.11	0.99, 1.24
Serum albumin (g/dl)	**log_2 AR**	-0.032	-0.07; 0.006	0.099	0.96	0.93, 1.01
	log_2 αENO	-0.076	-0.131; -0.020	0.008	0.92	0.87, 0.98

Supplement table 3. Prediction of clinical data after one year of follow-up by single antibody level at the time of diagnosis (linear regression). Predictors (IgG_4 antibody level) have been **log_2**-transformed. Linear regression of binary log-proteinuria at 1 year adjusted for baseline levels. Each doubling of AR or αENO at baseline is respectively associated with 12% and 11% greater probability of doubling proteinuria at 1 year (anti-log or exp[beta]). Linear regression of serum albumin (on the natural scale) at 12 months adjusted for baseline levels. Betas represent the change in serum albumin at one year (in g/dL) per each doubling of AR or αENO. No correlation was found with SOD2, NEP and PLA2r antibodies. CI: confidence interval.