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Multi-autoantibody Signature and Clinical Outcome in Membranous Nephropathy

Gian Marco Ghiggeri^{1*}, Barbara Seitz-Polski^{2,3*}, Joana Justino², Christelle Zaghrini², Christine Payré², Vesna Brglez^{2,3}, Guillaume Dolla², Alberto Sinico⁴, Francesco Scolari⁵, Augusto Vaglio⁶, Marco Prunotto⁷, Giovanni Candiano⁸, Antonella Radice⁹, The Italian Study Group for Membranous Nephropathy¹⁰, Maurizio Bruschi^{8*} and Gérard Lambeau^{2*}

¹Istituto Giannina Gaslini IRCCS, Department of Pediatric and Hemato-Oncology Science, Nephrology, Dialysis and Transplantation Unit, Genoa, Italy;

²Université Côte d'Azur, CNRS, Institut de Pharmacologie Moléculaire et Cellulaire (IPMC), Valbonne Sophia Antipolis, France;

³Université Côte d'Azur, Département d'Immunologie, CHU de Nice, France;

⁴University Bicocca, Department of Medicine and Surgery, Milan, Italia;

⁵University of Brescia, Montichiari Hospital, Nephrology and Dialysis Unit, Montichiari, Italy;

⁶University of Parma, Department of Clinical Medicine, Nephrology and Health Sciences, Parma, Italy;

⁷School of Pharmaceutical Sciences, University of Geneva, Geneva, Switzerland;

⁸Istituto Giannina Gaslini IRCCS, Molecular Nephrology Laboratory, Genoa, Italy;

⁹Microbiology Institute, ASST Santi Paolo e Carlo, S. Carlo Borromeo Hospital, Milan, Italy

¹⁰The Italian Study Group for Membranous Nephropathy (see expanded list).

****** GMG, BSP, MB and GL contributed equally to this work.

¹⁰The Italian Study Group for Membranous Nephropathy:

Gian Marco Ghiggeri, Nephrology, Dialysis, and Transplantation, Istituto Giannina Gaslini IRCCS, Genoa, Italy; coordinator of the group and PI of the study;

Antonella Radice, Microbiology Institute, ASST Santi Paolo e Carlo, S. Carlo Borromeo Hospital, Milan, Italy;

Alberto Sinico, Department of Medicine and Surgery, University Bicocca, Milan, Italy;

Francesco Scolari, Nephrology and Dialysis Unit, University of Brescia and Montichiari Hospital, Brescia, Italy;

Loreto Gesualdo, Division of Nephrology, University of Bari, Bari, Italy;

Gabriella Moroni, Division of Nephrology and Dialysis, IRCCS Foundation-Ospedale Maggiore, Mangiagalli, Regina Elena, Milan, Italy;

Riccardo Magistroni, Department of Nephrology, University of Modena, Modena, Italy;

Domenico Santoro, Division of Nephrology, University of Messina, Messina, Italy;

Antonello Pani, Division of Nephrology and Dialysis, Brotzu Hospital, Cagliari, Italy;

Augusto Vaglio, Landino Allegri and Isabella Pisani, Department of Clinical Medicine, Nephrology, and Health Sciences, University of Parma, Parma, Italy;

Antonio Granata, Division of Nephrology and Dialysis, San Giovanni di Dio Hospital, Agrigento, Italy;

Giuseppe Grandagliano, Ospedali Riuniti, Foggia, Italy;

Davide Rolla, Division of Nephrology and Dialysis, San Andrea Hospital, La Spezia, Italy;

Giuliano Boscutti, Division of Nephrology and Dialysis, Cattinara Hospital, Trieste, Italy;

Lucia Mardin bianco, Division of Nephrology and Dialysis, Gorizia Hospital, Italy;

Statistical control and analysis

Maurizio Bruschi, Molecular Nephrology Laboratory, Istituto Giannina Gaslini IRCCS, Via Gerolamo Gaslini 5, 16147 Genova;

Pietro Ravani, Division of Nephrology, University of Calgary, 1403-29th Street, NW, Calgary, Alberta T2N 2T9, Canada.

Local coordination

Monica Bodria, Division of Nephrology, Dialysis and Transplantation, Istituto Giannina Gaslini IRCCS, Genoa, Italy;

Giovanni Candiano, Division of Nephrology, Dialysis and Transplantation, Istituto Giannina Gaslini IRCCS, Genoa, Italy.

Correspondence:

Dr Gian Marco Ghiggeri, Dipartimento Integrato di Scienze Pediatriche e Emato-Oncologiche, U.O.C. Nefrologia, Dialisi e Trapianto, Istituto Giannina Gaslini IRCCS, Via Gerolamo Gaslini 5, 16147 Genova, Email: gmarcoghiggeri@gaslini.org

Dr Gérard Lambeau, Institut de Pharmacologie Moléculaire et Cellulaire, UMR 7275 CNRS et Université Côte d'Azur, Valbonne Sophia Antipolis, France. Email: lambeau@ipmc.cnrs.fr

Running Title: Multi-autoantibody composition in Membranous Nephropathy

Abstract

Background and objectives Patients with membranous nephropathy can have circulating autoantibodies against membrane-bound (PLA2R1 and THSD7A) and intracellular (AR, SOD2 and α ENO) podocyte autoantigens. We studied their combined association with clinical outcome.

Design, setting, participants, & measurements Serum levels of anti-PLA2R1, anti-THSD7A, anti-AR, anti-SOD2 and anti- α ENO autoantibodies were determined in 285 patients at diagnosis and during follow-up using standardized and homemade assays. Maintenance of eGFR >60 mL/min/1.73 m² and remission of proteinuria (<0.3 / <3.5 g/day) after 12 months were the clinical outcome targets.

Results At diagnosis, 182 (64%), 8 (3%) and 95 (33%) patients were anti-PLA2R1+, anti-THSD7A+ and double-negative, respectively. The prevalence of a detectable antibody to at least one intracellular antigen was similarly distributed in anti-PLA2R1+ (n=118, 65%) and double negative (n=64, 67%) patients. Positivity for anti-PLA2R1, anti-SOD2 and anti- α ENO antibodies and higher titers at diagnosis were independently to each other associated with poor clinical outcome. Combined positivity for anti-PLA2R1, anti-SOD2 and anti- α ENO was associated with maximal risk (OR 5.5[1.2-24], p=0.01). In Kaplan-Meier analysis, patients anti-PLA2R1+/anti-SOD2+ or anti-PLA2R1+/anti- α ENO+ had reduced eGFR at 12 months compared to anti-PLA2R1+/anti-SOD2- or anti- α ENO- patients. Similar associations were observed when considering treatment. Predictive tests (NRI and ROC-AUC) showed that combined assessment of antibodies improved classification of outcome in 22-34% of cases for partial remission of proteinuria and maintenance of normal eGFR. For patients with nephrotic syndrome at diagnosis, anti-SOD2 positivity and high anti-PLA2R1 titer were associated with lack of complete remission. Patients anti-PLA2R1-/intracellular- had the lowest proteinuria and the highest eGFR at diagnosis and the lowest risk of reducing eGFR at 12 months. Epitope spreading was present in 81% of anti-PLA2R1+ patients and was associated with increased positivity for intracellular antigens and poor eGFR at diagnosis and 12 months.

Conclusions Combined serological analysis of autoantibodies targeting membrane-bound and intracellular autoantigens identifies patients with poor clinical outcome.

Introduction

Membranous nephropathy is a common cause of nephrotic syndrome in adults (1-3). It is characterized by thickening of the glomerular basement membrane with a well-defined pattern of subepithelial immune complex deposits, usually including IgG4 autoantibodies against various autoantigens. The clinical outcome of membranous nephropathy is variable (4). About one third of patients does not require immunosuppressive therapy and reach spontaneous remission while two thirds require immunosuppressive treatment (such as cyclosporine, cytotoxic agents or anti-CD20 antibodies) and respond or not to treatment, with possible progression to end-stage kidney disease over 5-10 years (5-10).

Antibodies against various podocyte autoantigens have been shown to play a role in the pathogenesis of various forms of membranous nephropathy. In rare familial cases of alloimmune membranous nephropathy, autoantibodies were identified against neutral endopeptidase (11). These autoantibodies developed in mothers deficient for neutral endopeptidase and crossed the placenta to induce membranous nephropathy in the fetus.

This elegant demonstration of a first causative autoantibody of membranous nephropathy was followed by the identification of phospholipase A2 receptor 1 (PLA2R1) as the major autoantigen in adult membranous nephropathy, with circulating autoantibodies present in about 70% of patients (12). Genetic studies further supported a causal role of PLA2R1 based on association of the disease with 6p21 *HLA-DQA1* and 2q24 *PLA2R1* loci in cohorts of Caucasian (13) and Asian (14) patients. Multiple epitopes have been identified in the CysR, CTLD1, CTLD7 and CTLD8 domains of PLA2R1 (15-20), which may be reminiscent of a mechanism of epitope spreading reflecting the maturation of the immune response from CysR to other PLA2R1 domains (21, 22). Epitope spreading has also been documented in the Heymann rat nephritis model where megalin is the major autoantigen (23).

Thrombospondin type-1 domain containing 7A (THSD7A) was reported as a second autoantigen in adult membranous nephropathy, with circulating anti-THSD7A autoantibodies present in about 3% of a different group of patients (24).

Autoantibodies targeting different intracellular podocyte autoantigens including aldose reductase (AR) (25), superoxide dismutase (SOD2) (26) and α -enolase (α ENO) (27) have been identified in a significant number of patients with membranous nephropathy (28).

Finally, three new antigenic markers of autoimmunity (exostosin1/exostosin2 and NELL-1) have been very recently identified in immune deposits, with exostosins being more present in

secondary cases of membranous nephropathy (*i.e.* class V lupus nephritis) (29, 30). The exact prevalence and significance of autoantibodies against these new autoantigens in the pathogenesis of membranous nephropathy remain to be determined, with circulating autoantibodies currently found only for NELL-1, and with different prevalence between American and European cohorts of patients (29, 30).

In this study, we aimed to 1) detect autoantibodies directed against the well-characterized membrane-bound autoantigens PLA2R1 and THSD7A and the intracellular autoantigens SOD2, AR and α ENO in a large retrospective cohort of 285 patients with membranous nephropathy at diagnosis and after 6 and 12 months of follow-up, and 2) evaluate the clinical outcome of patients stratified into different groups based on their combined positivity or not for multiple autoantibodies directed against the different autoantigens.

Materials and Methods

Study design and patient population

This is a cross-sectional study in patients with MN assessing the relationships between detection of different autoantibodies at baseline and eGFR and proteinuria at 12 months. We included 285 patients at the time of a histopathological finding of idiopathic or primary membranous nephropathy (Table S1) and followed them with clinical visits for at least 12 months. They were enrolled in the frame of two studies: 244 patients were enrolled in Italy (Italian Study Group on Membranous Nephropathy-EudraCT 2011-003942-41) and 41 in France (Cohort SOURIS NCT02199145). Criteria for enrollment were (1) biopsy-proven diagnosis of membranous nephropathy associated with proteinuria >0.3 g/day, (2) normal complement profile, (3) negative tests for ANA, anti-dsDNA, ANCA, cryoglobulins and absence of viral markers (hepatitis B surface antigen and HIV), (4) absence of clinical and biochemical signs of cancer and (5) serum samples available at diagnosis and during follow up for determination of antibody levels. Clinical studies were approved by the relevant institutional review boards in the different hospitals and were conducted according to the principles of the Declaration of Helsinki, with written informed consent obtained from all patients. The study was presented at the Ethical Committee of IRCCS Giannina Gaslini. Serum was also obtained from 50 healthy donors recruited at the same institutions that participated to the sample recruitment. Healthy donors had at least one normal urine analysis and normal clinical tests in the prior six months (Table S1).

Detection of circulating autoantibodies

Anti-PLA2R1 autoantibodies (detection of total IgG) against the full-length extracellular domain of PLA2R1 were measured with the standardized ELISA from Euroimmun (Medizinische Labordiagnostika AG, Lübeck, Germany) (33). Anti-PLA2R1 autoantibodies (detection of IgG4 subclass) against the specific epitope-containing domains CysR, CTLD1 and CTLD7 were determined as described (19) using recombinant soluble forms of each domain harboring a HA-tag and produced in HEK293 cells. Anti-THSD7A autoantibodies (detection of IgG4 subclass) against the full-length extracellular domain of THSD7A were determined by ELISA as described (34). Positivity was confirmed by western-blot (24) and indirect immunofluorescence (35) assays. Autoantibodies against the intracellular antigens AR, SOD2 and α ENO (detection of IgG4 subclass) were determined by dot-blot using recombinant proteins as immobilized antigens as described (26, 27). Further details on the detection methods and source of antigens are given in the supplemental material. For anti-PLA2R1 total IgG, we used the normal limit of positivity of

20 RU/mL given by the manufacturer (33). For anti-THSD7A and intracellular autoantibodies, normal limits were calculated from ROC curves as described (34, 36). High and low levels of autoantibodies were defined as the titers above median and between median and limit of normality, respectively (Table S2).

Outcome

Clinical outcome was evaluated on the basis of proteinuria levels at diagnosis versus after 12 months of follow-up, and was defined as complete remission (proteinuria <0.3 g/day) or partial remission (proteinuria <3.5 g/day) according to Thompson *et al* (31). Failure to achieve clinical remission was defined by persistence of proteinuria >3.5 g/day. Kidney function (eGFR) was evaluated based on the CKD-EPI creatinine equation (32).

Statistical analysis

Autoantibody levels were expressed as median and interquartile range. The differences in serum concentration of all autoantibodies within patient groups were analyzed using non-parametric U Mann-Whitney test for unpaired samples; Spearman's correlation was used to evaluate correlations among circulating levels of the various types of autoantibodies. The receiver-operating characteristic (ROC) curve analysis was used to discriminate between healthy people and MN patients based on levels of each autoantibody at diagnosis. The best cut-offs were selected as the values that minimized the geometric distance from 100% sensitivity and 100% specificity on the ROC curves. In these analyses, discrimination is assessed as the ability of the test (performance) to distinguish the presence of the outcome beyond chance, defined when the area under the curves (AUC) are significantly >0.5. Odds ratios (OR) and 95% confidence intervals (CI) were tested to determine the associations between autoantibodies positivity or high titer and proteinuria and eGFR using 2x2 contingency tables. Fisher's exact test was used to determine the statistical significance. The P-values were corrected for multiple comparisons with alpha error values of 5%. We used the Kaplan-Meier method to construct survival curves for time to events according to the level of proteinuria. Differences in outcomes were estimated using the log-rank (Mantel-Cox) test and their hazard ratio; *p*-values ≤0.05 were considered as significant. The performance of single antibody and their synergism for predicting clinical outcome were assessed by two-category net reclassification index (NRI) and area under the curve of receiver operating characteristic analysis (AUC-ROC) (36). We used R for all statistical analyses (<http://www.R-project.org/>).

Results

Patients and predictors of clinical outcome

We included 285 patients with biopsy-proven membranous nephropathy whose the clinical characteristics are shown in Tables 1 and S1, according to PLA2R1 and intracellular antibody positivity. Most patients were men (n=194, 68%). At diagnosis, 67% of patients had nephrotic syndrome. Seventy-nine percent of patients were treated with immunosuppressive treatments: steroids (33%), cytotoxic drugs (32%), rituximab (12%), and cyclosporine (25%). Antiproteinuric treatment with angiotensin converting enzyme inhibitors (ACEi) was added to the above therapy. Twenty-one percent of patients received only ACEi. Patients with nephrotic syndrome (n=177, 62%) had persistent high proteinuria after antiproteinuric treatment, with evidence for deterioration of kidney function (eGFR). When the cohort was analyzed as a whole, gender and nephrotic proteinuria at diagnosis were the only two clinical factors associated with the risk of failure to achieve complete remission of proteinuria after 12 months of follow-up (Figure S1), in agreement with previous findings (31).

Individual autoantibodies: distribution and levels

All patients were tested at diagnosis for the presence of circulating autoantibodies recognizing PLA2R1 and THSD7A by specific ELISAs (33, 34). The presence of circulating autoantibodies specifically recognizing the CysR, CTLD1 and CTLD7 domains of PLA2R1 were also analyzed, defining non-spreaders (patients with detectable reactivity limited to CysR) and spreaders (patients with detectable reactivity to CysR, CTLD1 and/or CTLD7 domains) (19, 37). Patients were also tested for the presence of autoantibodies recognizing the intracellular autoantigens SOD2, AR and α ENO (26, 27). Patients with single or multiple positivities to any of these intracellular antigens were referred to as Intracellular⁺ while patients with no detectable reactivity were referred to as Intracellular⁻. The same analyses were repeated after 6 and 12 months (Figures S2a-S2e).

Among all patients, 182 (64%) were positive for anti-PLA2R1 (PLA2R1⁺ patients, Figures 1a and S2a). The median level of anti-PLA2R1 autoantibodies was 122 RU/mL (IQR 60-267) at baseline and decreased after 6 and 12 months of follow-up (Figure S2a). At baseline, 118 (65%) PLA2R1⁺ patients were positive for one or more intracellular antigens, defining PLA2R1⁺/Intracellular⁺ patients while 64 (35%) were negative, defining PLA2R1⁺/Intracellular⁻ patients (Table 1, Figures 1a and S3a). Interestingly, the percentage of Intracellular⁺ patients was higher in patients with high titer of anti-PLA2R1 autoantibodies (Figure 1b). Furthermore, PLA2R1⁺ patients defined as

spreaders had higher anti-PLA2R1 titers than non-spreaders (Figure S3b) and were also more positive for intracellular autoantigens (Figure S3c and Table S3), in particular for α ENO (Figure S3d).

Among patients, only eight (3%) were positive for anti-THSD7A (THSD7A⁺, Figures 1a and S2b). One was double-positive for PLA2R1 and THSD7A (Figure S2b). Seven of these patients had additional autoantibodies against at least one intracellular autoantigen (Figures 1a and S3e). Because of their low prevalence, anti-THSD7A⁺ patients were not considered for further analysis.

Thus, among all patients, 95 patients (33%) were identified as double-negative for PLA2R1 and THSD7A. Among these latter patients, 64 (67%) were also positive for one or more intracellular antigens, defining double-negative/Intracellular⁺ patients while 31 (33%) were negative, defining double-negative/Intracellular⁻ patients (Table 1, Figure 1a and S3f).

Overall, autoantibodies against AR, SOD2 and α ENO were detected in 85, 85 and 117 patients, respectively (Figures S2c, S2d and S2e). The median levels of each intracellular autoantibody decreased at six and 12 months of follow-up (Figures S2c, S2d and S2e). Positivity and titer levels for anti-PLA2R1, anti-SOD2, anti-AR and anti- α ENO autoantibodies at baseline did not correlate to each other (Figure S3g).

Association of the different antibodies with clinical outcome

Considering all patients, positivity for anti-PLA2R1, anti-SOD2 and anti- α ENO autoantibodies at diagnosis was variably and independently associated with complete or partial remission of proteinuria and eGFR after 12 months (Table 2). Concomitant positivity of the three antibodies had the highest odds ratio for complete remission of proteinuria after 12 months (OR 5.5 [1.2-24], $p=0.01$). High titers of anti-PLA2R1 and anti-SOD2 above the median (Table S2, $n=91$ and $n=43$, respectively) were similarly associated with lack of partial or complete remission of proteinuria after 12 months compared to those with low titers (Table 2). Kaplan-Meier analysis showed that combined positivity for anti-PLA2R1 and anti-SOD2 or anti- α ENO was associated with a higher risk of low eGFR (<60 ml/min/1.73 m²) after 12 months compared to positivity for only anti-PLA2R1 (Figure 2). Additionally, high titers of anti-PLA2R1 and anti- α ENO were associated with reduced eGFR after 12 months, as compared to low titers (Table 2). Considering only patients with nephrotic syndrome at diagnosis (67% of the whole cohort), the associations were different (Table 3). Only positivity for anti-SOD2 antibodies and concomitant positivity for anti-PLA2R1 and anti-SOD2 were associated with persistence of proteinuria after 12 months. High titers of anti-PLA2R1 were also associated with persistence of proteinuria. Considering treatment, positivity and

high levels of anti-PLA2R1 and anti-SOD2 were the main features associated with lack of partial and complete remission after 12 months in patients who had been treated with cytotoxic drugs, cyclosporine A or rituximab (Table S4).

We further compared the added value of anti-SOD2 and anti- α ENO over anti-PLA2R1 to predict clinical outcome using net reclassification improvement (NRI) and area under the receiver-operating-characteristic curve (AUC-ROC) analysis (38). In NRI analysis, the association of two antibodies allowed the reclassification of patients who developed or maintained proteinuria <3.5 g/day after 12 months in 34% and 22% of cases negative for both PLA2R1 and SOD2 or α ENO, respectively (Table 4). The same association allowed reclassification of eGFR >60 mL/min/1.73 m² in 27% and 24% of patients at 12 months, respectively (Table 4). AUC-ROC analysis discriminated the outcome after 12 months in respect to the titer of a single antibody or its association. In this case, the association of more than one antibody slightly increased the discrimination for patients who developed eGFR <60 mL/min/1.73 m² (Table 4).

Stratification of patients based on combined positivity for multiple autoantibodies and association with clinical outcome

Based on combined positivity for autoantibodies against PLA2R1 and intracellular antigens at diagnosis and excluding THSD7A⁺ patients, we stratified the remaining 277 patients into four groups (Table 1). Hundred eighteen patients were anti-PLA2R1⁺/Intracellular⁺ (*i.e.* with additional single or multiple positivity for autoantibodies against intracellular antigens), 64 patients were anti-PLA2R1⁺/Intracellular⁻, 64 patients were anti-PLA2R1⁻/Intracellular⁺, and 31 patients were anti-PLA2R1⁻/Intracellular⁻. At diagnosis, the four groups did not differ for age or sex and had the same percentage of histology classes (Table 1). All patients received comparable therapy (Table 1). Proteinuria at diagnosis was higher in the two groups anti-PLA2R1⁺ versus those anti-PLA2R1⁻ (Table 1 and Figure 3a). Proteinuria after 12 months was lower and eGFR higher in anti-PLA2R1⁻/Intracellular⁻ patients versus others (Table 1 and Figures 3a and 3b).

Differences in outcomes were estimated using Kaplan-Meier curves and the log-rank (Mantel-Cox) test and their hazard ratio. Forty-five percent of anti-PLA2R1⁻/Intracellular⁻ patients reached complete remission of proteinuria after 12 months, as compared to 16, 16 and 28% for the other groups (Figure 4a). More anti-PLA2R1⁻/Intracellular⁺ patients had a worse clinical outcome after 12 months as compared to anti-PLA2R1⁻/Intracellular⁻ patients (Figure 4b). Odds ratios and 95% confidence intervals were utilized to determine the association with reduced eGFR after 12 months. Patients anti-PLA2R1⁺/Intracellular⁺ and anti-PLA2R1⁻/Intracellular⁺ had about four-fold

higher risk of developing eGFR <60 mL/min/1.73 m² after 12 months, as compared to patients PLA2R1⁺/Intracellular⁺ (Figure 4c). We finally analyzed whether the combined positivity for PLA2R1 epitope spreading and intracellular antigens may be associated with clinical outcome (Table S5). The concomitance of spreading and anti- α ENO positivity was associated with low eGFR at diagnosis and after 12 months (Figure S4) whereas eGFR at diagnosis in non-spreaders was only barely modified by positivity for anti- α ENO autoantibodies (not shown).

Discussion

This is the largest retrospective study cohort of membranous nephropathy patients with the combined detection of autoantibodies against the two major membrane-bound antigens PLA2R1 and THSD7A and the three intracellular autoantigens AR, SOD2 and α ENO. As expected, PLA2R1⁺ patients represent the majority of the cohort (64%) while THSD7A⁺ patients represent only 3%, in line with recent studies (34, 35, 39). Our results confirmed that high titers of anti-PLA2R1 autoantibodies are associated with poor outcome in terms of remission of proteinuria and eGFR (40-45). We also confirmed that epitope spreading was associated with reduced eGFR after 12 months (19, 37).

In this study, we add to the above points that the presence of circulating anti-SOD2 and anti- α ENO autoantibodies was independently associated with poor outcome in terms of partial and complete remission of proteinuria and eGFR. Furthermore, anti-SOD2, anti- α ENO and anti-AR autoantibodies are not correlated to anti-PLA2R1 autoantibodies, and similar percentages of patients were positive for one or more intracellular antigens, irrespective of positivity (n=118, 65%) or not (n=64, 67%) for PLA2R1. Further analysis by Kaplan-Meier curves showed worse clinical outcome for patients positive for anti-PLA2R1 and anti-SOD2 or anti- α ENO while NRI and AUC-ROC suggested that intracellular autoantibodies might have an added value over anti-PLA2R1 for refinement of association with clinical outcome. We thus conclude that the positivity for intracellular autoantibodies has an added value over anti-PLA2R1 positivity to predict adverse clinical outcome. It remains to be seen if this might be also true for other membrane-bound antigens such as THSD7A or the new extracellular autoantigens, in particular NELL-1.

Based on the concomitant presence or absence of anti-PLA2R1 and anti-intracellular autoantibodies, we stratified patients into four groups. Extending what was reported above for anti-PLA2R1 positivity, another novel finding is that patients negative for the two categories of autoantibodies have a reduced proteinuria at diagnosis and a better clinical outcome after 12 months of follow-up, in comparison to other patients. Thus, in anti-THSD7A negative patients, the simultaneous measurement of the two categories of autoantibodies (anti-PLA2R1 and anti-SOD2 and anti- α ENO) at baseline might be an additive factor to better evaluate clinical outcome. In this setting, positivity to intracellular antigens may represent *per se* a negative factor associated with poor clinical outcome. However, we acknowledge limitations in our study including the use of a retrospective cohort with patients with different ages, different clinical characteristics and

treatments, and homemade assays to measure autoantibodies to intracellular autoantigens. Our results should thus be confirmed in prospective studies and/or randomized clinical trials.

The second main finding reported here is that positivity for autoantibodies targeting the intracellular autoantigens, especially anti- α ENO autoantibodies, is maximal in PLA2R1⁺ patients with high anti-PLA2R1 titer or those defined as spreaders. The combined positivity associates with a reduction of eGFR, being worse in patients with high anti-PLA2R1 titer (Table 1) and in spreaders (Figure S4) positive for anti- α ENO autoantibodies. The lower positivity of anti- α ENO autoantibodies or other intracellular autoantigens in patients with low anti-PLA2R1 titer or defined as non-spreaders suggests that the formation of autoantibodies against intracellular antigens represents a secondary phenomenon that occurs after the formation of autoantibodies against PLA2R1 (and possibly THSD7A) as primary triggers of membranous nephropathy. We thus propose that anti-SOD2 and anti- α ENO autoantibodies may appear as a second wave of autoantibodies in the pathogenesis of membranous nephropathy. This hypothesis is also supported by the *in vitro* and *in vivo* data obtained in the mouse model of THSD7A-associated membranous nephropathy where SOD2 was induced after podocyte injury and in carbonic anhydrase II-associated membranous nephropathy (46, 47).

Anti-SOD2 and anti- α ENO autoantibodies are not unique to membranous nephropathy and their occurrence has been described in lupus nephritis (27, 48). However, the IgG subclass is different in membranous nephropathy and lupus nephritis, with IgG4 in membranous nephropathy but IgG2 in lupus nephritis. Therefore, the same autoantigen can trigger the formation of autoantibodies with different IgG isotypes depending on the type of disease. For lupus nephritis, it has been speculated that exposure of methyl-oxidized α ENO and/or the DNA-oxidized α ENO by neutrophils to TLR9 is a possible mechanism leading to the production of anti- α ENO antibodies (48, 49).

SOD2 is a key antioxidant enzyme playing a major role in protecting cells from oxidative injury (50). It is activated as the main antioxidant pathway after formation of autoimmune deposits in glomeruli. Formation of anti-SOD2 autoantibodies may modify the antioxidant protective effect of SOD2 and worsen the clinical outcome.

α ENO is a glycolytic enzyme that catalyzes dehydration of 2-phospho-D-glycerate to phosphoenolpyruvate and produces pyruvate and ATP in the Embden-Meyerhof-Parnas pathway (51). α ENO has also an extracellular localization where it functions as a plasminogen receptor (52). Upon binding to α ENO, plasminogen is transformed into plasmin that degrades extracellular matrix

components and activates metalloproteases (51, 53). The presence of anti- α ENO autoantibodies may reduce its anti-fibrotic role and favor the accumulation of ECM.

Overall, the results of this study show a multi-autoantibody composition in membranous nephropathy, with antibodies that may act in concert, leading to high proteinuria and low eGFR. Based on the possible functions of SOD2 and α ENO after podocyte injury both *in vitro* and *in vivo* (46, 47), we propose a multi-hit pathogenic mechanism of membranous nephropathy that first involves the formation of anti-PLA2R1 (or anti-THSD7A or autoantibodies to another similar "primary" autoantigen), that then progresses by a mechanism of both intramolecular (such as within PLA2R1) and intermolecular epitope spreading along which i) the anti-PLA2R1 titer increases and ii) is exacerbated by a second wave of autoantibodies targeting intracellular autoantigens, leading for instance to the production of anti-SOD2 and/or anti- α ENO autoantibodies. In patients negative for anti-PLA2R1 and anti-THSD7A autoantibodies, an autoantibody targeting a still unknown membrane-bound autoantigen would take a role similar to anti-PLA2R1 or anti-THSD7A antibodies, possibly represented by patients positive for NELL-1, exostosins or other unknown antigens (29, 30).

In conclusion, our study first confirms the relevance of detecting and measuring anti-PLA2R1 (and anti-THSD7A) autoantibodies as fundamental tools to diagnose and identify patients with membranous nephropathy and at risk of severe disease. The study further shows the added value of assessing autoantibodies targeting intracellular podocyte antigens, combined or not with positivity for the above membrane-bound autoantigens. Altogether, our findings pave the way for more personalized medicine in membranous nephropathy.

Author Contributions

G.M.G, B.S.P. and G.L. designed the study; J.J., C.Z., C.P., V.B., G.D., C.M., M.B., G.C and A.S. carried out experiments; G.M.G, B.S.P., M.B., M.P. and G.L. analyzed the data; G.M.G, B.S.P., J.J., M.B. and G.L. made the figures; G.M.G, B.S.P., J.J., M.B. and G.L. drafted and revised the paper; A.S., F.S., A.V., M.P. and B.S.P. had a major role in the inclusion of patients; all authors approved the final version of the manuscript.

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Disclosures

Some coauthors are co-inventors on the patents “Diagnostics for membranous nephropathy” (GL), “Methods and kits for monitoring membranous nephropathy” (BSP and GL), and “Prognosis and monitoring of membranous nephropathy based on the analysis of PLA2R1 epitope profile and spreading” (BSP, GD and GL). GMG is inventor on a patent on the use of anti-SOD2 and anti-ENO antibodies as biomarkers in membranous nephropathy (EP 3097419).

Supplemental Material

This article contains the following supplemental material:

1. *Supplemental Methods*
2. *Supplemental references*
3. *Supplemental Tables and Figures*

Table S1. Epidemiology, histological stage, clinical characteristics and treatment of patients with membranous nephropathy (MN) versus controls (Healthy donors) at diagnosis and after 12 months of follow-up.

Table S2. Quantitative data for limits of positivity and distribution of serum levels for each autoantibody.

Table S3. Detailed autoantibody positivity for intracellular antigens in non-spreaders and spreaders PLA2R1+ patients.

Table S4. 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.

Table S5. Clinical characteristics at diagnosis and after 12 months of follow-up for PLA2R1+ patients stratified according to epitope profiles.

Figure S1. Prognosis clinical factors associated with complete remission.

Figure S2. Circulating levels of the various autoantibodies at diagnosis and during follow-up.

Figure S3. Multi-autoantibody composition in the cohort of patients with membranous nephropathy.

Figure S4. Kidney function (eGFR) in spreaders positive (n=63) or not (n=84) for anti- α ENO autoantibodies.

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Legend to Figures

Figure 1. Distribution of circulating autoantibodies against the membrane-bound autoantigens (PLA2R1 and THSD7A) further divided according to the combined positivity for intracellular autoantigens (AR, SOD2 and α ENO). (a) Patients were first divided according to the presence of circulating autoantibodies targeting the membrane-bound autoantigens PLA2R1 or THSD7A. The three groups of patients (PLA2R1⁺, THSD7A⁺ and PLA2R1⁻/THSD7A⁻) were further divided according to positivity for autoantibodies targeting any of the three intracellular podocyte autoantigens (AR, SOD2, α ENO). (b) Stratification of PLA2R1⁺ patients according to anti-PLA2R1 titers below and above the median. *The percentage of patients positive for intracellular autoantibodies was higher in those with high anti-PLA2R1 titers (p=0.02, Fisher exact test).

Figure 2. Additive effect of anti-SOD2+ and anti- α ENO+ to anti-PLA2R1+ for decreased eGFR after 12 months of follow-up. Kaplan-Meier curve showing the proportion of patients in the different groups who reached the endpoint of reduction of kidney function (eGFR <60 mL/min/1.73 m²) after 12 months of follow-up based on the concomitant positivity or not for anti-PLA2R1 and anti-SOD2 (a) or anti- α ENO (b). The Kaplan-Meier curve of the difference was calculated with the Log-rank (Mantel-Cox) test and the hazard ratios with confidence intervals for the different groups.

Figure 3. Clinical outcome of proteinuria and eGFR according to circulating autoantibodies defining four groups of patients. (a) Proteinuria and (b) eGFR values (data are presented as median with IQR) at diagnosis and after 12 months of follow-up for patients divided in four groups based on the concomitant positivity or not for anti-PLA2R1 (PLA2R1⁺ or PLA2R1⁻) and anti-intracellular antigen antibodies (Intracellular⁺ or Intracellular⁻). Proteinuria (a) and eGFR (b) values are presented at diagnosis (open bars) and after 12 months (grey bars) for all patients (overall), for patients with nephrotic syndrome (NS) or not (non NS) at diagnosis and for patients who had been treated with an immunosuppressive treatment (IS-treated with cyclophosphamide, cyclosporine A or rituximab) after 12 months of follow-up (black bars).

Figure 4. Clinical outcome (complete remission and eGFR) according to circulating autoantibodies defining four groups of patients. (a) Kaplan-Meier curve showing the proportion of patients in the different groups (based on the concomitant positivity or not for anti-PLA2R1

(P+/P-) and anti-intracellular antibodies (IC+/IC-) who reached the endpoint of complete remission (proteinuria <0.3 g/day) after 12 months of follow-up. The Kaplan-Meier curve of the difference was calculated with the Log-rank (Mantel-Cox) test and the hazard ratios with confidence intervals for the different groups. **(b)** Kaplan-Meier curve showing the proportion of patients in the different groups (based on the concomitant negativity for anti-PLA2R1 (P-) and anti-intracellular antigen antibodies negativity or positivity (IC+/IC-) who reached the endpoint of reduction of kidney function (eGFR <60 mL/min/1.73 m²) after 12 months of follow-up. **(c)** Odds ratios and confidence intervals were calculated for the association between the different groups and the risk of not reducing kidney function (eGFR <60 mL/min/1.73 m²) after 12 months of follow-up.

Table 1. Comparison of clinical characteristics at baseline (T₀) and at clinical outcome (T₁₂) of MN patients stratified into four groups according to their antibody profile. Patients both PLA2R1 and intracellular antibodies positive; patients PLA2R1 positive and intracellular antibodies negative; patients PLA2R1 negative and intracellular antibodies positive; and patients both PLA2R1 negative and intracellular antibodies negative. Data are reported as number and percentage or median with interquartile range. Mann-Whitney or Kruskal–Wallis tests were used for continuous variables, respectively for two or more of two unpaired samples, and chi-squared or Fisher’s exact tests were used for categorical variables. Two-tailed p-values ≤0.05 were considered as significant. *p=0.001 versus **A** and **B**; ** p=0.001 versus **A**, **B** and **C**. ACEi: Angiotensin converting enzyme inhibitors.

Characteristics	PLA2R1 ⁺ /Intracellular ⁺ (n=118)	PLA2R1 ⁺ /Intracellular ⁻ (n=64)	PLA2R1 ⁻ /Intracellular ⁺ (n=64)	PLA2R1 ⁻ /Intracellular ⁻ (n=31)
Sex (M/F)	84/34	45/19	47/17	21/10
Age (years)	61 (50-72)	63 (46-70)	57 (42-67)	55 (43-71)
MN histological stage				
(%)				
I	18.8	23.6	25.9	44.5
II	44.2	49.0	50.0	33.3
III	28.6	23.5	14.8	11.0
IV	8.4	3.9	9.3	11.1
Treatment (%)				
Steroids/ACEi [§]	80	78	85	78
Cyclosporine A	18	30	14	16
Cytotoxic	43	44	33	32
Rituximab	7	22	16	19
Clinical characteristics				
Proteinuria (g/day)				
T ₀	5.6(3.3-9.7)	6.0 (3.3-9.8)	3.4 (1.8-6.4)*	4.4 (2.6-6.4)*
T ₁₂	1.7 (0.4-4.4)	1.8(0.6-4.3)	1.3 (0.3-3.5)	0.5 (0.1-2.7)**
CKD-EPI				
(mL/min/1.73m ²)				
T ₀	69.4(46-99)	65.9(47-100)	72.4 (43-99)	89.2 (63-103)**
T ₁₂	73.5(48-99)	77.9(59-99)	67.2 (44-101)	95.5 (72-103)**

[§]Steroids and ACEi were given alone or in association with other drugs

Table 2. Two-way contingency table showing the association of antibody levels with indexes of kidney outcome and the interaction between autoantibodies against PLA2R1 and intracellular antigens for the whole cohort. The 2x2 contingency table reports the association with odds ratios (OR) and confidence interval (CI) between antibody titer and clinical outcome. Data are calculated from all patients excluding the eight anti-THSD7A positive patients (n=277). 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) or 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 for more than one antibody is indicated as P+/S+ and P+/S+/E+ (anti-PLA2R1+/anti-SOD2+/anti- α ENO+).

	Proteinuria >0.3 g/day at 12 months	Proteinuria ≤ 0.3 g/day at 12 months	OR (CI)	Proteinuria >3.5 g/day at 12 months	Proteinuria ≤ 3.5 g/day at 12 months	OR (CI)	eGFR ≤ 60 ml/min/1.73m ² at 12 months	eGFR>60 ml/min/1.73m ² at 12 months	OR (CI)
Anti-PLA2R1(P)									
Positive	149 (54%)	33 (12%)	2.6	55 (20%)	127 (46%)	1.7	67 (24%)	115(42%)	1.2
Negative	60 (22%)	35 (13%)	(1.5-4.6)	19 (7%)	76 (27%)	(0.9-3.1)	31 (11%)	64 (23%)	(0.7-2.1)
Anti-SOD2 (S)									
Positive	74 (27%)	11 (4%)	2.8	31 (11%)	54 (19%)	2	34 (12%)	51 (18%)	1.3
Negative	135 (49%)	57 (21%)	(1.4-5.7)	43 (16%)	149 (54%)	(1.1-3.5)	64 (23%)	128 (46%)	(0.8-2.3)
Anti-αENO(E)									
Positive	95 (34%)	22 (8%)	1.7	40 (14%)	77 (28%)	1.9	51 (18%)	66 (24%)	1.8
Negative	114 (41%)	46 (17%)	(0.98-3.1)	34 (12%)	126 (45%)	(1.1-3.3)	47 (17%)	113 (41%)	(1.1-3.1)
P+/S+ *									
Positive	55 (30%)	6 (3%)	2.6	22 (12%)	39 (21%)	1.5	24 (13%)	37 (20%)	1.2
Negative	94 (52%)	27 (15%)	(1.02-6.8)	33 (18%)	88 (48%)	(0.8-2.9)	43 (24%)	78 (43%)	(0.6-2.2)
P+/S+/E+ *									
Positive	39 (21%)	2 (1%)	5.5	16 (9%)	25 (14%)	1.7	16 (9%)	25 (14%)	1.1
Negative	110 (60%)	31 (17%)	(1.2-24)	39 (21%)	102 (56%)	(0.8-3.5)	51 (28%)	90 (49%)	(0.5-2.3)
Anti-PLA2R1(P)									
High titer	82 (45%)	9 (5%)	3.3	36 (20%)	55 (30%)	2.5	44 (24%)	47 (26%)	2.8
Low titer	67 (37%)	24 (13%)	(1.4-7.5)	19 (10%)	72 (40%)	(1.3-4.8)	23 (13%)	68 (37%)	(1.5-5.2)
Anti-SOD2 (S)									
High titer	40 (47%)	3 (4%)	4.6	22 (26%)	21 (25%)	3.8	19 (22%)	24 (28%)	1.4
Low titer	27 (32%)	15 (18%)	(1.1-19)	9 (11%)	33 (39%)	(1.5-9.9)	15 (18%)	27 (32%)	(0.6-3.4)
Anti-αENO(E)									
High titer	49 (42%)	10 (9%)	1.3	26 (22%)	32 (27%)	2.6	32 (27%)	27 (23%)	2.4
Low titer	46 (39%)	12 (10%)	(0.5-3.2)	14 (12%)	45 (38%)	(1.2-5.8)	19 (16%)	39 (33%)	(1.1-5.1)
High P+/S+ **									
High titer P/ S+	21 (34%)	3 (5%)	0.6	8 (13%)	16 (26%)	0.8	10 (16%)	14 (23%)	1.2
High titerP/S-	34 (56%)	3 (5%)	(0.1-3.3)	14 (23%)	23 (38%)	(0.3-2.4)	14 (23%)	23 (38%)	(0.4-3.3)

*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 (highP titer and S positivity) were compared to high P titer and S negativity.

Table 3. Two-way contingency table showing the association of antibody levels with indexes of kidney outcome and the interaction between autoantibodies against PLA2R1 and intracellular antigens for nephrotic patients at diagnosis. The 2x2 contingency table reports the association with odds ratios (OR) and confidence interval (CI) between antibody titer and clinical outcome. Data are calculated from the subset of patients with nephrotic syndrome excluding the eight anti-THSD7A positive patients (n=177). 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) or partial (< 3.5 g/day) remission) and eGFR after 12 months. The lower section shows the association of high versus low levels of each antibody with the same parameters of proteinuria and eGFR. The additive effect of positivity for more than one antibody is indicated as P+/S+ and P+/S+/E+.

	Proteinuria >0.3 g/day at 12 months	Proteinuria ≤ 0.3 g/day at 12 months	OR (CI)	Proteinuria >3.5 g/day at 12 months	Proteinuria ≤ 3.5 g/day at 12 months	OR (CI)	eGFR ≤ 60 ml/min/1.73m ² at 12 months	eGFR>60 ml/min/1.73m ² at 12 months	OR (CI)
Anti-PLA2R1 (P)									
Positive	109 (62%)	22 (12%)	1.5	44 (25%)	87 (49%)	1.6	53 (30%)	78(44%)	1.3
Negative	35 (20%)	11 (6%)	(0.7-3.5)	11 (6%)	35 (20%)	(0.7-3.5)	16 (9%)	30 (17%)	(0.6-2.6)
Anti-SOD2 (S)									
Positive	57 (32%)	6 (3%)	2.9	24 (14%)	39 (22%)	1.6	29 (16%)	34 (19%)	1.6
Negative	87 (49%)	27 (15%)	(1.1-7.6)	31 (18%)	83 (47%)	(0.8-3.2)	40 (23%)	74 (42%)	(0.8-2.9)
Anti-αENO (E)									
Positive	67 (38%)	11 (6%)	1.7	29 (16%)	49 (28%)	1.7	36 (20%)	42 (24%)	1.7
Negative	77 (44%)	22 (12%)	(0.8-3.8)	26 (15%)	73 (41%)	(0.9-3.1)	33 (19%)	66 (37%)	(0.9-3.1)
P+/S+ *									
Positive	45 (34%)	3 (2%)	4.4	19 (14%)	29 (22%)	1.5	21 (16%)	27 (21%)	1.2
Negative	64 (49%)	19 (14%)	(1.2-16)	25 (19%)	58 (44%)	(0.7-3.2)	32 (24%)	51 (39%)	(0.6-2.5)
P+/S+/E+ *									
Positive	30 (23%)	2 (2%)	3.8	15 (11%)	17 (13%)	2.1	13 (10%)	19 (15%)	1
Negative	79 (60%)	20 (15%)	(0.8-17)	29 (22%)	70 (53%)	(0.9-4.8)	40 (31%)	59 (45%)	(0.4-2.3)
Anti-PLA2R1 (P)									
High titer	65 (50%)	6 (5%)	3.9	26 (20%)	45 (34%)	1.3	33 (25%)	41 (31%)	1.5
Low titer	44 (34%)	16 (12%)	(1.4-11)	18 (14%)	42 (32%)	(0.6-2.8)	20 (15%)	37 (28%)	(0.7-3)
Anti-SOD2 (S)									
High titer	28 (44%)	5 (8%)	0.2	14 (22%)	18 (29%)	1.6	15 (24%)	16 (25%)	1.2
Low titer	29 (46%)	1 (2%)	(0.02-1.7)	10 (16%)	21 (33%)	(0.6-4.6)	14 (22%)	18 (29%)	(0.4-3.2)
Anti-αENO (E)									
High titer	33 (42%)	5 (6%)	1.2	15 (19%)	23 (29%)	1.2	15 (19%)	27 (35%)	0.4
Low titer	34 (44%)	6 (8%)	(0.3-4)	14 (18%)	26 (33%)	(0.5-3)	21 (27%)	15 (19%)	(0.15-1)
High P+/S+ **									
High titer P/ S+	17 (35%)	1 (2%)	1.2	8 (17%)	11 (23%)	1.2	6 (12%)	10 (21%)	1
High titer P/ S-	28 (58%)	2 (4%)	(0.1-14)	11 (23%)	18 (37%)	(0.4-3.9)	13 (27%)	17 (35%)	(0.3-3.4)

*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.

Table 4. Two-category net reclassification improvement (NRI) and area under the receiver-operating-characteristic curve (AUC-ROC) analysis for predictors of clinical outcome after 12 months in MN patients by adding intracellular autoantibodies to anti-PLA2R1 positivity. Data are calculated from all patients excluding the eight anti-THSD7A positive patients (n=277). For NRI, combined negativity of anti-PLA2R1 and anti-SOD2 antibodies allowed the reclassification of 34% of patients with proteinuria <3.5g/day (non-event) and 27% of cases with eGFR>60 mL/min/1.73 m²(non-event) after 12 months. Similar results were found for combined negativity of anti-PLA2R1 and anti-αENO. In both cases, the reclassification improvement was in comparison with anti-PLA2R1 positivity alone.

	Proteinuria >0.3 g/day	Proteinuria >3.5 g/day	eGFR<60 mL/min/1.73 m2
Events (n)	209	74	98
Nonevents (n)	68	203	179
Two-category NRI (n)			
Anti-PLA2R1 plus anti-SOD2			
NRI_{events}(%)	16	1	-2
NRI_{non-events}(%)	-35	34	27
NRI_{overall}(%)	-19	35	25
P value	0.002	0.00018	0.00016
Anti-PLA2R1 plus anti-αENO			
NRI_{events}(%)	13	1	13
NRI_{non-events}(%)	-37	22	24
NRI_{overall}(%)	-24	23	37
P value	0.003	0.0002	0.00013
AUC ROC(95%CI)			
Anti-PLA2R1	0.66(0.6-0.66) P=0.001	0.60(0.50-0.7) P=0.02	0.56(0.49-0.63) P=0.09
Anti-SOD2	0.60(0.52-0.67) P=0.02	0.60(0.51-0.7) P=0.02	0.56(0.50-0.64) P=0.09
Anti-αENO	0.57(0.5-0.65) P=0.09	0.60(0.52-0.7) P=0.02	0.54(0.50-0.61) P=0.1
Anti-PLA2R1 plus anti-SOD2	0.61(0.5-0.73) P=0.03	0.65(0.54-0.71) P=0.02	0.61(0.53-0.71) P=0.05
Anti-PLA2R1 plus anti-αENO	0.68(0.53-0.82) P=0.03	0.63(0.51-0.76) P=0.04	0.66(0.52-0.76) P=0.02

Table 1. Comparison of clinical characteristics at baseline (T₀) and at clinical outcome (T₁₂) in patients with iMN stratified into four subgroups according to their antibody profile: patients both PLA2R1 and intracellular antibodies positive; patients PLA2R1 positive and intracellular antibodies negative; patients PLA2R1 negative and intracellular antibodies positive; and patients both PLA2R1 negative and intracellular antibodies negative. Data are reported as number and percentage or median with interquartile range. Mann-Whitney or Kruskal–Wallis tests were used for continuous variables, respectively for two or more of two unpaired samples, and chi-squared or Fisher’s exact tests were used for categorical variables. Two-tailed p-values ≤0.05 were considered as significant. *p=0.001 versus **A** and **B**; ** p=0.001 versus **A**, **B** and **C**. ACEi: Angiotensin converting enzyme inhibitors.

Characteristics	PLA2R1 ⁺ /Intracellular ⁺ (n=118)	PLA2R1 ⁺ /Intracellular ⁻ (n=64)	PLA2R1 ⁻ /Intracellular ⁺ (n=64)	PLA2R1 ⁻ /Intracellular ⁻ (n=31)
Sex (M/F)	84/34	45/19	47/17	21/10
Age (years)	61 (50-72)	63 (46-70)	57 (42-67)	55 (43-71)
MN histological stage (%)				
I	18.8	23.6	25.9	44.5
II	44.2	49.0	50.0	33.3
III	28.6	23.5	14.8	11.0
IV	8.4	3.9	9.3	11.1
Treatment (%)				
Steroids/ACEi [§]	80	78	85	78
Cyclosporine A	18	30	14	16
Cytotoxic	43	44	33	32
Rituximab	7	22	16	19
Clinical characteristics				
Proteinuria (g/day)				
T ₀	5.6(3.3-9.7)	6.0 (3.3-9.8)	3.4 (1.8-6.4)*	4.4 (2.6-6.4)*
T ₁₂	1.7 (0.4-4.4)	1.8(0.6-4.3)	1.3 (0.3-3.5)	0.5 (0.1-2.7)**
CKD-EPI (mL/min/1.73m²)				
T ₀	69.4(46-99)	65.9(47-100)	72.4 (43-99)	89.2 (63-103)**
T ₁₂	73.5(48-99)	77.9(59-99)	67.2 (44-101)	95.5 (72-103)**

[§]Steroids and ACEi were given alone or in association with other drugs

Table 2. Two-way contingency table showing the association of antibody levels with indexes of kidney outcome and the interaction between autoantibodies against PLA2R1 and intracellular antigens for the whole cohort. The 2x2 contingency table reports the association with odds ratios (OR) and confidence interval (CI) between antibody titer and clinical outcome. Data are calculated from all patients excluding the eight anti-THSD7A positive patients (n=277). 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) or partial (< 3.5 g/day) remission) and of 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 for more than one antibody is indicated as P+/S+ and P+/S+/E+ (anti-PLA2R1+/anti-SOD2+/anti- α ENO+).

	Proteinuria >0.3 g/day at 12 months	Proteinuria ≤ 0.3 g/day at 12 months	OR (CI)	Proteinuria >3.5 g/day at 12 months	Proteinuria ≤ 3.5 g/day at 12 months	OR (CI)	eGFR ≤ 60 ml/min/1.73m ² at 12 months	eGFR>60 ml/min/1.73m ² at 12 months	OR (CI)
Anti-PLA2R1(P)									
Positive	149 (54%)	33 (12%)	2.6	55 (20%)	127 (46%)	1.7	67 (24%)	115(42%)	1.2
Negative	60 (22%)	35 (13%)	(1.5-4.6)	19 (7%)	76 (27%)	(0.9-3.1)	31 (11%)	64 (23%)	(0.7-2.1)
Anti-SOD2 (S)									
Positive	74 (27%)	11 (4%)	2.8	31 (11%)	54 (19%)	2	34 (12%)	51 (18%)	1.3
Negative	135 (49%)	57 (21%)	(1.4-5.7)	43 (16%)	149 (54%)	(1.1-3.5)	64 (23%)	128 (46%)	(0.8-2.3)
Anti-αENO(E)									
Positive	95 (34%)	22 (8%)	1.7	40 (14%)	77 (28%)	1.9	51 (18%)	66 (24%)	1.8
Negative	114 (41%)	46 (17%)	(0.98-3.1)	34 (12%)	126 (45%)	(1.1-3.3)	47 (17%)	113 (41%)	(1.1-3.1)
P+/S+ *									
Positive	55 (30%)	6 (3%)	2.6	22 (12%)	39 (21%)	1.5	24 (13%)	37 (20%)	1.2
Negative	94 (52%)	27 (15%)	(1.02-6.8)	33 (18%)	88 (48%)	(0.8-2.9)	43 (24%)	78 (43%)	(0.6-2.2)
P+/S+/E+ *									
Positive	39 (21%)	2 (1%)	5.5	16 (9%)	25 (14%)	1.7	16 (9%)	25 (14%)	1.1
Negative	110 (60%)	31 (17%)	(1.2-24)	39 (21%)	102 (56%)	(0.8-3.5)	51 (28%)	90 (49%)	(0.5-2.3)
Anti-PLA2R1(P)									
High titer	82 (45%)	9 (5%)	3.3	36 (20%)	55 (30%)	2.5	44 (24%)	47 (26%)	2.8
Low titer	67 (37%)	24 (13%)	(1.4-7.5)	19 (10%)	72 (40%)	(1.3-4.8)	23 (13%)	68 (37%)	(1.5-5.2)
Anti-SOD2 (S)									
High titer	40 (47%)	3 (4%)	4.6	22 (26%)	21 (25%)	3.8	19 (22%)	24 (28%)	1.4
Low titer	27 (32%)	15 (18%)	(1.1-19)	9 (11%)	33 (39%)	(1.5-9.9)	15 (18%)	27 (32%)	(0.6-3.4)
Anti-αENO(E)									
High titer	49 (42%)	10 (9%)	1.3	26 (22%)	32 (27%)	2.6	32 (27%)	27 (23%)	2.4
Low titer	46 (39%)	12 (10%)	(0.5-3.2)	14 (12%)	45 (38%)	(1.2-5.8)	19 (16%)	39 (33%)	(1.1-5.1)
High P+/S+ **									
High titer P/ S+	21 (34%)	3 (5%)	0.6	8 (13%)	16 (26%)	0.8	10 (16%)	14 (23%)	1.2
High titerP/S-	34 (56%)	3 (5%)	(0.1-3.3)	14 (23%)	23 (38%)	(0.3-2.4)	14 (23%)	23 (38%)	(0.4-3.3)

*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 (highP titer and S positivity) were compared to high P titer and S negativity.

Table 3. Two-way contingency table showing the association of antibody levels with indexes of kidney outcome and the interaction between autoantibodies against PLA2R1 and intracellular antigens for nephrotic patients at diagnosis. The 2x2 contingency table reports the association with odds ratios (OR) and confidence interval (CI) between antibody titer and clinical outcome. Data are calculated from the subset of patients with nephrotic syndrome excluding the eight anti-THSD7A positive patients (n=177). 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) or partial (< 3.5 g/day) remission) and of eGFR after 12 months. The lower section shows the association of high versus low levels of each antibody with the same parameters of proteinuria and eGFR. The additive effect of positivity for more than one antibody is indicated as P+/S+ and P+/S+/E+.

	Proteinuria >0.3 g/day at 12 months	Proteinuria ≤ 0.3 g/day at 12 months	OR (CI)	Proteinuria >3.5 g/day at 12 months	Proteinuria ≤ 3.5 g/day at 12 months	OR (CI)	eGFR ≤ 60 ml/min/1.73m ² at 12 months	eGFR>60 ml/min/1.73m ² at 12 months	OR (CI)
Anti-PLA2R1 (P)									
Positive	109 (62%)	22 (12%)	1.5	44 (25%)	87 (49%)	1.6	53 (30%)	78(44%)	1.3
Negative	35 (20%)	11 (6%)	(0.7-3.5)	11 (6%)	35 (20%)	(0.7-3.5)	16 (9%)	30 (17%)	(0.6-2.6)
Anti-SOD2 (S)									
Positive	57 (32%)	6 (3%)	2.9	24 (14%)	39 (22%)	1.6	29 (16%)	34 (19%)	1.6
Negative	87 (49%)	27 (15%)	(1.1-7.6)	31 (18%)	83 (47%)	(0.8-3.2)	40 (23%)	74 (42%)	(0.8-2.9)
Anti-αENO (E)									
Positive	67 (38%)	11 (6%)	1.7	29 (16%)	49 (28%)	1.7	36 (20%)	42 (24%)	1.7
Negative	77 (44%)	22 (12%)	(0.8-3.8)	26 (15%)	73 (41%)	(0.9-3.1)	33 (19%)	66 (37%)	(0.9-3.1)
P+/S+ *									
Positive	45 (34%)	3 (2%)	4.4	19 (14%)	29 (22%)	1.5	21 (16%)	27 (21%)	1.2
Negative	64 (49%)	19 (14%)	(1.2-16)	25 (19%)	58 (44%)	(0.7-3.2)	32 (24%)	51 (39%)	(0.6-2.5)
P+/S+/E+ *									
Positive	30 (23%)	2 (2%)	3.8	15 (11%)	17 (13%)	2.1	13 (10%)	19 (15%)	1
Negative	79 (60%)	20 (15%)	(0.8-17)	29 (22%)	70 (53%)	(0.9-4.8)	40 (31%)	59 (45%)	(0.4-2.3)
Anti-PLA2R1 (P)									
High titer	65 (50%)	6 (5%)	3.9	26 (20%)	45 (34%)	1.3	33 (25%)	41 (31%)	1.5
Low titer	44 (34%)	16 (12%)	(1.4-11)	18 (14%)	42 (32%)	(0.6-2.8)	20 (15%)	37 (28%)	(0.7-3)
Anti-SOD2 (S)									
High titer	28 (44%)	5 (8%)	0.2	14 (22%)	18 (29%)	1.6	15 (24%)	16 (25%)	1.2
Low titer	29 (46%)	1 (2%)	(0.02-1.7)	10 (16%)	21 (33%)	(0.6-4.6)	14 (22%)	18 (29%)	(0.4-3.2)
Anti-αENO (E)									
High titer	33 (42%)	5 (6%)	1.2	15 (19%)	23 (29%)	1.2	15 (19%)	27 (35%)	0.4
Low titer	34 (44%)	6 (8%)	(0.3-4)	14 (18%)	26 (33%)	(0.5-3)	21 (27%)	15 (19%)	(0.15-1)
High P+/S+ **									
High titer P/ S+	17 (35%)	1 (2%)	1.2	8 (17%)	11 (23%)	1.2	6 (12%)	10 (21%)	1
High titer P/ S-	28 (58%)	2 (4%)	(0.1-14)	11 (23%)	18 (37%)	(0.4-3.9)	13 (27%)	17 (35%)	(0.3-3.4)

*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.

Table 4. Two-category net reclassification improvement (NRI) and area under the receiver-operating-characteristic curve (AUC-ROC) analysis for predictors of clinical outcome after 12 months in MN patients by adding intracellular autoantibodies to anti-PLA2R1 positivity. Data are calculated from all patients excluding the eight anti-THSD7A positive patients (n=277). For NRI, combined negativity of anti-PLA2R1 and anti-SOD2 antibodies allowed the reclassification of 34% of patients with proteinuria <3.5g/day (non-event) and 27% of cases with eGFR>60 mL/min/1.73 m²(non-event) after 12 months. Similar results were found for combined negativity of anti-PLA2R1 and anti-αENO. In both cases, the reclassification improvement was in comparison with anti-PLA2R1 positivity alone.

	Proteinuria >0.3 g/day	Proteinuria >3.5 g/day	eGFR<60 mL/min/1.73m2
Events (n)	209	74	98
Nonevents (n)	68	203	179
Two-category NRI (n)			
Anti-PLA2R1 plus anti-SOD2			
NRI_{events}(%)	16	1	-2
NRI_{non-events}(%)	-35	34	27
NRI_{overall}(%)	-19	35	25
P value	0.002	0.00018	0.00016
Anti-PLA2R1 plus anti-αENO			
NRI_{events}(%)	13	1	13
NRI_{non-events}(%)	-37	22	24
NRI_{overall}(%)	-24	23	37
P value	0.003	0.0002	0.00013
AUC ROC(95%CI)			
Anti-PLA2R1	0.66(0.6-0.66) P=0.001	0.60(0.50-0.7) P=0.02	0.56(0.49-0.63) P=0.09
Anti-SOD2	0.60(0.52-0.67) P=0.02	0.60(0.51-0.7) P=0.02	0.56(0.50-0.64) P=0.09
Anti-αENO	0.57(0.5-0.65) P=0.09	0.60(0.52-0.7) P=0.02	0.54(0.50-0.61) P=0.1
Anti-PLA2R1 plus anti-SOD2	0.61(0.5-0.73) P=0.03	0.65(0.54-0.71) P=0.02	0.61(0.53-0.71) P=0.05
Anti-PLA2R1 plus anti-αENO	0.68(0.53-0.82) P=0.03	0.63(0.51-0.76) P=0.04	0.66(0.52-0.76) P=0.02

Figure 1

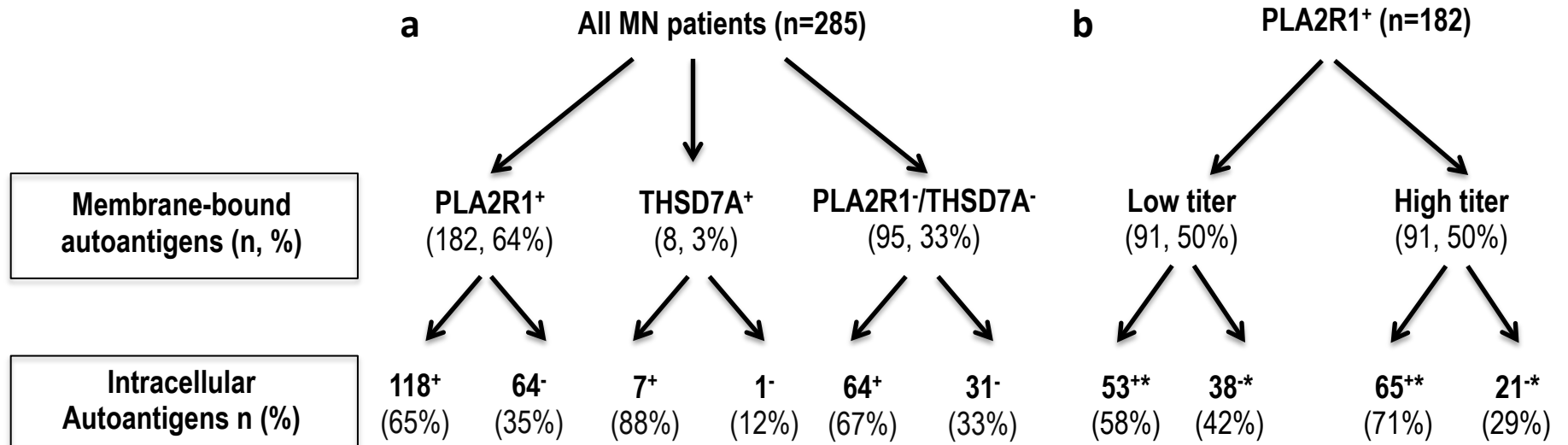


Figure 2

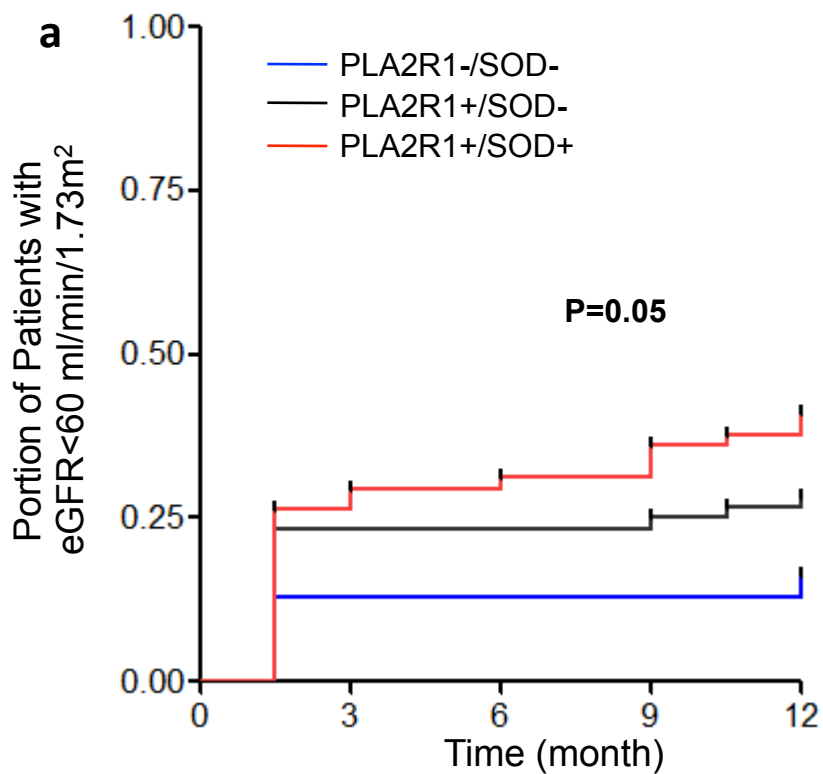
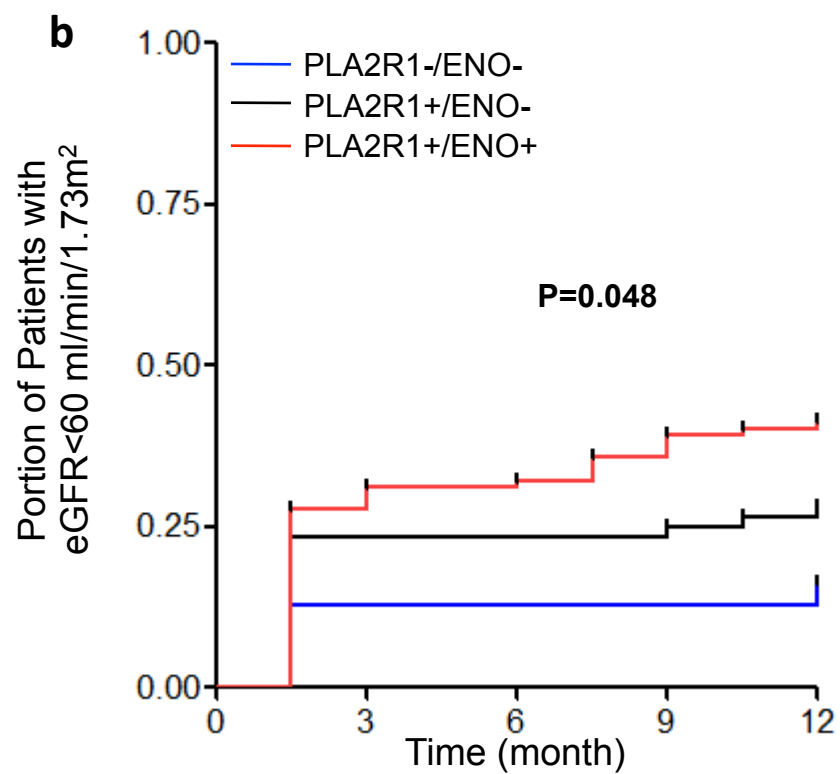
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Figure 3a

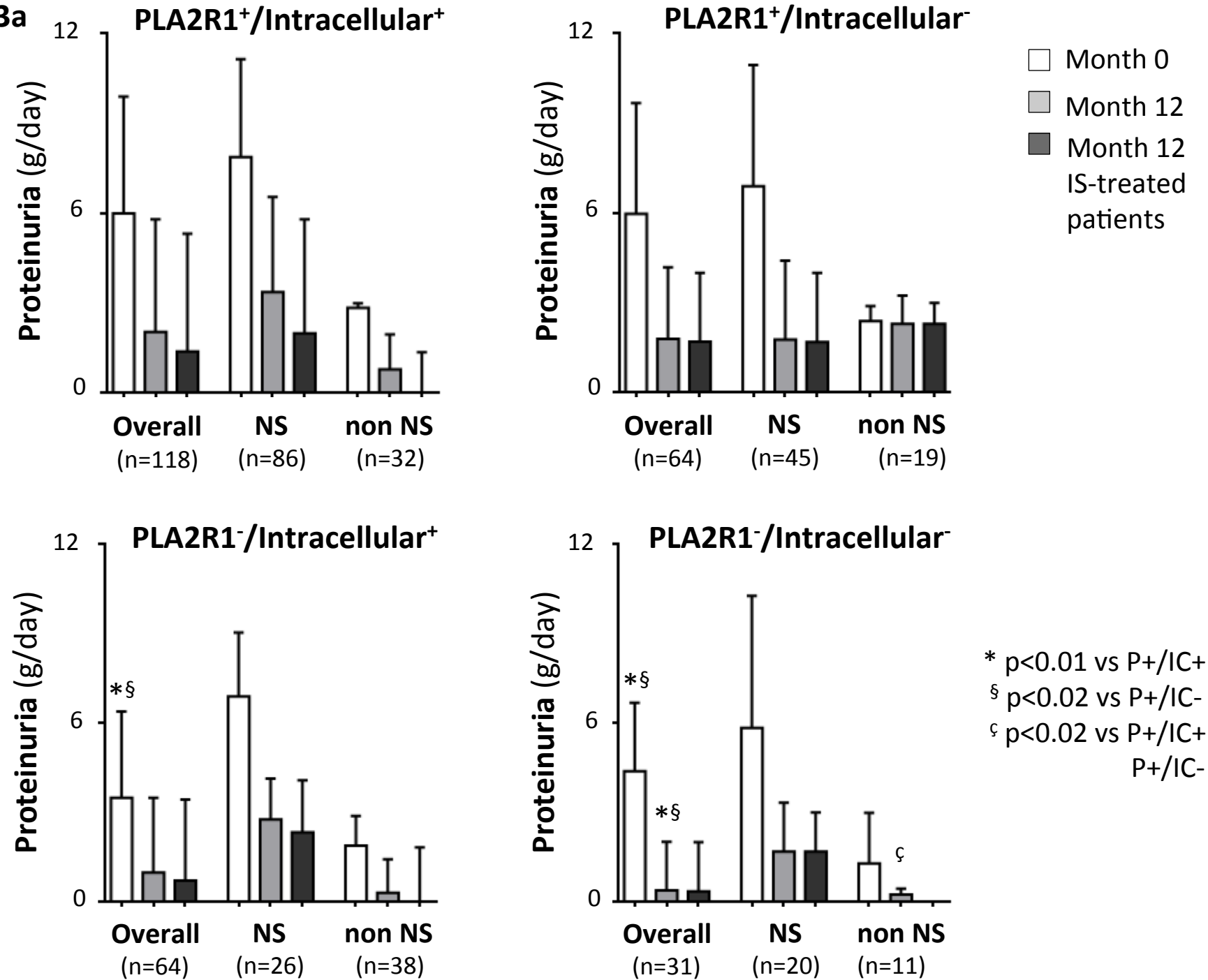


Figure 3b

□ Month 0
■ Month 12
■ IS-treated patients

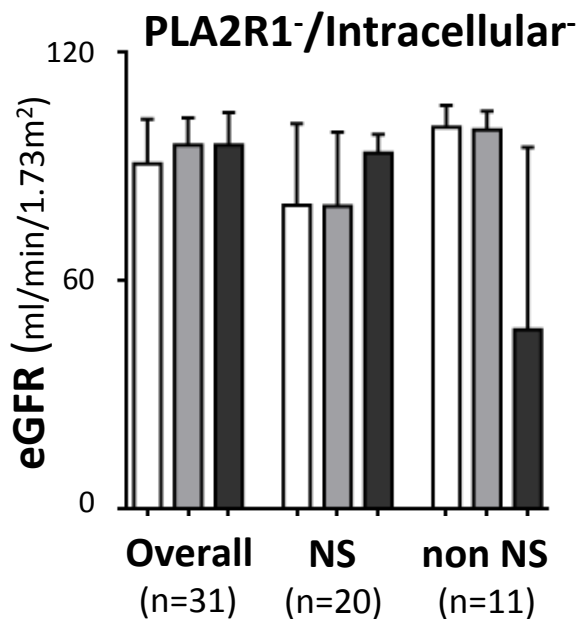
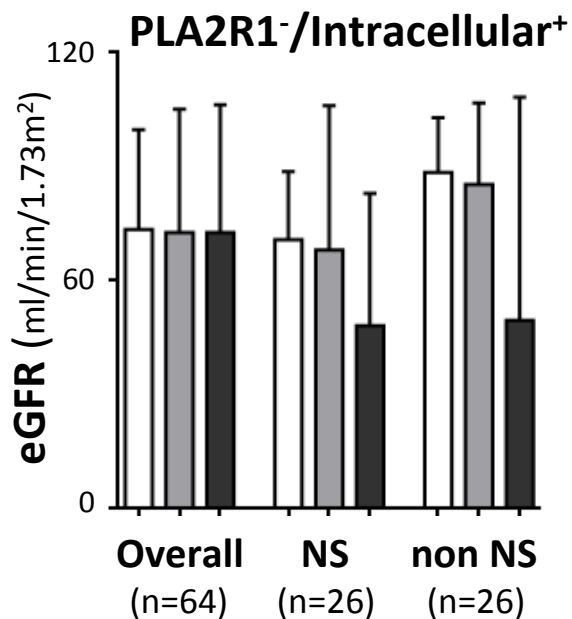
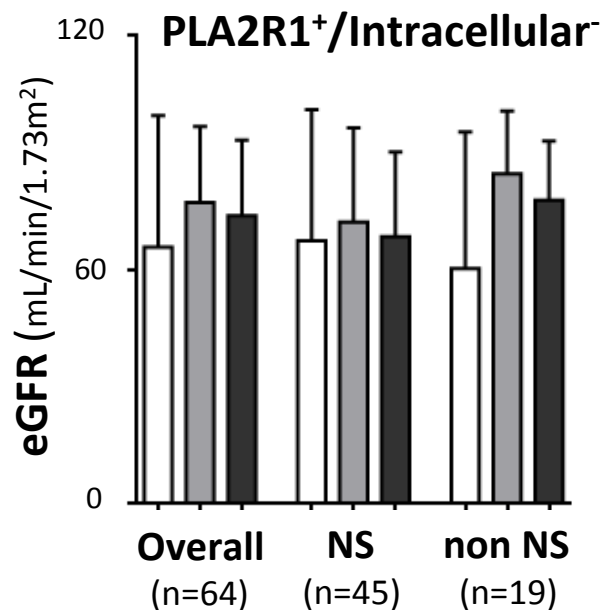
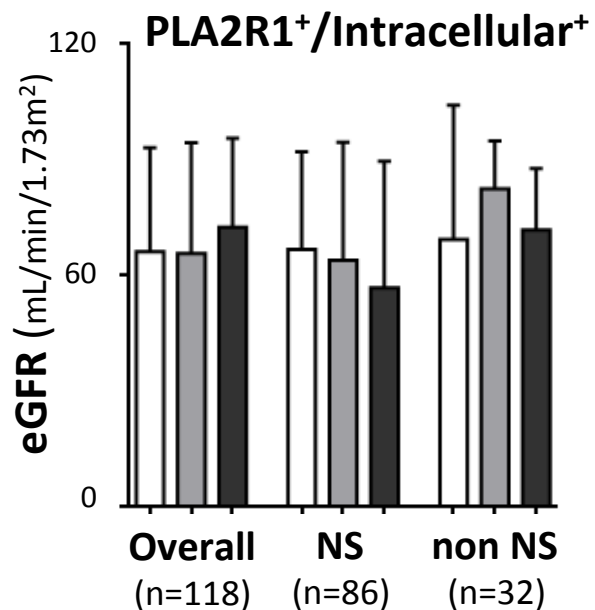
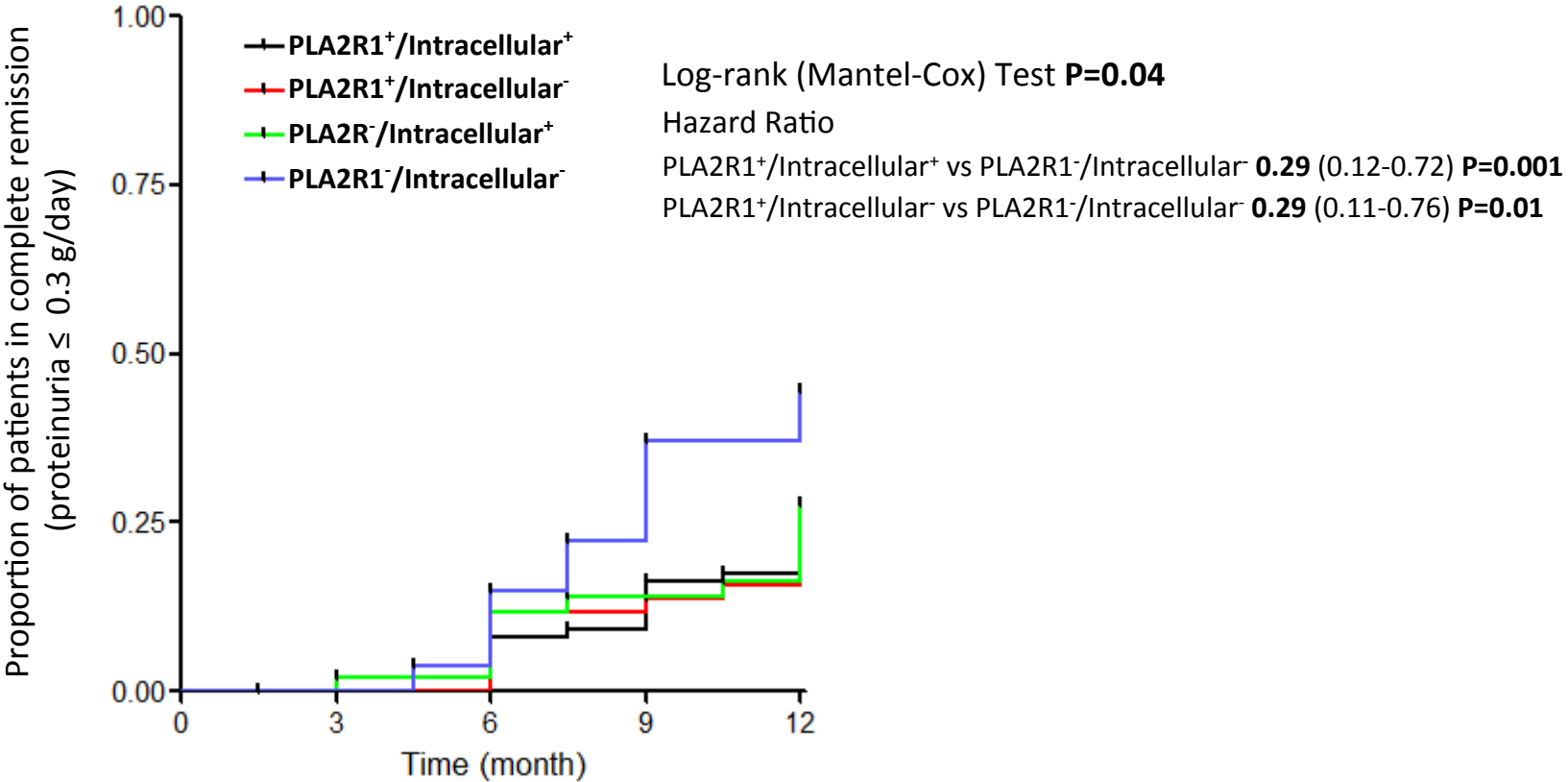
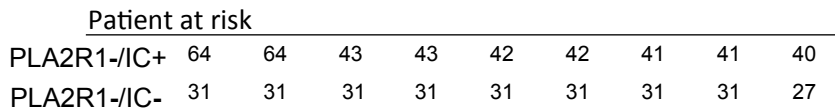


Figure 4a



Patients at risk					
PLA2R1 ⁺ /Intracellular ⁺	118	118 (100%)	108 (92%)	100 (85%)	99 (84%)
PLA2R1 ⁺ /Intracellular ⁻	64	64 (100%)	62 (97%)	56 (88%)	54 (84%)
PLA2R ⁻ /Intracellular ⁺	64	62 (97%)	60 (94%)	56 (87%)	46 (72%)
PLA2R1 ⁻ /Intracellular ⁻	31	31 (100%)	26 (84%)	19 (61%)	17 (55%)

All patients



Portion of Patients with eGFR < 60 ml/min/1.73m²

Time (month)

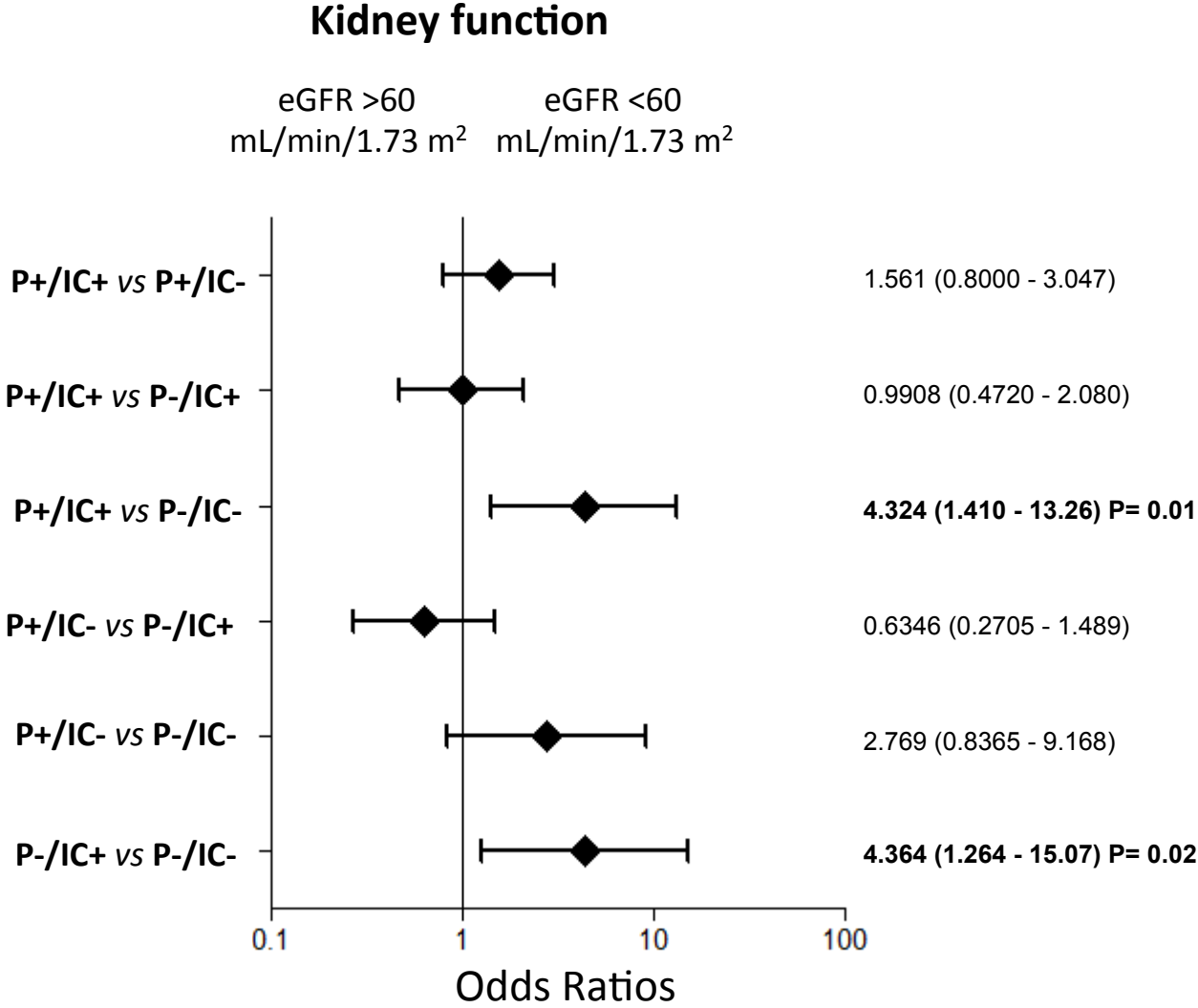
PLA2R1-/IC-
PLA2R1-/IC+

P=0.05

Time (month)	PLA2R1-/IC- (Proportion)	PLA2R1-/IC+ (Proportion)
0	0.00	0.00
1	0.15	0.40
3	0.15	0.43
9	0.15	0.47
12	0.20	0.47

[illegible]

Figure 4c



SUPPLEMENTAL MATERIAL

Multi-Autoantibody Signature and Clinical Outcome in Membranous Nephropathy

Gian Marco Ghiggeri, Barbara Seitz-Polski, Joana Justino, Christelle Zaghrini, Christine Payré, Vesna Brglez, Guillaume Dolla, Alberto Sinico, Francesco Scolari, Augusto Vaglio, Marco Prunotto, Giovanni Candiano, Antonella Radice, The Italian Study Group for Membranous Nephropathy, Maurizio Bruschi and Gérard Lambeau

Table of Contents

1. Supplemental Methods

Recombinant proteins.

Antibodies.

Dot-blot for anti-AR, anti-SOD2 and anti- α ENO autoantibodies.

Anti-PLA2R1 epitopes.

2. Supplemental references

3. Supplemental Tables and Figures

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 of follow-up.

Supplemental Table 2. Quantitative data for limits of positivity and distribution of serum levels for each autoantibody.

Supplemental Table 3. Detailed autoantibody positivity for intracellular antigens in non-spreaders and spreaders PLA2R1+ patients.

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.

Supplemental Table 5. Clinical characteristics at diagnosis and after 12 months of follow-up for PLA2R1+ patients stratified according to epitope profiles.

Supplemental figure 1. Prognosis clinical factors associated with complete remission.

Supplemental figure 2. Circulating levels of the various autoantibodies at diagnosis and during follow-up.

Supplemental figure 3. Multi-autoantibody composition in the cohort of patients with membranous nephropathy.

Supplemental figure 4. Kidney function (eGFR) in spreaders positive (n=63) or not (n=84) for anti- α ENO autoantibodies.

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); recSOD2: full-length recombinant protein with GST tag, Abnova Corporation (Taipei, Taiwan); rec α ENO: 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- α ENO*: 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- α ENO autoantibodies. For anti-AR, anti-SOD2 and anti- α ENO 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 μ L 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 μ L using a multi-channel pipette. The vacuum was applied until all the samples were adsorbed. The same operation was repeated five times with 150 μ L 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 μ g/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 described³ 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 mM Tris pH 8.0 (100 μ L/well) at 4°C overnight. Plates were blocked for 2h with SeramunBlock (Seramun Diagnostica). Cell medium from HEK293 cells transfected with soluble forms of PLA2R1 domains (10-100 μ L/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 μ L/well). After 2h incubation at room temperature on a plate shaker, plates were washed and HRP-conjugated anti-IgG4 secondary antibodies (Southern Biotech) diluted 1:7,500 in SeramunStab ST plus (Seramun Diagnostica) was added (100 μ L/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- α ENO (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- α ENO+).

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

*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).

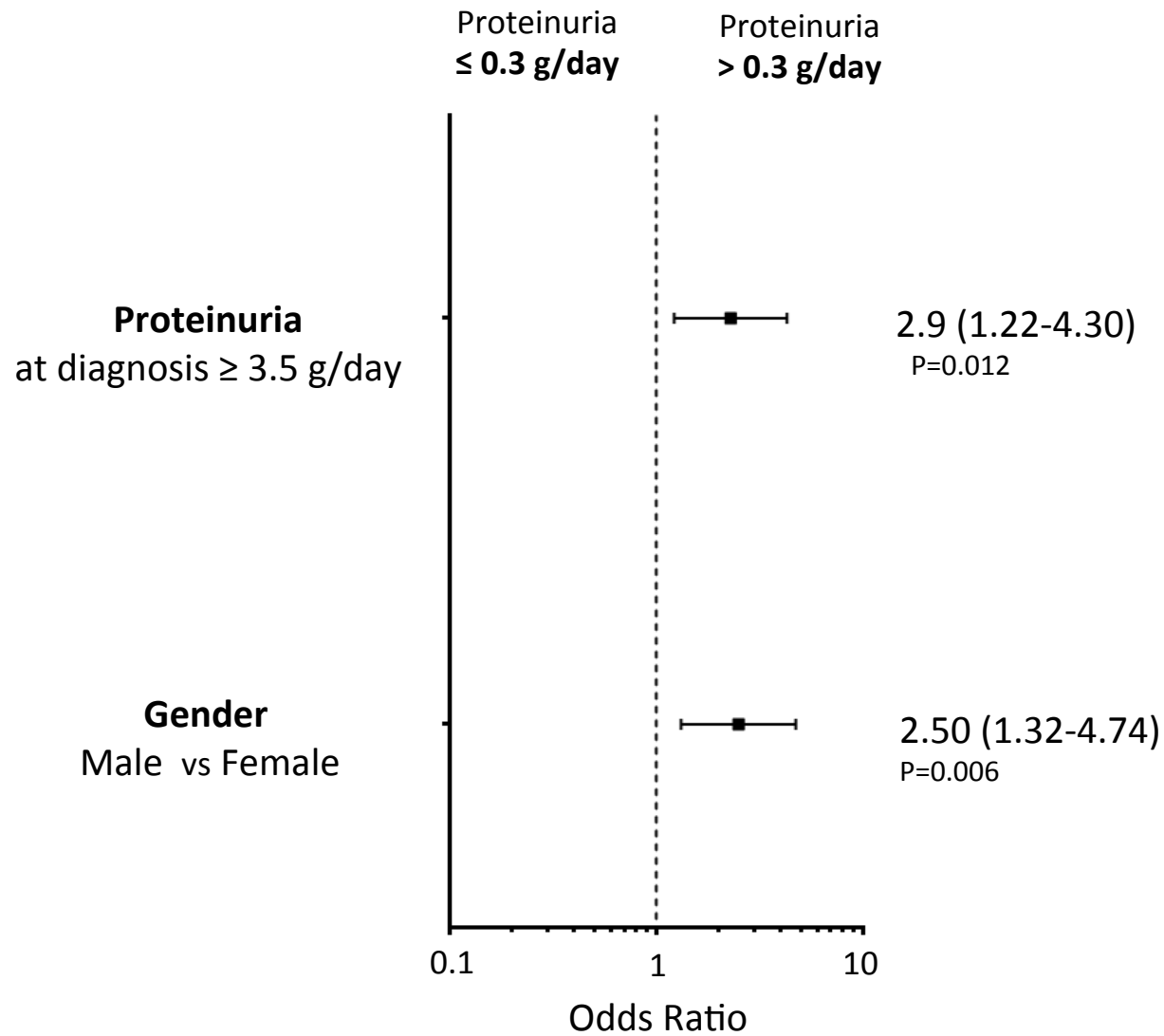
Supplemental figure 2. Circulating levels of the various autoantibodies at diagnosis and during follow-up. Circulating levels of (a) anti-PLA2R1, (b) anti-THSD7A and (c-e) anti-intracellular antigen autoantibodies (anti-AR, anti-SOD2, anti- α ENO) were determined at the time of diagnosis and after 6 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/mL⁷; 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- α ENO 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 α ENO 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- α ENO) and composite anti-intracellular podocyte autoantigens (AR+SOD2+; AR+ α ENO+; SOD2+ α ENO+; AR+SOD2+ α ENO+) for separate groups of spreaders are given in Table S3. (d) Percentage of patients positive for anti- α ENO 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

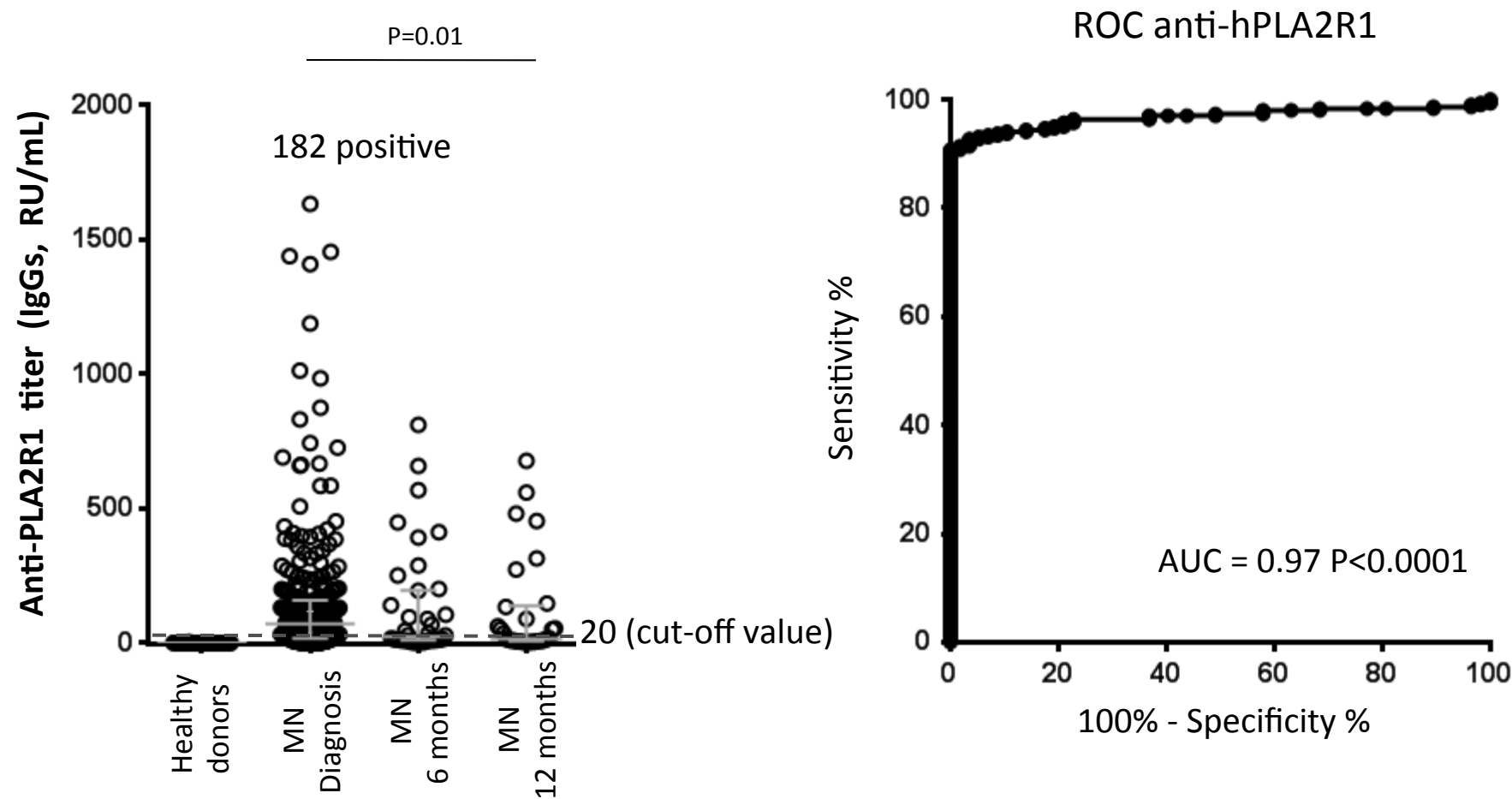
correlations between serum levels. This analysis was based on Spearman's correlation. The coefficient values are depicted by a pseudo-color scale extending from 0.1 (light gray or blue) to 0.89 (red). Moreover, the tree dendrogram displays the results of an unsupervised hierarchical clustering analysis 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- α ENO autoantibodies. eGFR at diagnosis and after 12 months in spreaders is modified by additional positivity for anti- α ENO 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.

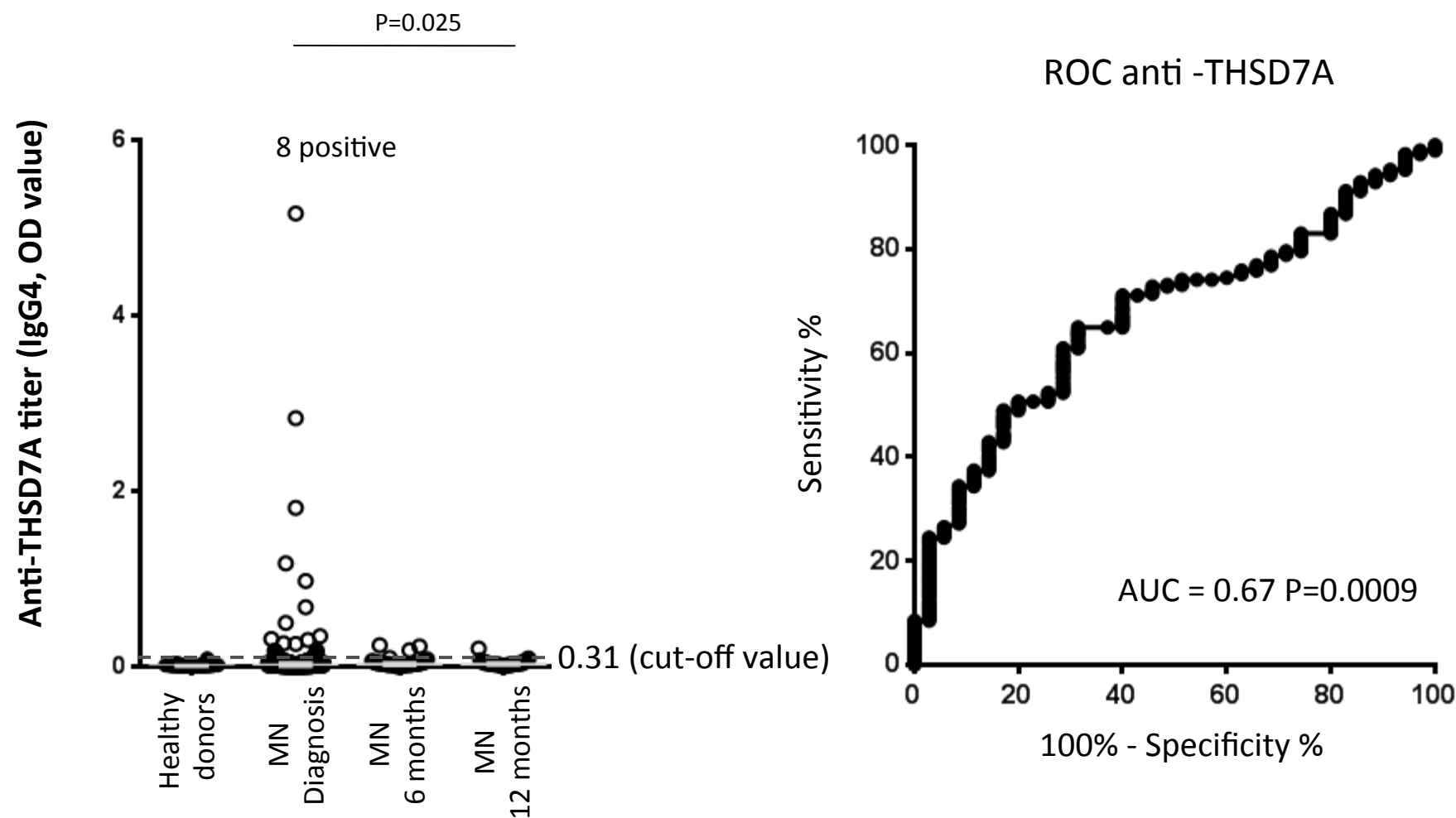
Supplemental Figure 1



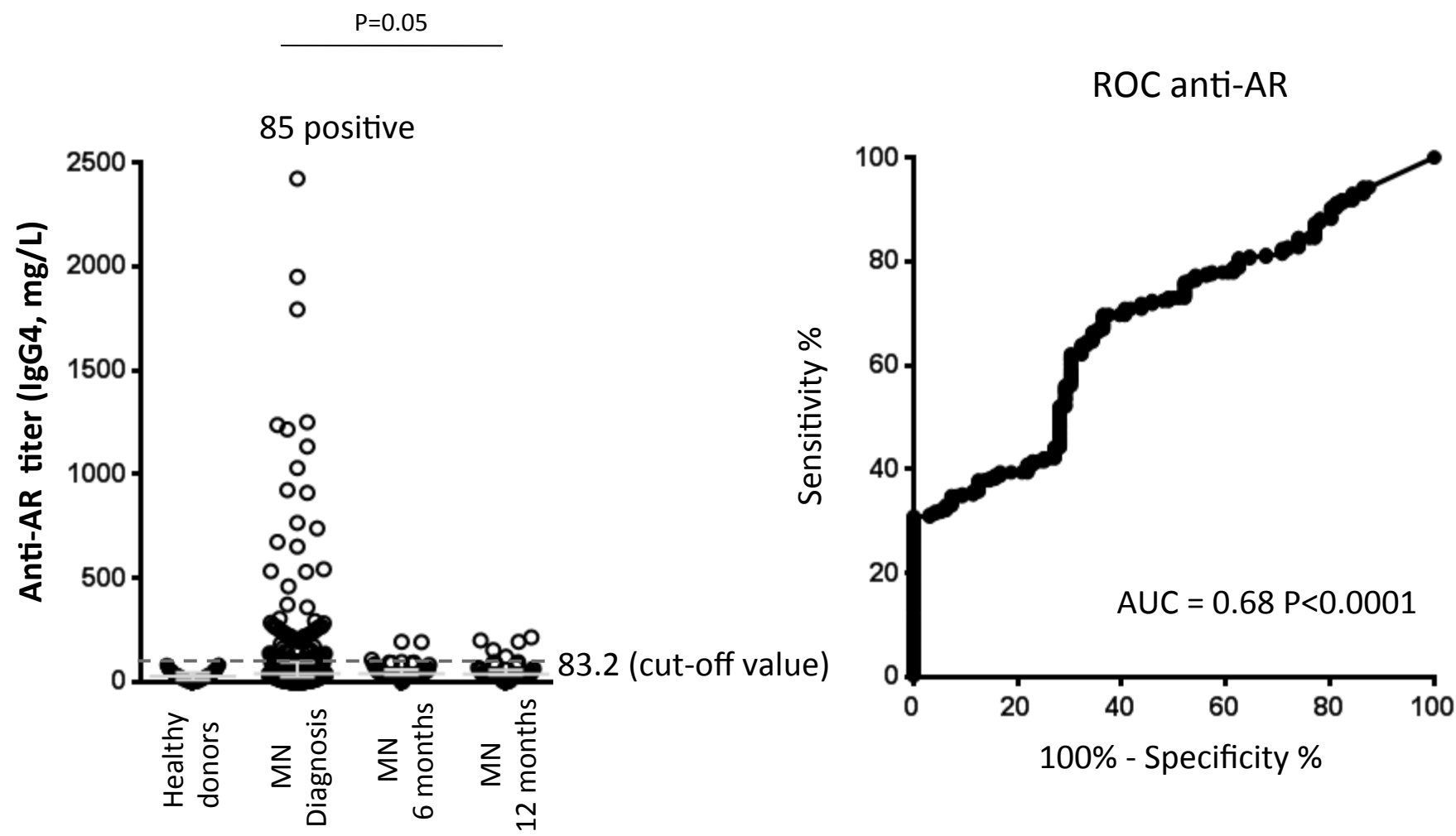
Supplemental Figure 2a



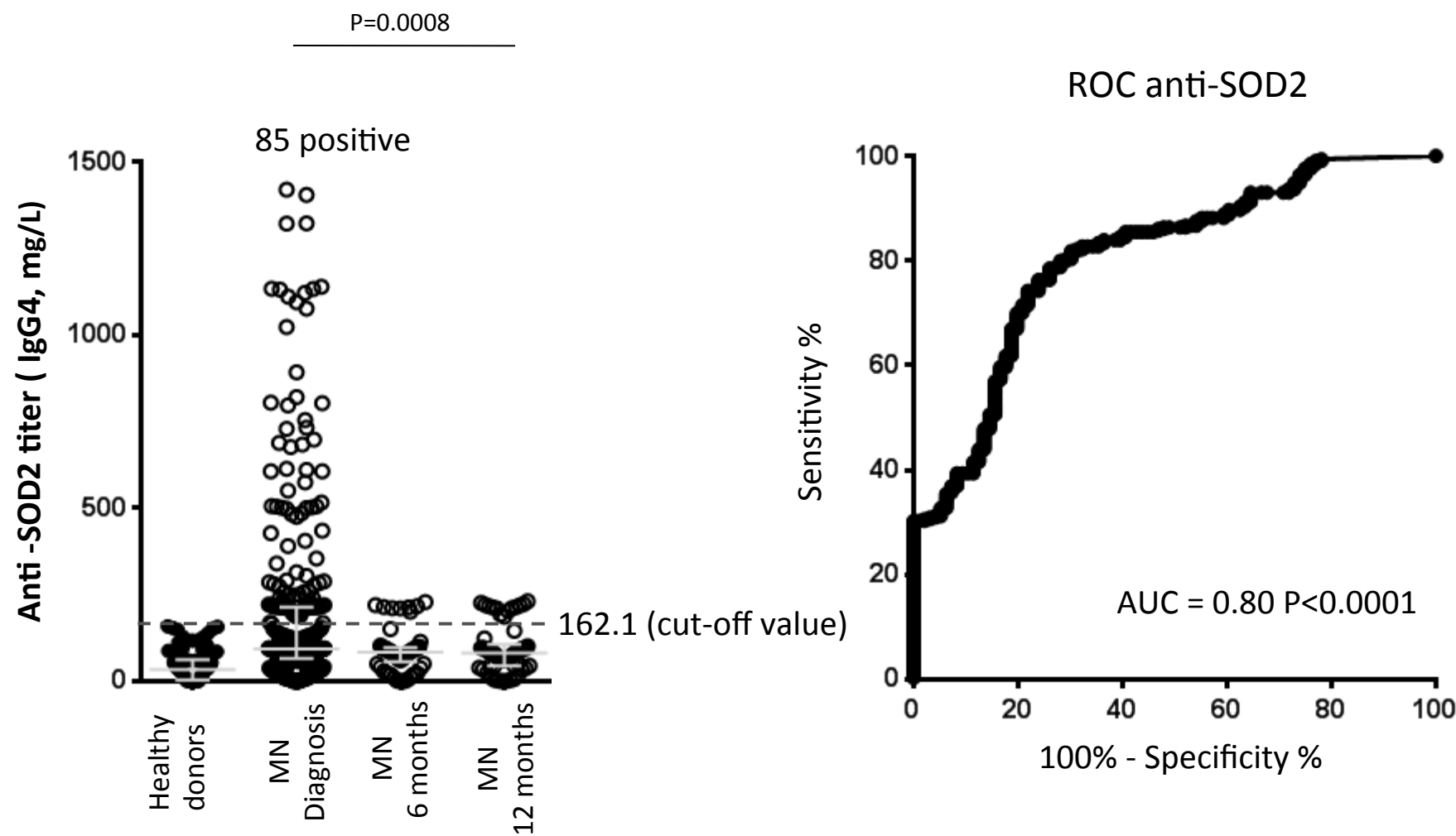
Supplemental Figure 2b



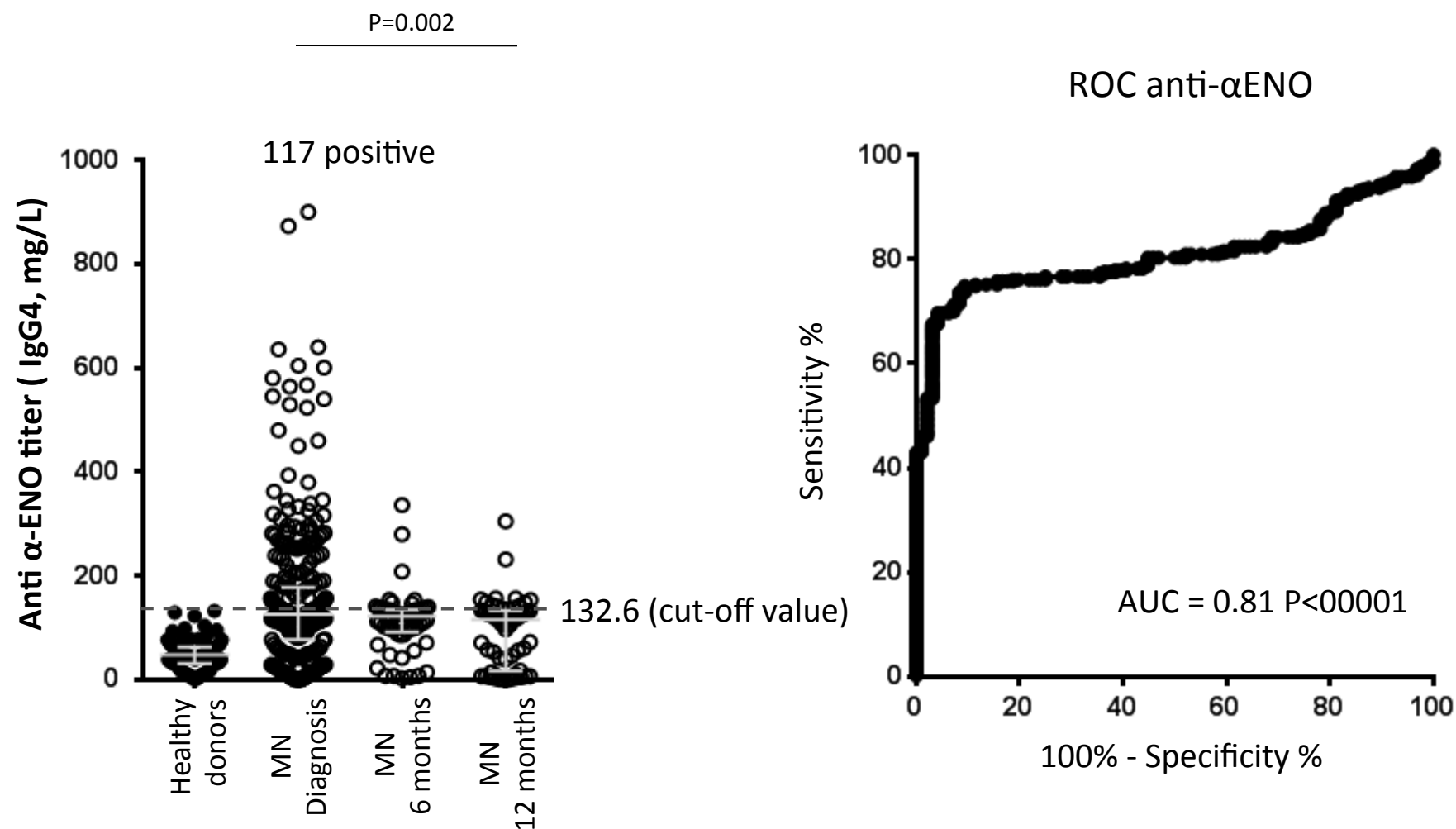
Supplemental Figure 2c



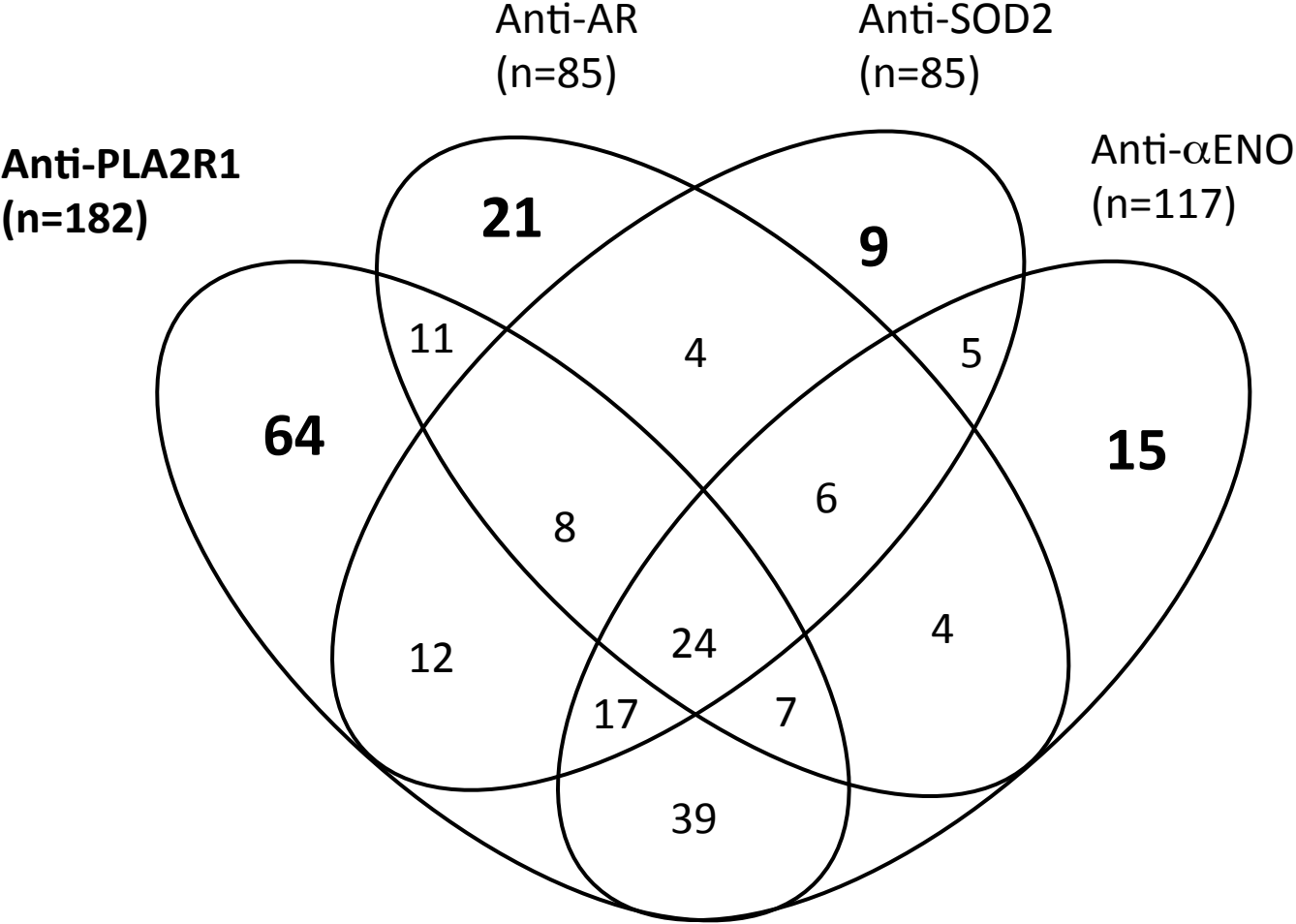
Supplemental Figure 2d



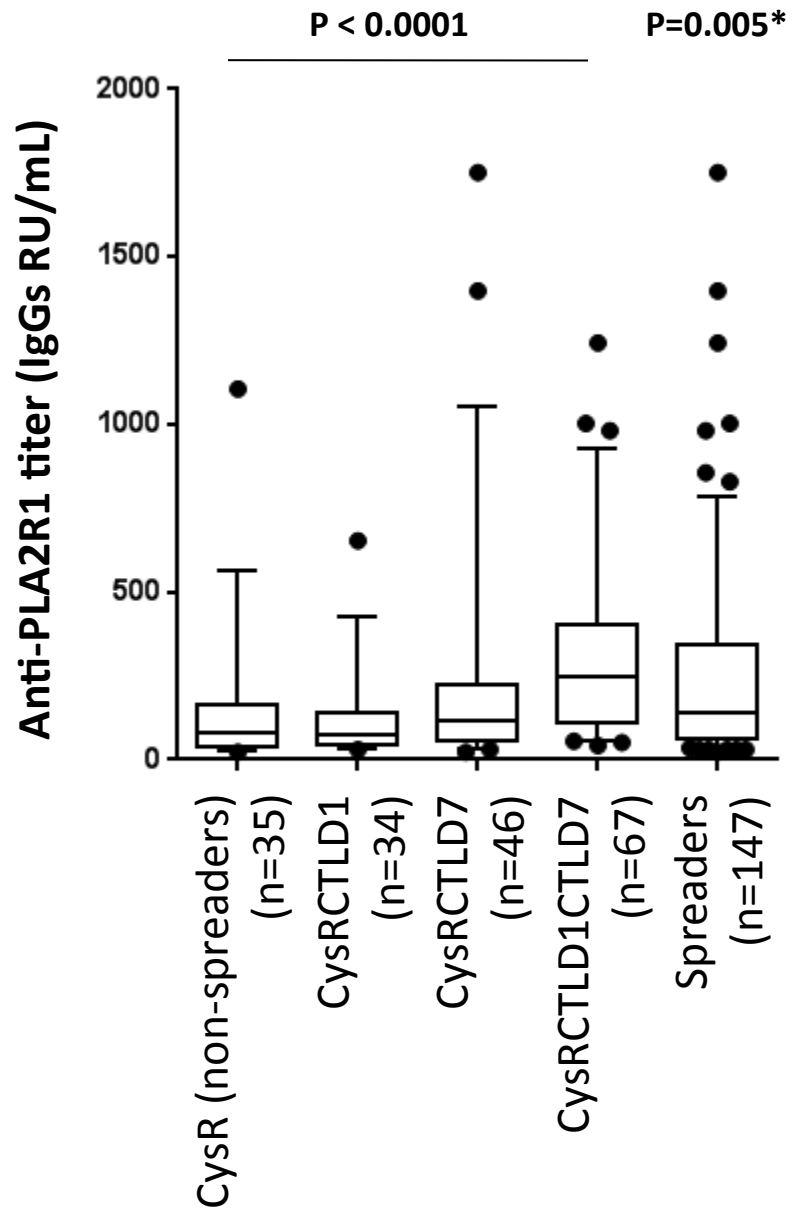
Supplemental Figure 2e



Supplemental Figure 3a

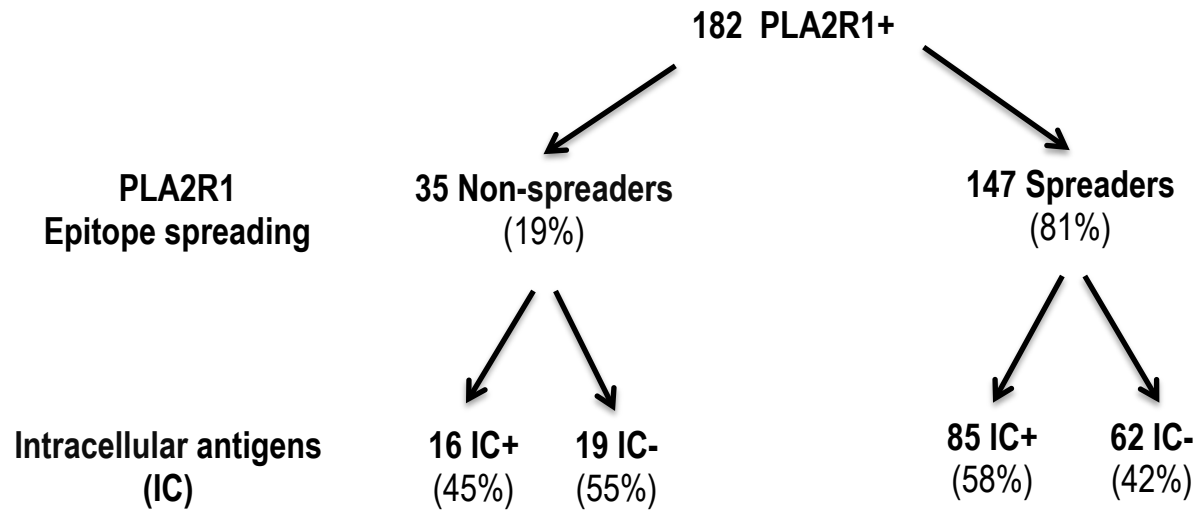


Supplemental Figure 3b

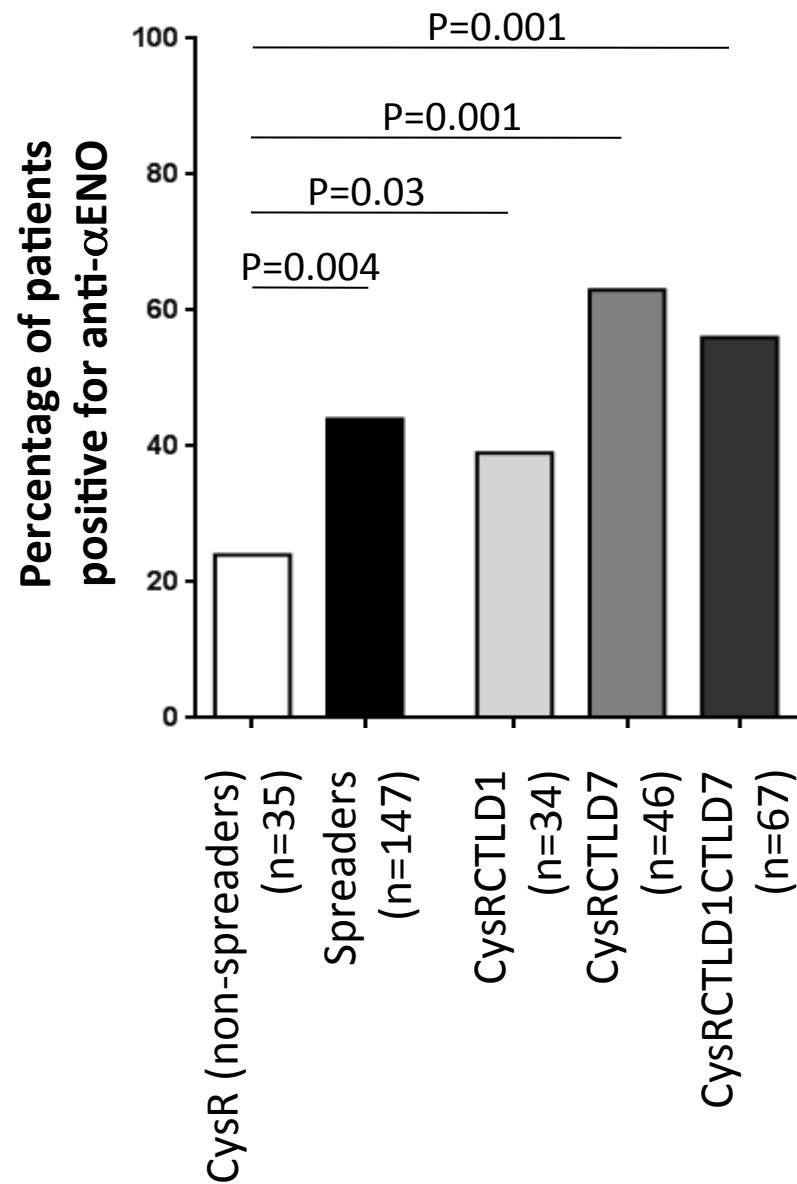


* Comparison non-spreaders versus spreaders

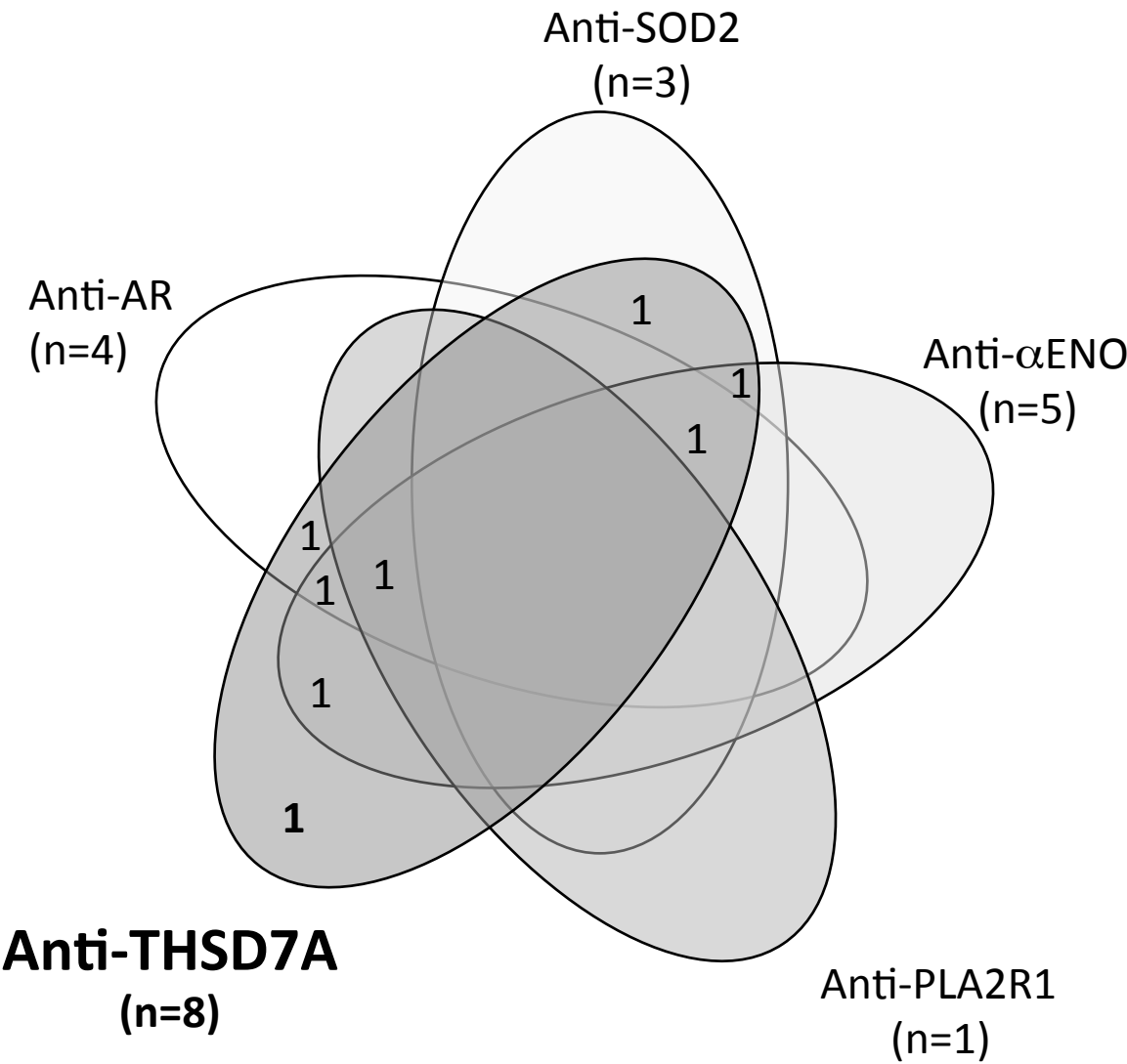
Supplemental Figure 3c



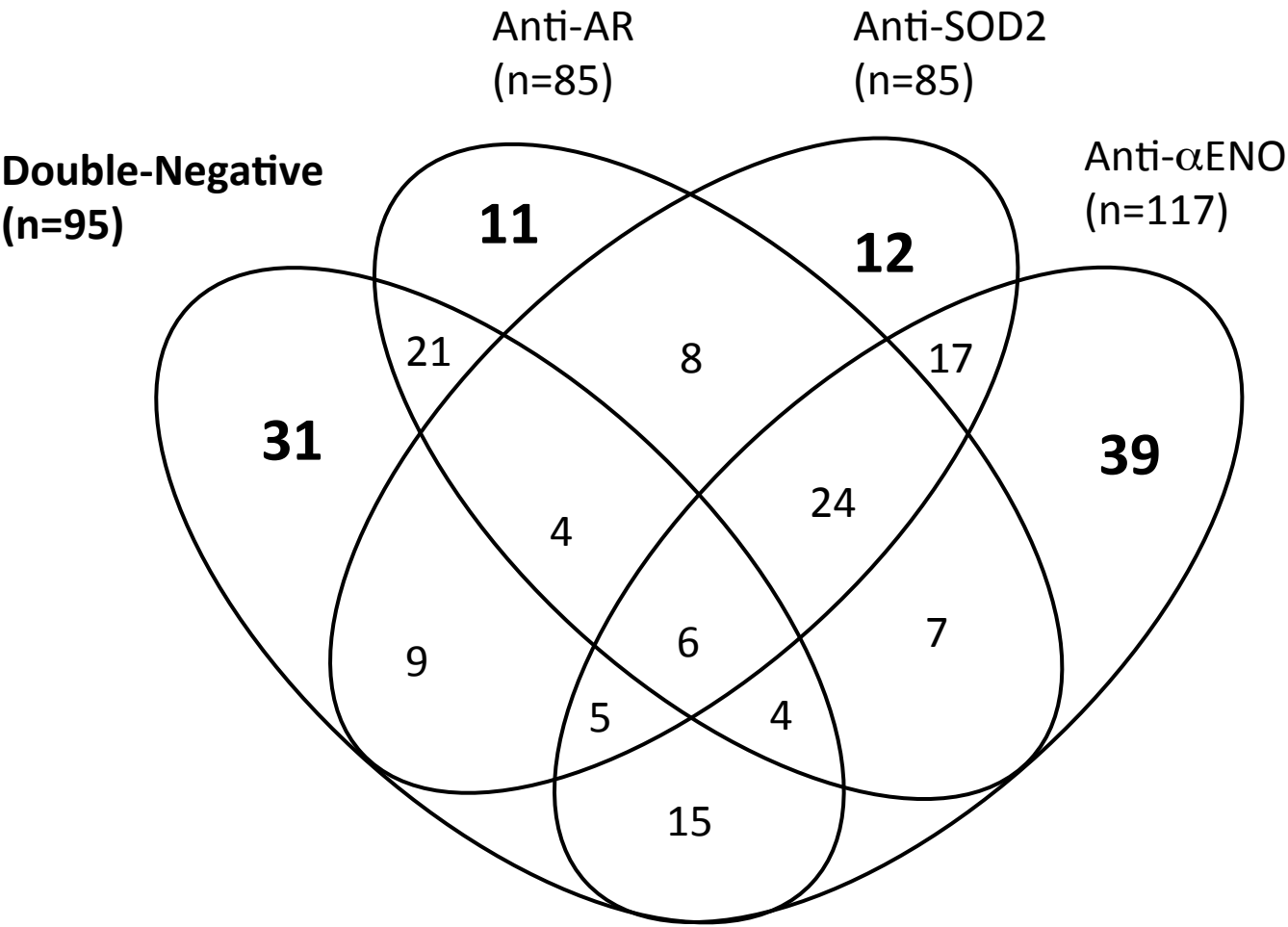
Supplemental Figure 3d



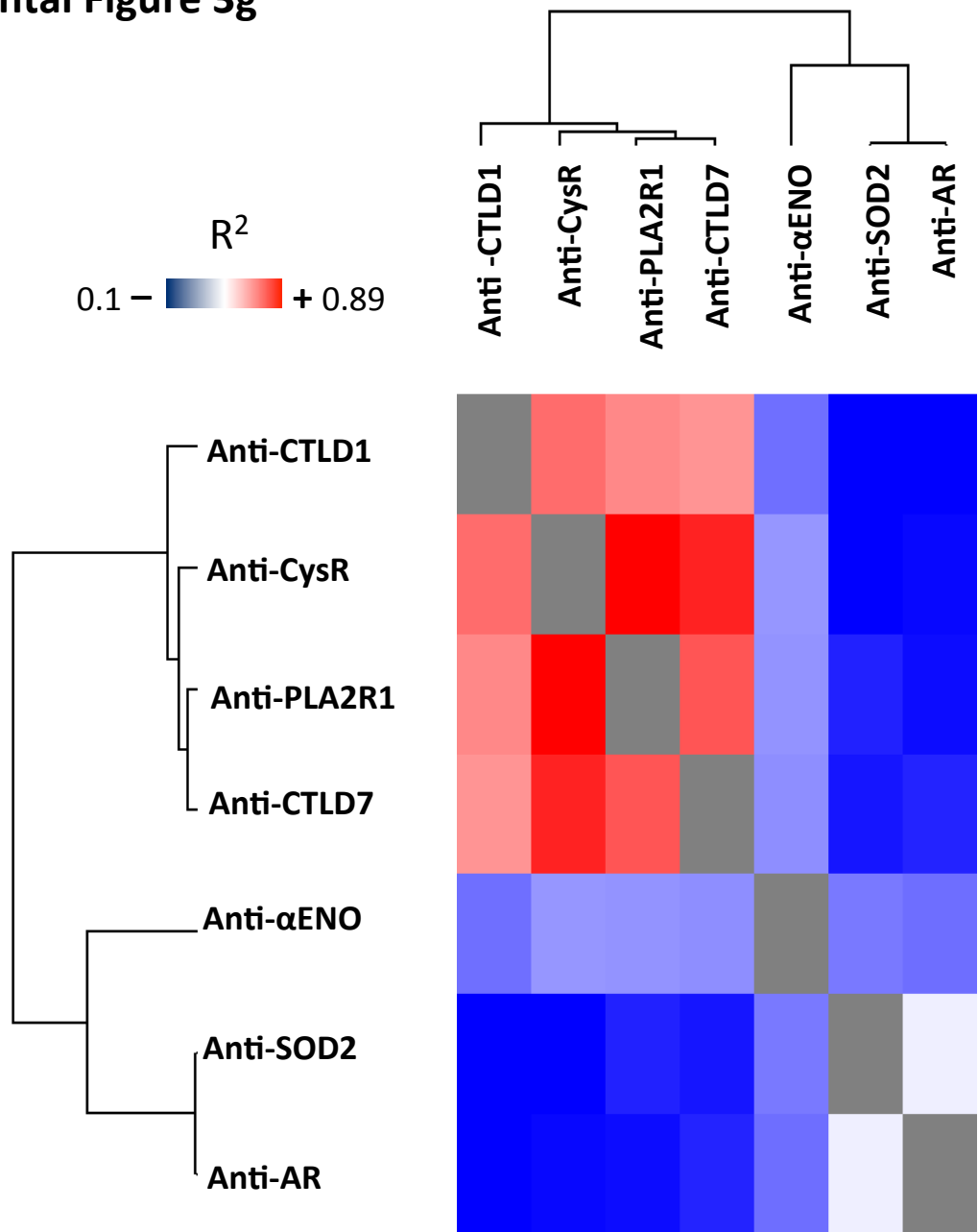
Supplemental Figure 3e



Supplemental Figure 3f



Supplemental Figure 3g



Supplemental Figure 4

