Multi-Autoantibody Signature and Clinical Outcome in Membranous Nephropathy

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1. Supplemental Methods

Recombinant proteins. Recombinant proteins utilized for dot-blot were obtained from the companies as follows: *recAR:* full-length recombinant protein with GST tag, Abnova

Corporation (Taipei, Taiwan); rec*SOD2:* full-length recombinant protein with GST tag, Abnova Corporation (Taipei, Taiwan); *recαENO:* full-length recombinant protein with GST tag, Abnova Corporation (Taipei, Taiwan).

Antibodies. Antibodies for specific proteins were utilized for the calibration curve. They were obtained from the following companies: *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 Morpho Sys Ltd, (Endeavour House, Kidlington Oxford, UK). HRP-conjugated secondary antibodies utilized for dot-blot and western-blot were obtained from the following companies: purified mouse monoclonal antibody to human IgG4 (Clone: HP6025) Southern Biotech (Birmingham, AL, USA).

Dot-blot for anti-AR, anti-SOD2 and anti-αENO autoantibodies. For anti-AR, anti-SOD2 and anti-αENO autoantibody determination, we utilized dot-blot and recombinant proteins as fixed antigen following procedures already described in details [1, 2]. The assays were 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. Equal amounts of protein (300 ng) were placed in 80 µl of TBS. After removal of 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 µL using a multi-channel pipette. The vacuum was applied until all the samples were adsorbed. The same operation was repeated five times with 150 µL 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 incubated for six hours at room temperature. At the end of incubation, the membrane was washed six times in 0.15% (v/v) (TBS-T). Incubation with HRP-conjugated anti-human IgG4 (0.5 µg/mL) in 1% w/v BSA in TBS-T was performed for two hours at room temperature. The membrane was then washed four times, 15 min each, with Tween-TBS prior to developing the immunoreaction with SuperSignal West Pico Chemiluminescent substrate (Thermo scientific, Rockford, IL, USA). Chemiluminescence was detected by VersaDoc and computed with QuantityOne software (Bio-Rad) and given as relative optical density [OD unit].

Anti-PLA2R1 epitopes. Anti-PLA2R1 epitope autoantibodies were determined as described[3] using recombinant and soluble forms of CysR, CTLD1 and CTLD7 domains with HA-tag. Plates were coated with anti-HA antibody (Sigma-Aldrich) diluted at 1:5,000 in 20 mMTris pH 8.0 (100 µL/well) at 4°Covernight. Plates were blocked for 2h with SeramunBlock (Seramun Diagnostica). Cell medium from HEK293 cells transfected with soluble forms of PLA2R1 domains (10-100 µL/well) were then added and incubated for 1 h. Plates were

washed and patients' sera diluted at 1:100 in PBS/0.1% non-fat dry milk were added (100 µL/well). After 2h incubation at room temperature on a plate shaker, plates were washed and HRP-conjugatedanti-IgG4 secondary antibodies (Southern Biotech) diluted 1:7,500 in SeramunStab ST plus (Seramun Diagnostica) was added (100 µL/well) and incubated for 1h at room temperature on a plate shaker. After four washes, tetramethylbenzidine was added, and the reaction was developed for 15 min and then stop with HCl 1.2N. The plates were read at 450 nm.

2. Supplemental References

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Supplemental Table 1. Epidemiology, histological stage, clinical characteristics and treatment of patients with membranous nephropathy (MN) versus controls (Healthy donors) at diagnosis and after 12 months.

N.A., not applicable. ACEi: Angiotensin converting enzyme inhibitors.

Characteristics	MN (n=285)	Controls (n=50)
Male gender (n (%))	194 (68)	35 (70)
Age (years)	61 (11-87)	52 (18-60)
Histological stage (n (%))		
I	68 (24)	N.A.
II	131 (46)	N.A.
III	63 (22)	N.A.
IV	23 (8)	N.A.
Clinical characteristics		
eGFR (mL/min/1.73 m^2)		
Diagnosis	74.2 (47-100)	104 (95-110)
Month 12	80 (43-104)	
Proteinuria (g/day)		
Diagnosis	5.3 (0.5-25)	0.1 (0.05-0.15)
Month 12	1.8 (0.1-33)	
Serum albumin (g/dL)		
Diagnosis	2.4 (1.0-4.2)	4.2 (4-4.5)
Month 12	3.5 (1.5-4.8)	
Treatment (n (%))		
Cytotoxic	90 (32)	N.A.
Cyclosporine A	72 (25)	N.A.
Rituximab	34 (12)	N.A.
Steroids	95 (33)	N.A.
ACEi[§]	147 (51)	N.A.

[§]ACEi was given alone or in association with other drugs. Twenty-one percent of patients received only ACEi.

Supplemental Table 2. Quantitative data for limits of positivity and distribution of serum levels for each autoantibody. Low levels correspond to values between the limit of positivity and the median; high levels correspond to values over the median. The number of patients in the two groups is shown under parenthesis.

Autoantibody	Limit of Positivity	Low level limit (n)	High level limit (n)
Anti-PLA2R1 (RU/mL)	20	20-122 (91)	122-1,751 (91)
Anti-SOD2 (mg/L)	162	162-286 (42)	286-1,404 (43)
Anti-AR (mg/L)	84	84-151 (43)	151-2,422 (43)
Anti-αENO (mg/L)	136	136-205 (58)	205-900 (58)

Supplemental Table 3. Detailed autoantibody positivity for intracellular antigens in non-spreaders and spreaders PLA2R1[+] patients. Patients positive for anti-PLA2R1 autoantibodies (n=182) were split according to their epitope profiles as "non-spreaders" (CysR only) or "spreaders" (CysRCTLD1, CysRCTLD7 and CysRCTLD1CTLD7). Spreaders and non-spreaders were further split for the presence of single (anti-AR, anti-SOD2, anti-αENO) or composite anti-intracellular antigen positivity (Intracellular[+] included all single and multiple positivities for intracellular antigens).

	Anti–PLA2R1[+]				
	Non-spreaders	**Spreaders**			
	CysR	**Overall**	**CysR CTLD1**	**CysR CTLD7**	**CysR CTLD1 CTLD7**
	(n=35)	**(n=147)**	**(n=34)**	**(n=46)**	**(n=67)**
Autoantibody positivity					
Anti-AR[+] (%)	7	4	1	3	6
Anti-SOD2^{+} (%)	0	7	14	3	3
Anti-αENO[+] (%)	8	15	17	30	22
Anti-AR[+]SOD2^{+} (%)	10	3	0	3	6
Anti-AR[+]αENO[+] (%)	2	7	6	3	12
Anti-SOD2^{+}αENO[+] (%)	4	13	8	17	16
Anti-AR[+]SOD2^{+}αENO[+] (%)	10	9	8	13	6
Anti-Intracellular[+] (%)	45	58	54	72	71

Supplemental Table 4. Two-way contingency table showing the association of antibodies levels with indexes of kidney outcome and the interaction between autoantibodies against PLA2R1 and intracellular antigens for patients treated with cytotoxic drugs, cyclosporine A or rituximab. The 2x2 contingency table reports the association with odds ratios (OR), confidence interval (CI) and p values between antibody titer and clinical outcome. The P values were corrected for multiple comparisons with an alpha error value of 5%. Data are calculated from all patients treated with immunosuppressants after excluding the eight anti-THSD7A positive patients (n=196). The upper section shows the association between positivity versus negativity of each autoantibody at diagnosis with clinical outcome of proteinuria (complete (≤0.3 g/day) and partial (<3.5 g/day) remission) and eGFR after 12 months. The lower section shows the association of high versus low titers of each antibody with the same parameters of proteinuria and eGFR. The additive effect of positivity of more than one antibody is indicated as P+/S+ and P+/S+/E+ (anti-PLA2R1+/anti-SOD2+/anti-αENO+).

	Proteinuria >0.3 g/day	Proteinuria ≤0.3 g/day	OR (CI)	P value	Proteinuria >3.5 g/day	Proteinuria ≤3.5 g/day	OR (CI)	P value	eGFR≤60 ml/min/1.73m2	eGFR>60 ml/min/1.73m2	OR (CI)	P value
Anti-PLA2R1 (P)												
Positive	100 (51%)	23 (12%)	2.5 (1.3-4.9)	0.01	34 (17%)	89 (45%)	1.6 (0.8-3.3)	0. 2	44 (22%)	79 (40%)	1.2 (0.6-2.2)	0.6
Negative	46 (20%)	27 (14%)			14 (7%)	59 (30%)			23 (12%)	50 (26%)		
Anti-SOD2 (S)												
Positive	59 (30%)	9 (5%)	3.1 (1.4-6.8)	0.005	22 (11%)	46 (23%)	1.9 (1-3.6)	0.08	26 (13%)	36 (18%)	1.6 (0.9-3)	0.1
Negative	87 (44%)	41 (21%)			26 (13%)	102 (52%)			41 (21%)	93 (47%)		
Anti-αENO (E)												
Positive	64 (33%)	15 (8%)	1.8 (0.9-3.6)	0.1	25 (13%)	54 (28%)	1.8 (0.9-3.4)	0.09	29 (15%)	42 (21%)	1.6 (0.9-2.9)	0.1
Negative	82 (42%)	35 (18%)			24 (12%)	93 (47%)			38 (19%)	87 (44%)		
P+/S+*												
Positive	37 (30%)	4 (3%)	2.8 (0.9-9)	0.09	15 (12%)	26 (21%)	1.9 (0.8-4.3)	0.1	16 (13%)	25 (20%)	1.2 (0.6-2.7)	0.7
Negative	63 (51%)	19 (15%)			19 (15%)	63 (51%)			28 (23%)	54 (44%)		
P+/S+/E+*												
Positive	27 (22%)	2 (2%)	3.9 (0.8-18)	0.1	10 (8%)	19 (15%)	1.5 (0.6-3.7)	0.3	26 (21%)	42 (34%)	1.3 (0.6-2.7)	0.6
Negative	73 (59%)	21 (17%)			24 (20%)	70 (57%)			18 (15%)	37 (30%)		
Anti-PLA2R1 (P)												
High titer	50 (41%)	8 (7%)	1.9 (0.7-4.8)	0. 2	20 (16%)	39 (32%)	1.8 (0.8-4.1)	0.2	25 (20%)	34 (28%)	1.7 (0.8-3.7)	0.2
Low titer	50 (41%)	15 (12%)			14 (11%)	50 (41%)			19 (15%)	45 (37%)		
Anti-SOD2 (S)												
High titer	32 (47%)	7 (10%)	0.3 (0.1-1.8)	0.3	16 (24%)	20 (29%)	3.5 (1.1-10)	0.04	16 (26%)	18 (29%)	1.6 (0.6-4.5)	0.4
Low titer	27 (40%)	2 (3%)			6 (9%)	26 (38%)			10 (16%)	18 (29%)		
Anti-αENO (E)												
High titer	32 (41%)	8 (10%)	0.9 (0.3-2.7)	1	19 (24%)	25 (32%)	3.7 (1.3-11)	0.02	15 (21%)	23 (32%)	0.9 (0.3-2.3)	0.8
Low titer	32 (41%)	7 (9%)			6 (8%)	29 (37%)			14 (20%)	19 (27%)		
High P+/S+**												
High titer P/ S+	13 (32%)	2 (5%)	0.5 (0.1-4.3)	0.6	6 (15%)	9 (22%)	1.2 (0.3-4.7)	0.7	6 (15%)	9 (22%)	1 (0.3-3.9)	1
High titer P/ S-	24 (58%)	2 (5%)			9 (22%)	17 (41%)			10 (24%)	16 (39%)		

*In these cases, P+/S+ were compared to P+/S- and P+/S+/E+ were compared to P+/S-/E-.

** In this case, high P/S (high P titer and S positivity) were compared to high P titer and S negativity.

Supplemental Table 5. Clinical characteristics at diagnosis and after 12 months of follow-up for PLA2R1$^+$ patients stratified according to epitope profiles. Data are reported as median with interquartile range. Mann-Whitney or Kruskal–Wallis tests were used for continuous variables and Chi-squared or Fisher's exact tests for categorical variables. Two-tailed p-values ≤0.05 were considered as significant (ns: not significant). ACEi: Angiotensin converting enzyme inhibitors. Note the columns "Anti-CysR" and "Non-spreaders" are identical, but presented twice for clarity.

Characteristics	Anti-CysR (n=35)	Anti-CysRCTLD1 (n=34)	Anti-CysRCTLD7 (n=46)	Anti-CysRCTLD1CTLD7 (n=67)	p value	Non-spreaders (n=35)	Spreaders (n=147)	p value
Male gender (%)	69	62	70	78	ns	69	71	ns
Age (years)	58 (41-67)	67 (60-73)	55 (40-67)	64 (52-74)	0.04*	58 (41-67)	63 (49-75)	ns
Histological stage (%)								
I	18	41	41	35		18	38	
II	65	38	35	49		65	42	
III	18	19	23	16		18	18	
IV	0	4	3	0		0	2	
Treatment (%)								
ACEi§	41	31	16	25		41	24	
Steroids	45	40	65	40		45	45	
Cyclosporine A	23	23	30	15		23	22	
Cytotoxic	41	38	63	36		41	45	
Rituximab	11	10	8	18		11	13	
Clinical parameters								
Serum creatinine (mg/dL)								
Diagnosis	1 (0.9-1.3)	1.2 (0.9-1.4)	0.9 (0.8-1.2)	1.2 (1-1.4)	ns	1(0.9-1.3)	1.1 (0.9-1.4)	ns
Month 12	0.9 (0.8-1.4)	1.2 (0.9-1.6)	1 (0.9-1.2)	1.1 (0.9-1.4)	ns	0.9(0.8-1.4)	1 (0.9-1.5)	ns
Proteinuria (g/day)								
Diagnosis	6 (4.9-7.6)	4.6 (3-8)	6 (3.5-9.9)	7 (4.4-10.9)	ns*	6 (4.9-7.6)	6 (3.6-10)	ns
Month 12	1.2 (0.2-4)	1 (0.4-4.2)	2.4 (1.1-5.8)	2.8 (0.6-5.4)	ns*	1.2 (0.2-4)	2.2 (0.6-5.7)	ns
eGFR (mL/min/1.73m^2)								
Diagnosis	69.8 (48-109)	57.9 (40-75)	77.5 (55-107)	64.9 (44-87)	0.03*	69.8 (48-109)	65.8 (45-93)	ns
Month 12	89.5 (48-105)	60.2 (44-87)	79.9 (64-102)	62.3 (47-90)	0.04*	89.5 (48-105)	68.6 (48-90)	ns

*Kruskal-Wallis test comparing the whole line of data (ie. anti-CysR, CysRCTLD1, CysRCTLD7 and CysRCTLD1CTLD7).

§ACEi was given alone or in association with other drugs.

3. Supplemental Figures

Supplemental figure 1. Prognosis clinical factors associated with complete remission. Odds ratios and confidence intervals were calculated for the association of gender and nephrotic proteinuria (>3.5 g/day) at diagnosis with the risk of not achieving complete remission of proteinuria (<0.3 g/day) after 12 months of follow-up. Patients with nephrotic syndrome at the onset (n=177) were compared to sub-nephrotic patients (n=100).
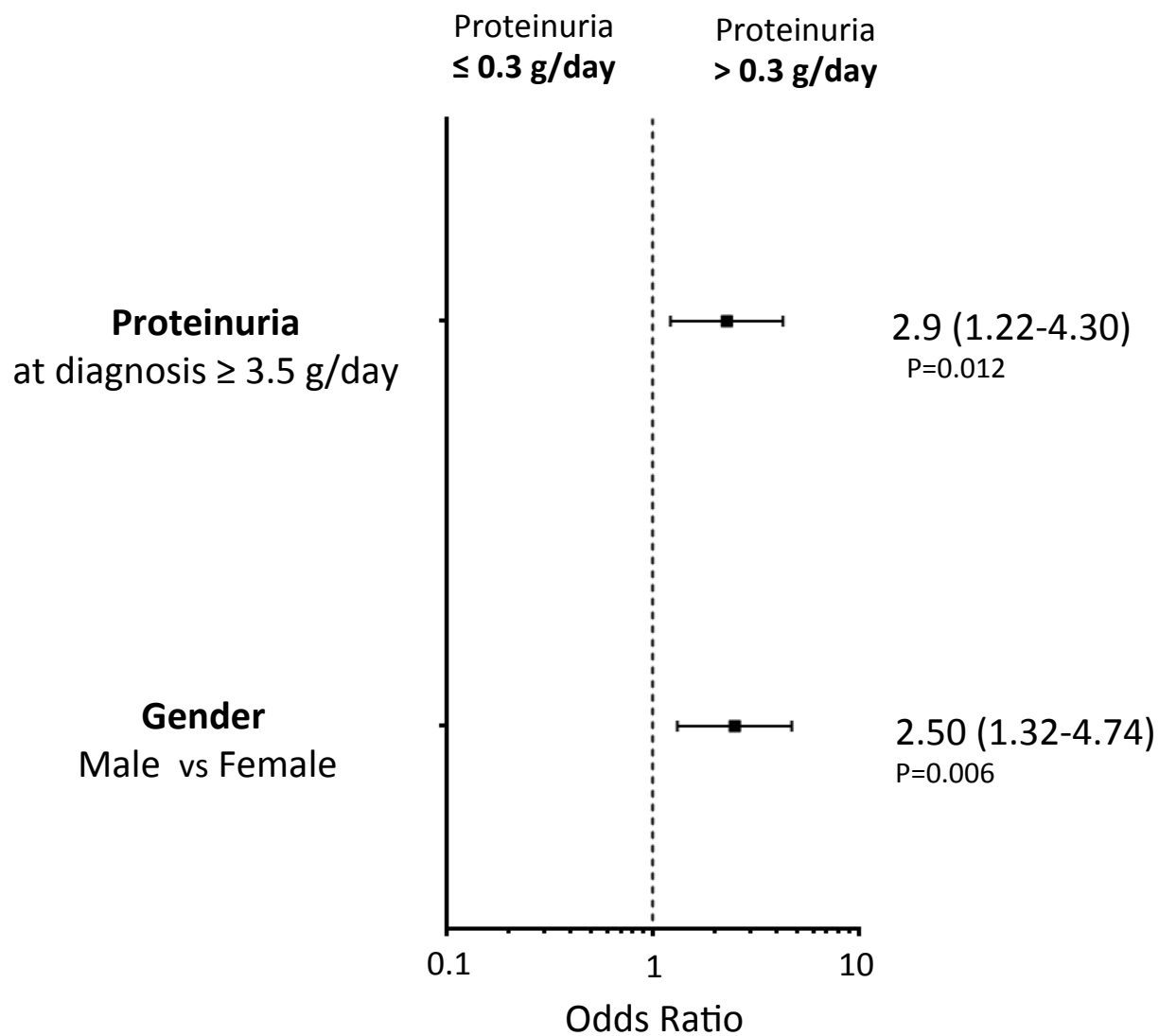
Supplemental figure 2. Circulating levels of the various autoantibodies at diagnosis and during follow-up. Circulating levels of (**a**) anti-PLA2R1, (**b**) anti-THSD7A and (**c-e**) anti-intracellular antigen autoantibodies (anti-AR, anti-SOD2, anti-αENO) were determined at the time of diagnosis and after 6 and 12 months during which the majority of patients received at least one drug. The dashline indicates the cut-off or limit of positivity that was calculated for each autoantibody; continuous lines indicate the interquartile ranges. For normal limits of anti-PLA2R1 total IgG, we used the limit of 20 RU/mL[7]; the receiver-operating characteristic (ROC) curve analysis was used to evaluate the discrimination capacity of each autoantibody.

Supplemental figure 3. Multi-autoantibody composition in the cohort of patients with membranous nephropathy. (a,e and f) Venn diagrams showing the composition of autoantibodies for anti-SOD2, anti-AR and anti-αENO positivity for groups of anti-PLA2R1 positive patients (a) anti-THSD7A positive patients (e) and anti-PLA2R1/anti-THSD7A double negative patients (f). Compared to Figure 1a, the Venn diagrams provide more details about the mutual combination of autoantibodies. (b) Associations between PLA2R1 epitope domain positivity (defining spreaders versus non-spreaders) and anti-PLA2R1 titer. Levels of anti-PLA2R1 are shown for patients defined as non-spreaders (CysR) and positive for autoantibodies towards additional epitope domains (CysRCTLD1, CysRCTLD7 and CysRCTLD1CTLD7). (c) Distribution of patients positive or not for circulating autoantibodies against the intracellular autoantigens AR, SOD2 and αENO in patients positive for anti-PLA2R1 and divided as "non-spreaders" (CysR only) and "spreaders" (CysRC1, CysRC7 and CysRC1C7). Details on positivity for single (anti-AR, anti-SOD2, anti-αENO) and composite anti-intracellular podocyte autoantigens (AR+SOD2+; AR+αENO+; SOD2+αENO+; AR+SOD2+αENO+) for separate groups of spreaders are given in Table S3. (d) Percentage of patients positive for anti-αENO autoantibodies in spreaders versus non-spreaders. (g) Quantitative correlations between the different types of autoantibodies. Circulating serum levels of each autoantibody were reported in Figure S2. The heatmap gives an overview of the quantitative
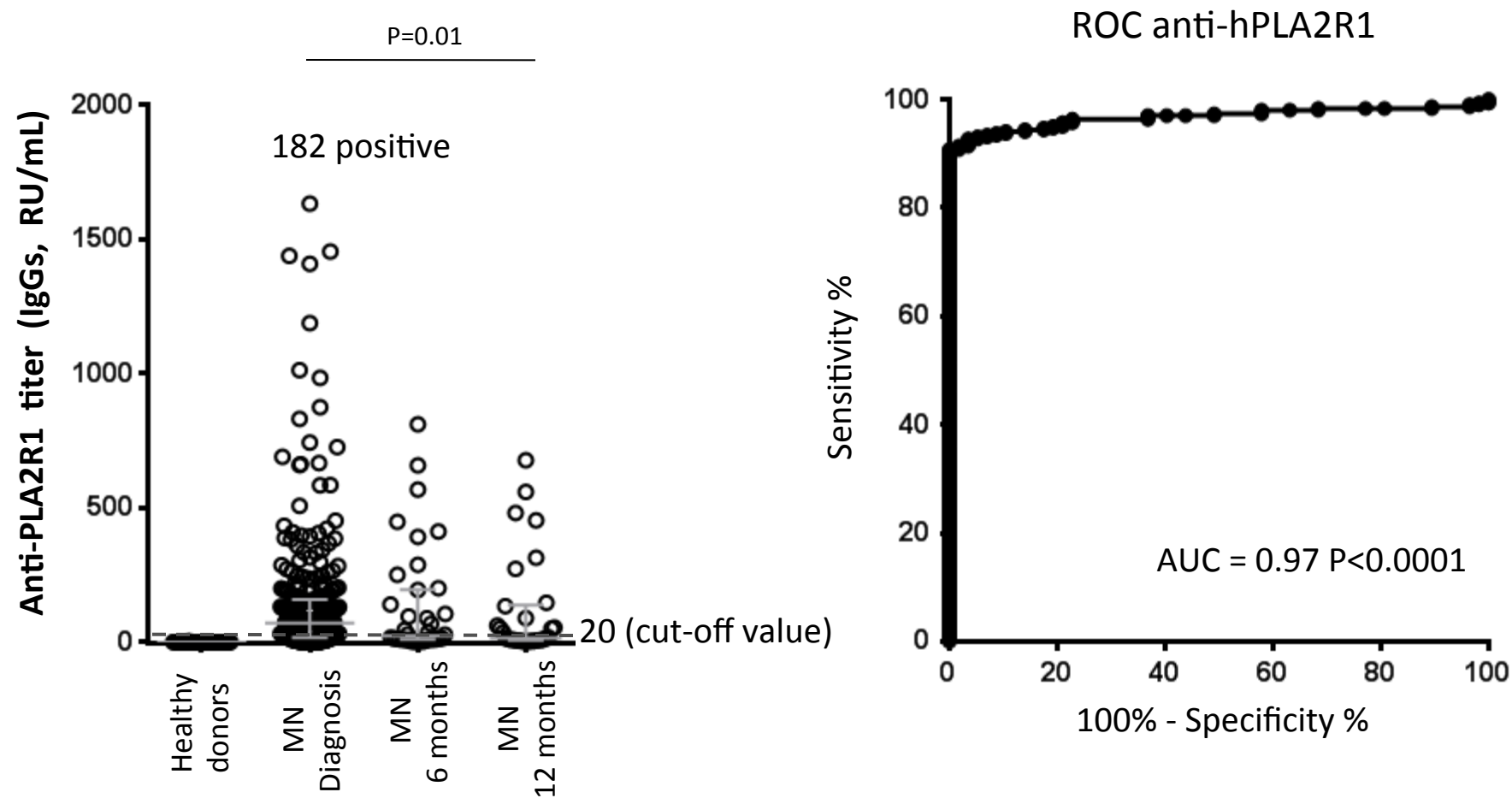
correlations between serum levels. This analysis was based on Spearman's correlation. The coefficient values are depicted by a pseudo-color scale extending from 0.1 (light gray or blue) to 0.89 (red). Moreover, the tree dendrogram displays the results of an unsupervised hierarchical clustering analysis placing similar Spearman's coefficient values near to each other.

Supplemental figure 4. Kidney function (eGFR) in spreaders positive (n=63) or not (n=84) for anti-αENO autoantibodies. eGFR at diagnosis and after 12 months in spreaders is modified by additional positivity for anti-αENO autoantibodies. Data are reported as median with interquartile range. Mann-Whitney or Kruskal–Wallis tests were used to compare eGFR. Two-tailed p-values <0.05 were considered as significant.

Supplemental Figure 1

Proteinuria ≤ 0.3 g/day   Proteinuria > 0.3 g/day

Proteinuria
at diagnosis ≥ 3.5 g/day

2.9 (1.22-4.30)
P=0.012

Gender
Male vs Female

2.50 (1.32-4.74)
P=0.006

0.1   1   10

Odds Ratio
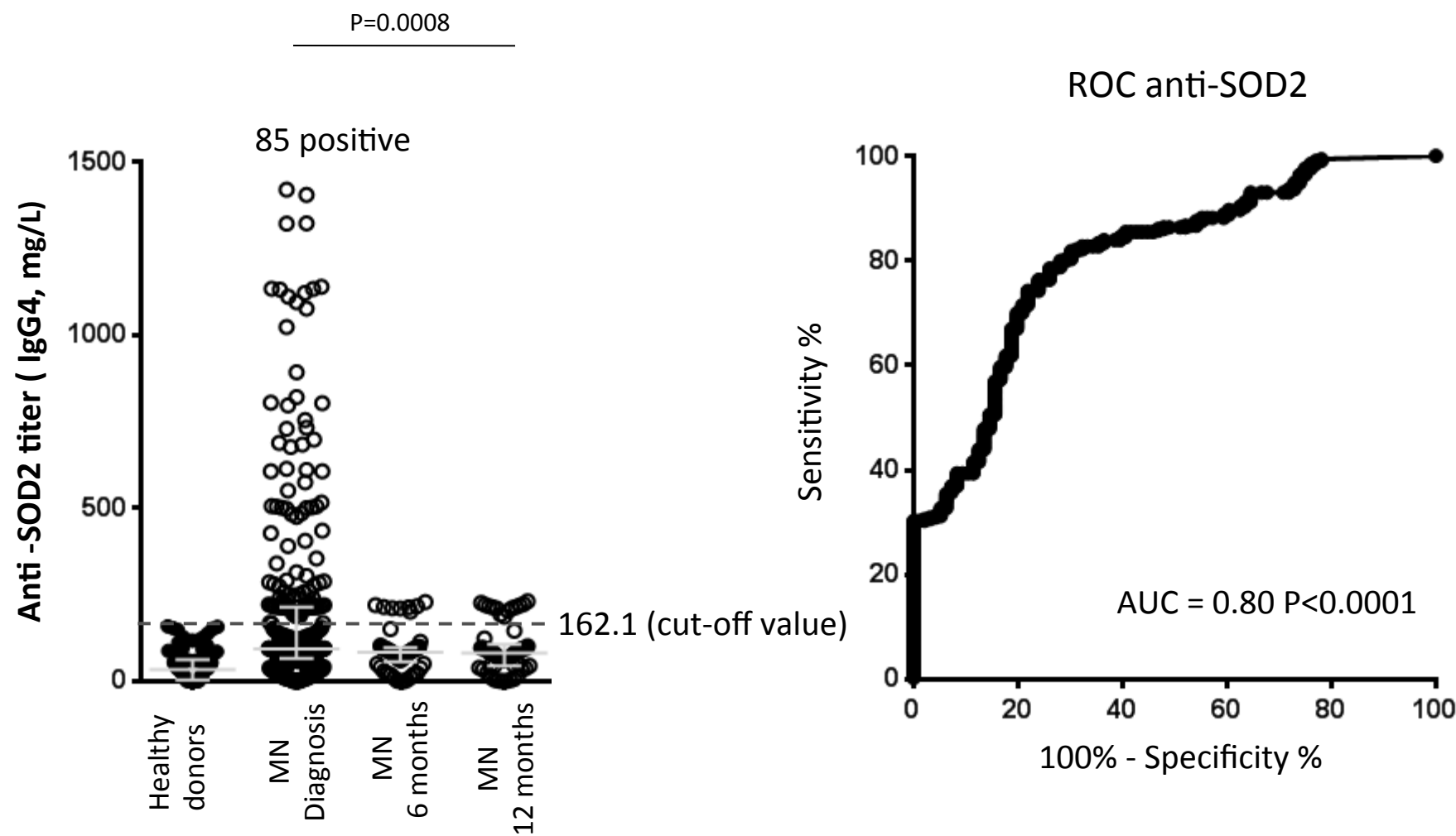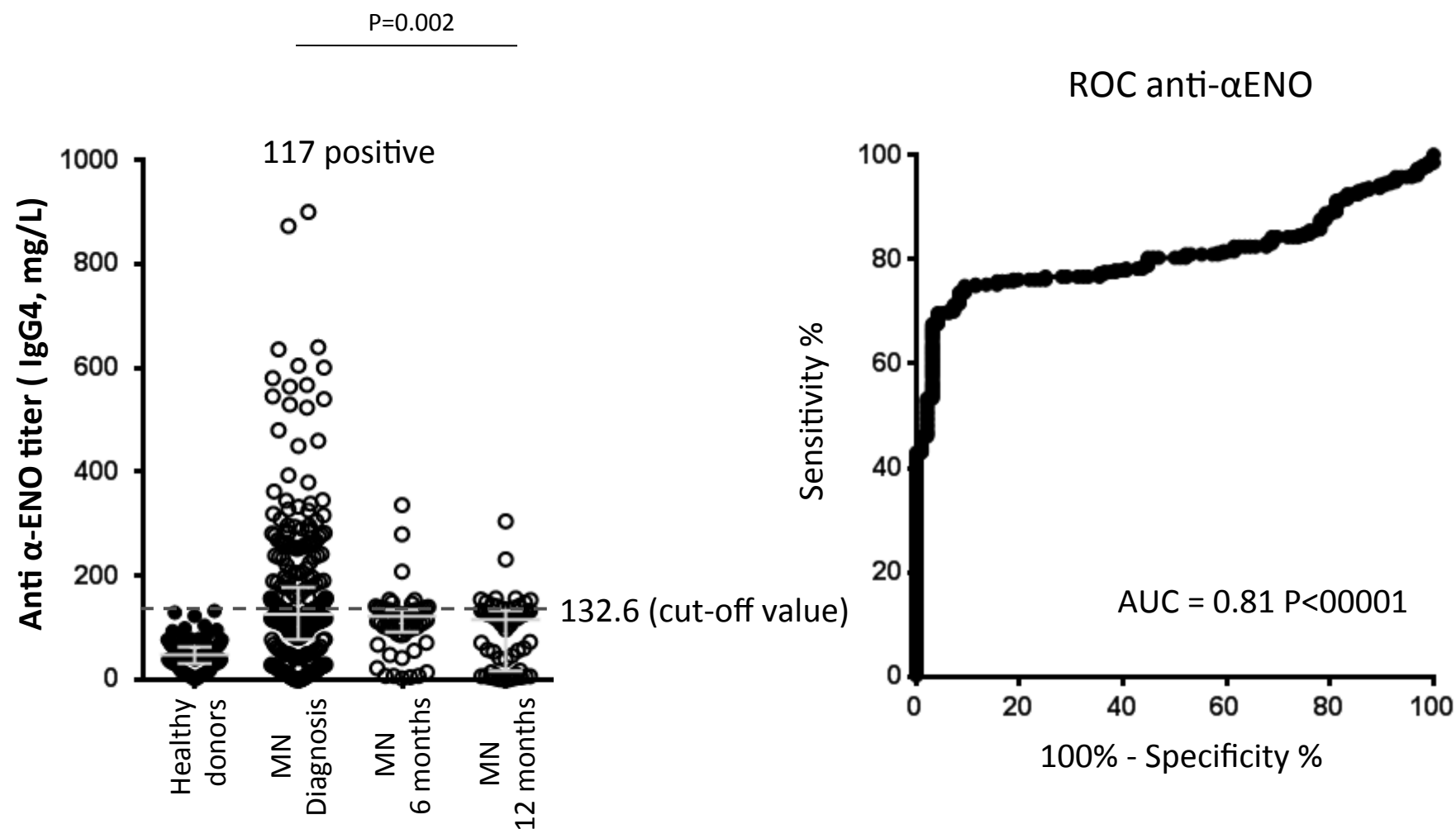
Supplemental Figure 2a

Supplemental Figure 2b
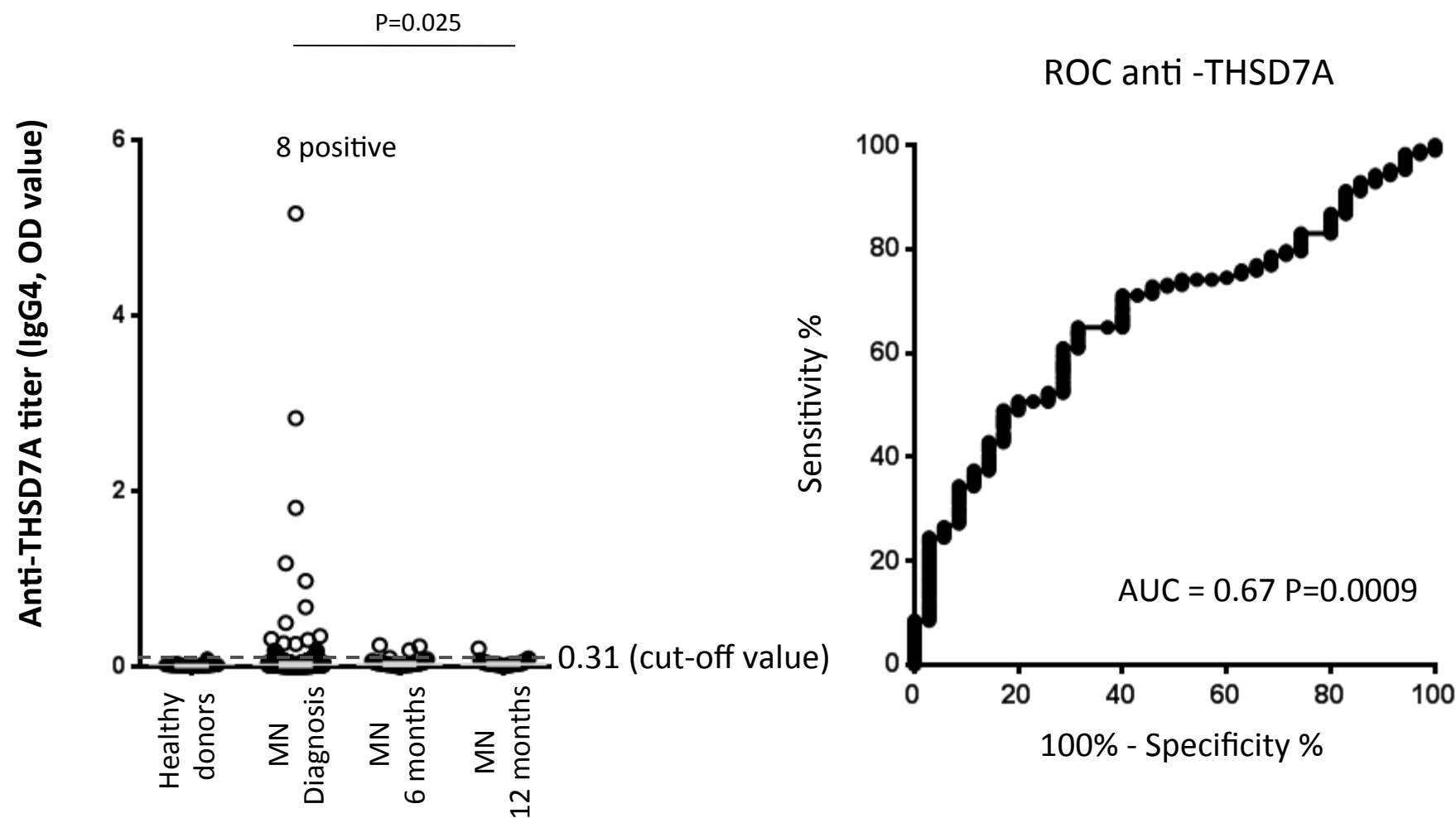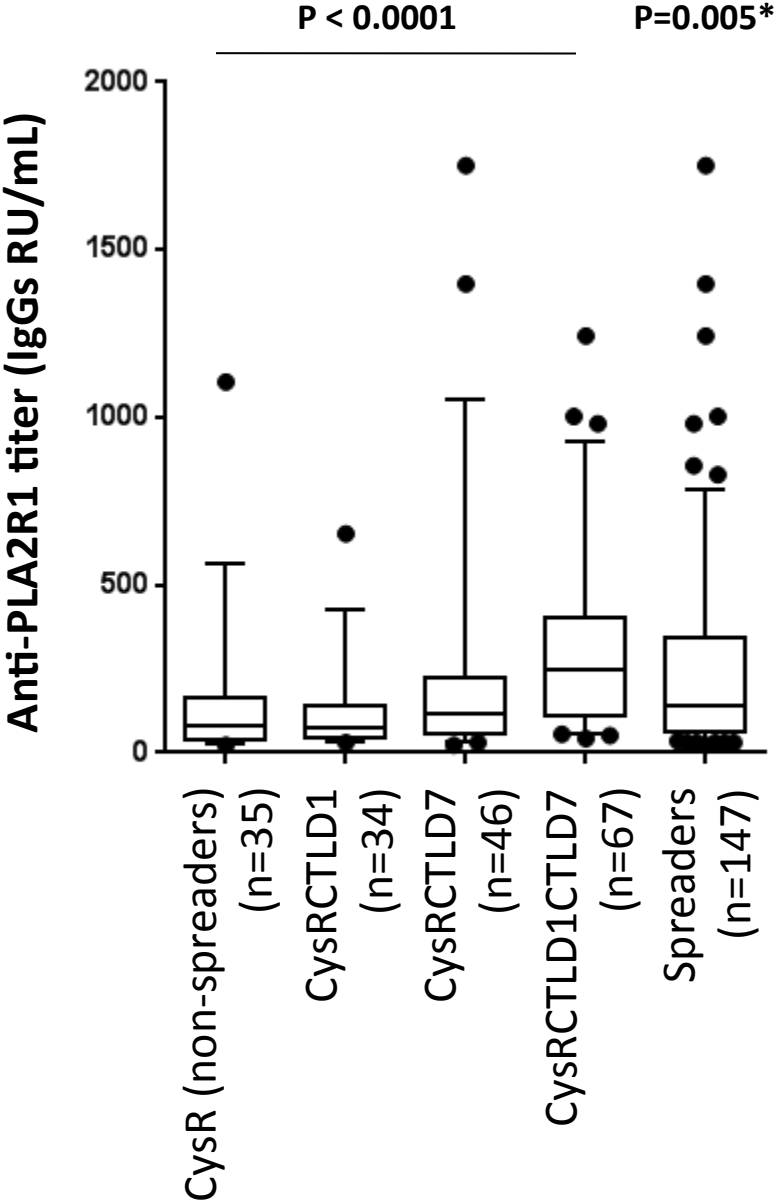
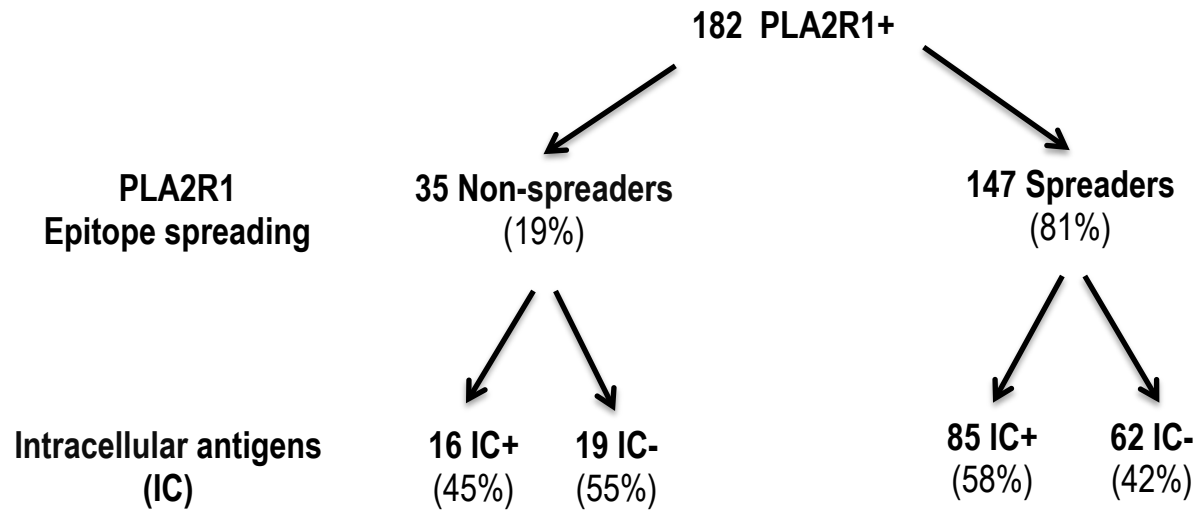## Supplemental Figure 2c
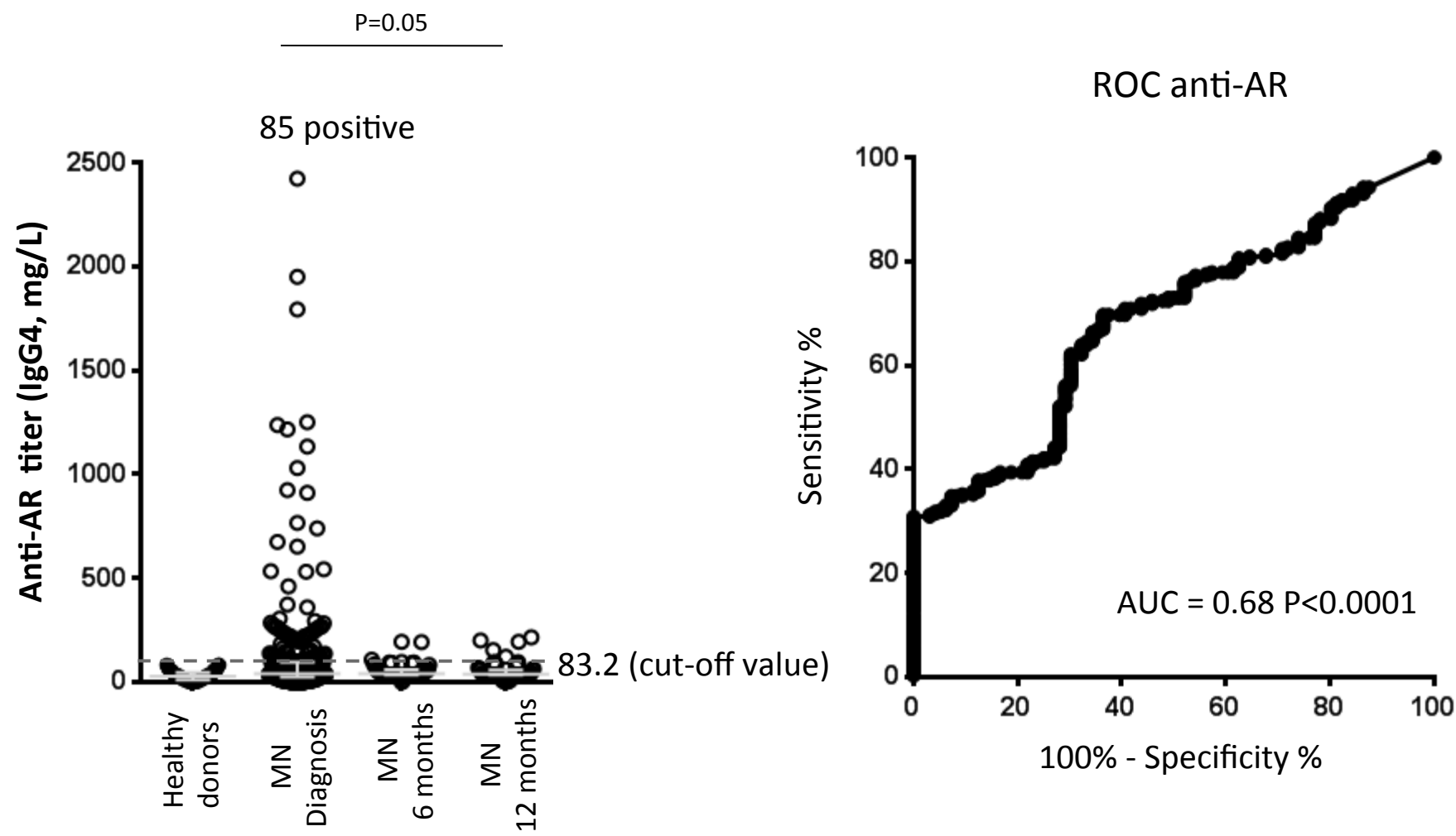
Supplemental Figure 2d

Supplemental Figure 2e

P=0.002

117 positive

Anti α-ENO titer (IgG4, mg/L)

132.6 (cut-off value)

Healthy donors

MN Diagnosis

MN 6 months

MN 12 months

ROC anti-αENO

Sensitivity %

100% - Specificity %

AUC = 0.81 P<00001

Supplemental Figure 3a
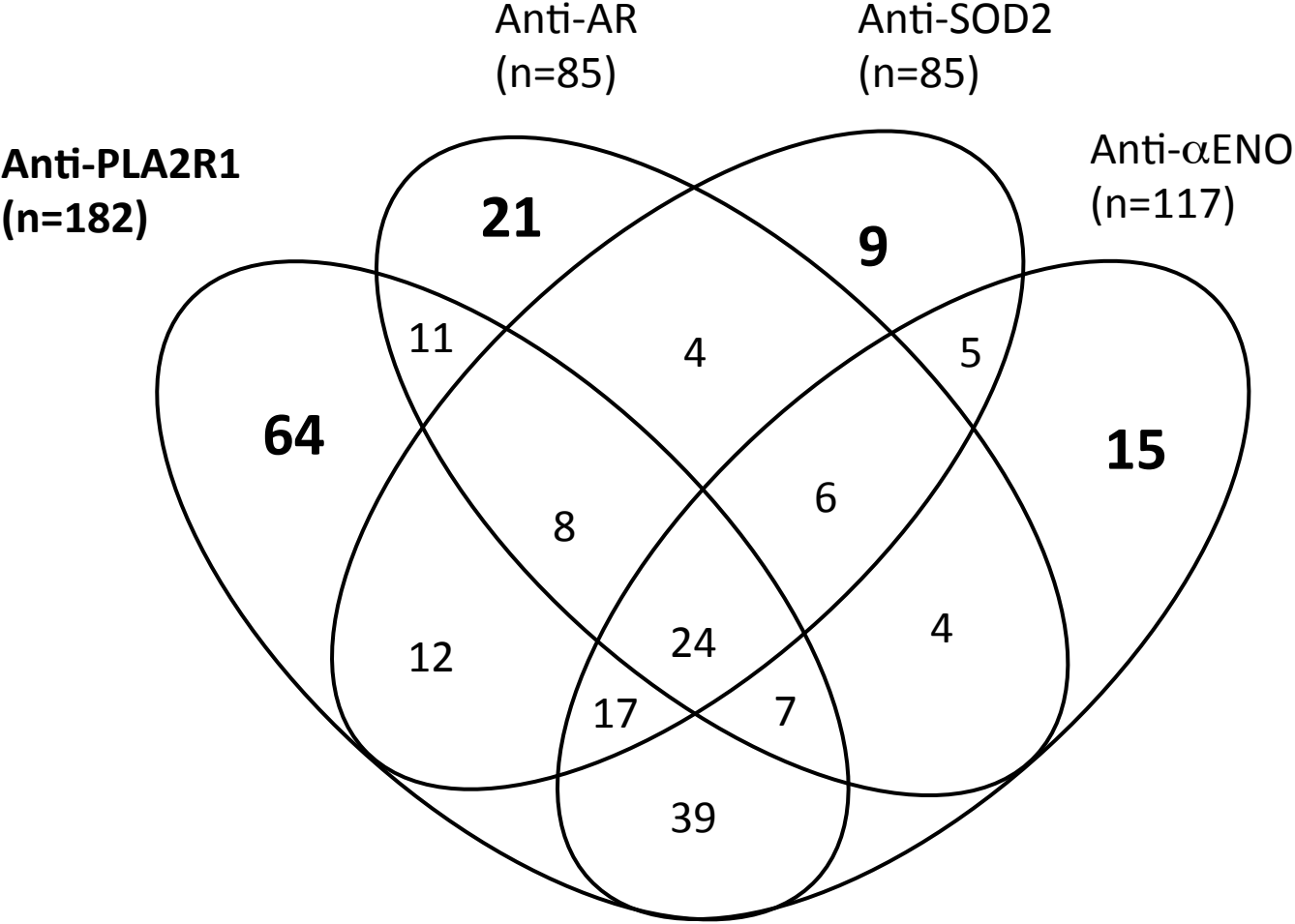
Supplemental Figure 3b

P < 0.0001   P=0.005*

* Comparison non-spreaders versus spreaders
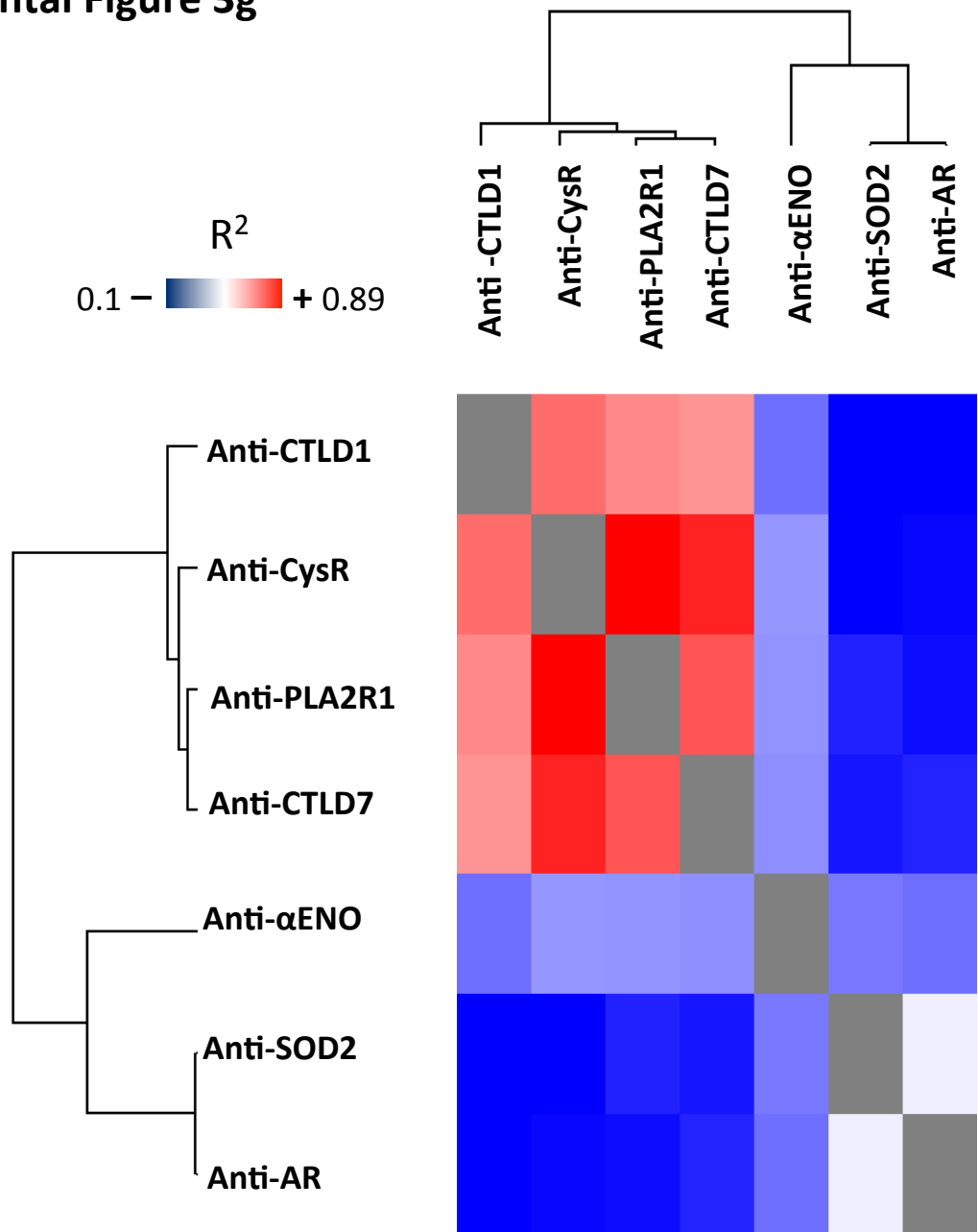
Supplemental Figure 3c

Supplemental Figure 3d

Supplemental Figure 3e

Anti-SOD2
(n=3)

Anti-AR
(n=4)

Anti-αENO
(n=5)

Anti-THSD7A
(n=8)

Anti-PLA2R1
(n=1)

1
1
1
1
1
1
1
1
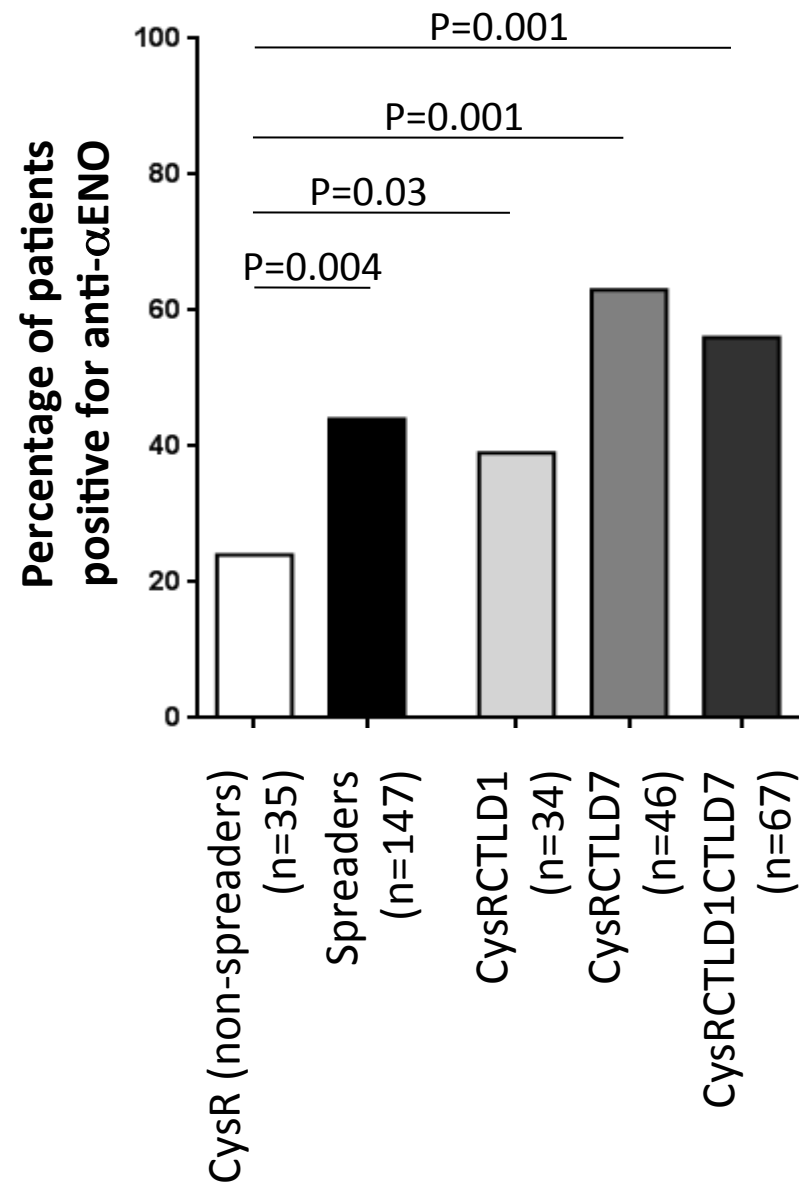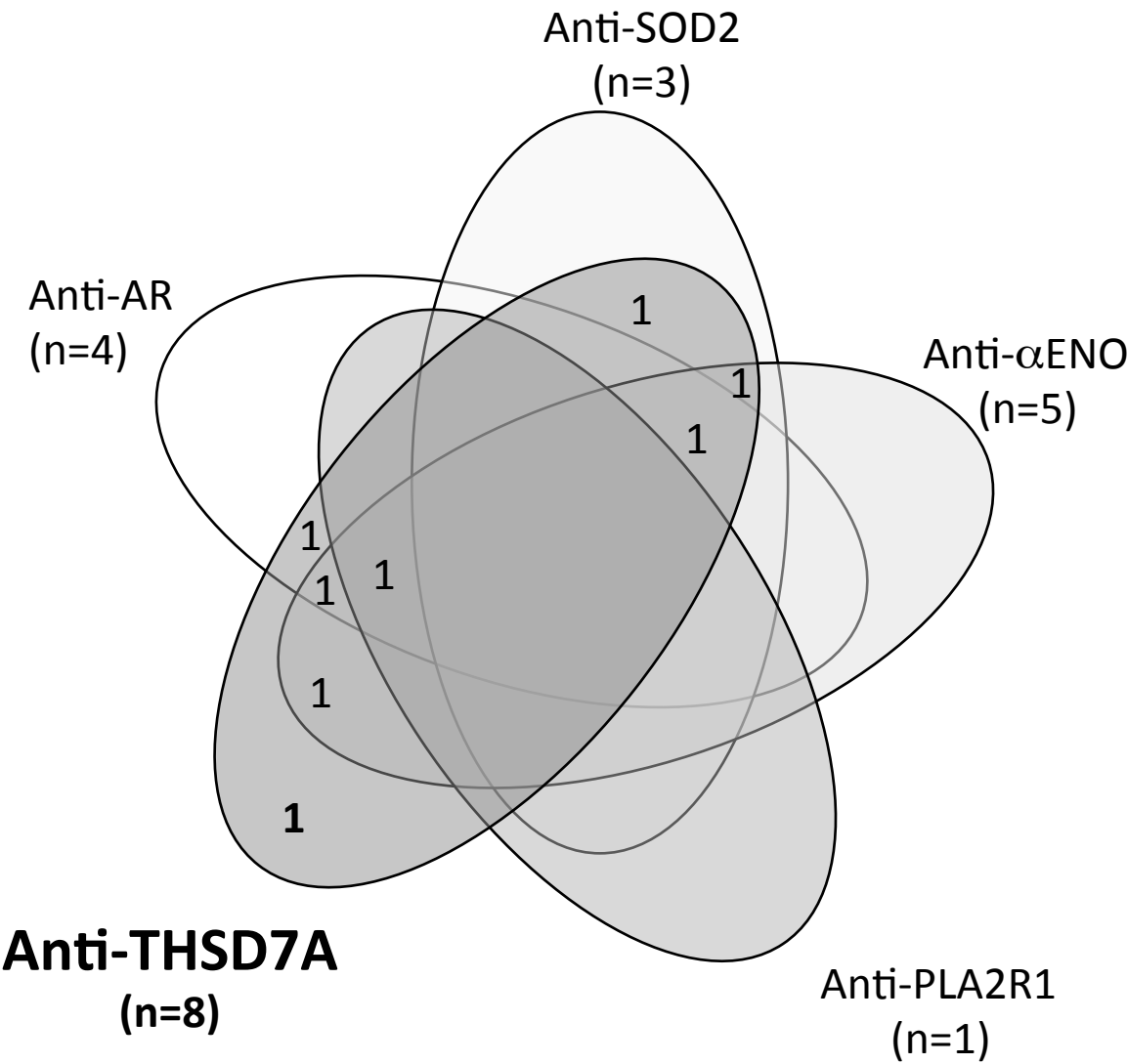
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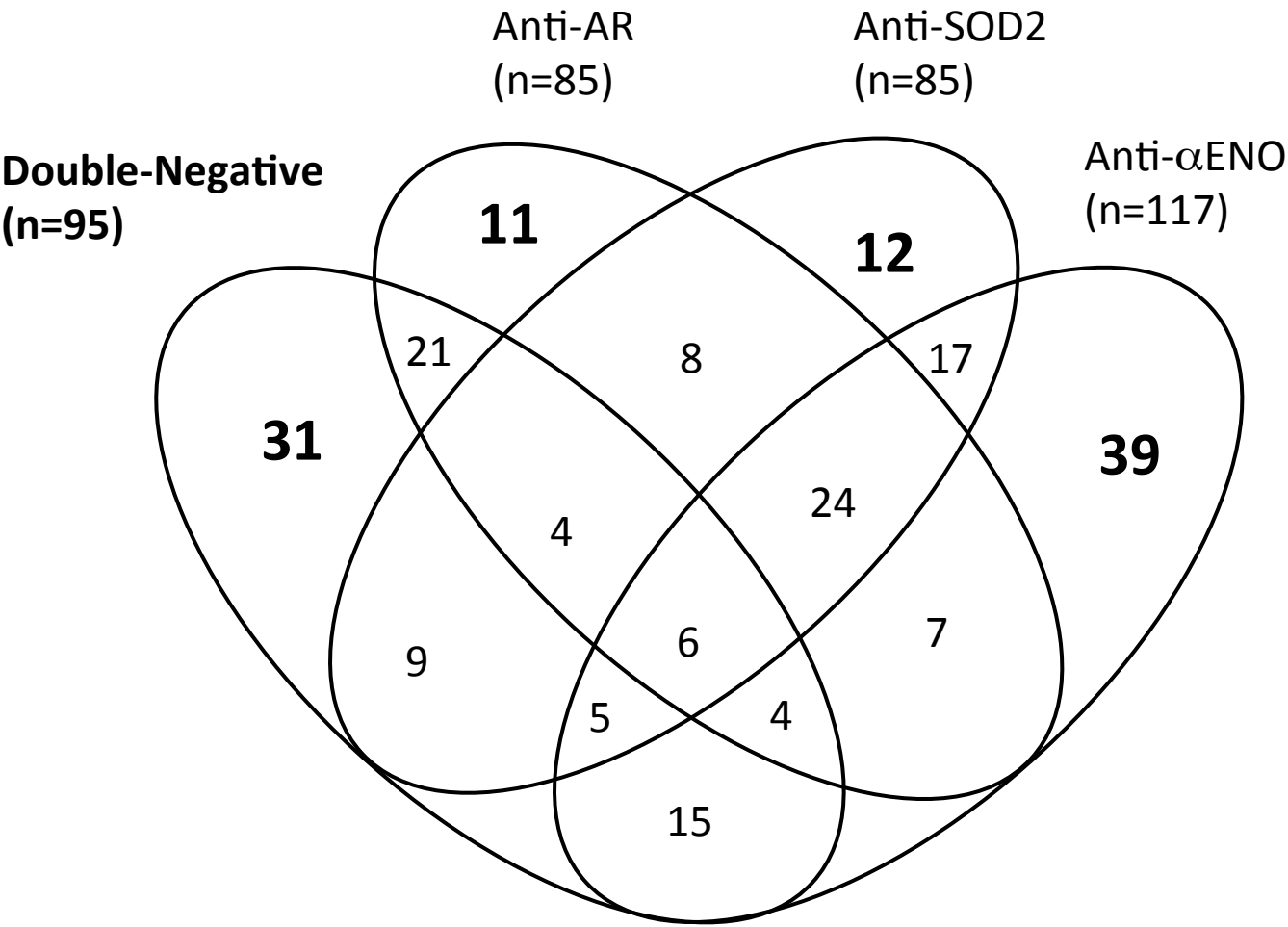
Supplemental Figure 3g

Supplemental Figure 4