IMMUNOPATHOLOGICAL AND GENETIC OBSERVATIONS IN MEMBRANOUS NEPHROPATHY

Dóra Bajcsi MD

PhD Thesis

Szeged

2026

University of Szeged, Albert Szent-Györgyi Medical School

Doctoral School of Multidisciplinary Medicine

IMMUNOPATHOLOGICAL AND GENETIC OBSERVATIONS IN MEMBRANOUS NEPHROPATHY

PhD Thesis

Dóra Bajcsi, MD

Supervisor:

Professor Béla Iványi, MD.

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1. Publications

Publications related to this thesis:

Dóra Bajcsi, László Bitó, Sándor Turkevi-Nagy, Tibor Nyári, Éva kemény, Péter Légrády, György Ábrahám, Béla Iványi: The Value of PLA2R Antigen and IgG Subclass Staining Relative to Anti-PLA2R Seropositivity in the Differential Diagnosis of Membranous Nephropathy. *BMC Nephrol* 2023, 24(1): 230, doi:10.1186/s12882-023-03273-4. **D/Q rank: Q2, impact factor: 2,7**

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Impact factor of relevant publications: 7,6

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Scientometrics:

Number of full publications: 26 – in English: 9, in Hungarian: 17

Number of first-author publications: 6

Other publications: 4 book chapters (1 first author), 3 MANET protocols (1 first author)

Cumulative IF: 23,808

Hirsch index (MTMT, total): 7; independent Hirsch index: 6.

Number of independent citations: 88

List of abbreviations

ANCA: anti-neutrophil cytoplasmic antibody

DNA: deoxyribonucleic acid

FITC: fluorescein isothiocyanate

GBM: glomerular basement membrane

GWAS: genome-wide association study

HbsAg: hepatitis B surface antigen

HBV: hepatitis B virus

HCV: hepatitis C virus

HIV: human immunodeficiency virus

IF: immunofluorescence

IRF4: interferon regulatory factor 4

Jo-1 antibody: myositis specific autoantibody directed against the histidyl-tRNA synthetase

MN: membranous nephropathy

NELL1: neural epidermal growth-factor-like 1 protein

NFKB1: nuclear factor kappa B1

NSAIDs: nonsteroidal antiinflammatory drugs

PCR: polymerase chain reaction

PLA2R: phospholipase A2 receptor

pMN: primary membranous nephropathy

sMN: secondary membranous nephropathy

SLE: systemic lupus erythematosus

SNP: single nucleotide polymorphisms

SSA: Sjögren's-syndrome-related antigen A

T-ALL: T-cell acute lymphoblastic leukemia

THSD7A: thrombospondin type-1 domain containing protein 7A

2. Introduction

2.1. Membranous nephropathy – epidemiology, classification, pathomechanism

Membranous nephropathy (MN) is a rare immune-complex mediated glomerular disease. Its incidence is about 8 to 12 cases per 1 million worldwide [1]. MN is the main cause of nephrotic syndrome in Caucasian adults [2]. The majority of patients were generally between 50 and 60 years at the time of diagnosis [3]. The morphological lesion is characterized by thickened glomerular capillaries with basement membrane spikes along the glomerular basement membrane noted with light microscopy, having a granular staining pattern for IgG and complement 3 along the glomerular capillary loops on immunofluorescence, and subepithelial electron dense deposits on the subepithelial side of the glomerular basement membrane (GBM) observed with electron microscopy [4]. The deposits undergo a series of changes of incorporation into the GBM, and this progression is described electron microscopically in four stages. MN is classified clinically as primary MN (pMN) or secondary MN (sMN). The clinical course of pMN ranges from spontaneous remissions to progressive disease.

pMN is responsible for 75-80% of cases of MN diagnosed in a kidney biopsy, and the vast majority are associated serologically with autoantibodies against a transmembrane glycoprotein of podocytes, the phospholipase A2 receptor (PLA2R) [5], discovered in 2009. The initial anti-PLA2R antibody response is directed against the immunodominant cysteine-rich domain of PLA2R protein, and then with epitope spreading, other domains, such as the C-type lectin domain1 and the C-type lectin domain7 are also targeted [6]. The predominant anti-PLA2R antibodies are of the IgG4 subclass, but other subclasses, such as IgG1 and IgG3, are present in lower amounts [5,7]. During the evaluation of kidney biopsies, global and intense peripheral granular staining of polytypic IgG and C3, along with PLA2R antigen-positivity in a similar staining and distribution pattern indicate PLA2R-associated MN. In 2014, the second pathogenetic pathway of adult pMN, the axis of thrombospondin type-1 domain containing protein 7A (THSD7A) autoantigen and anti-THSD7A antibodies was identified in anti-PLA2R-negative cases [8]. The PLA2R- and THSD7A-associated cases represent about 85% of pMN cases. Other MN subgroups were described quite recently, including the neural epidermal growth-factor-like 1 protein (NELL1)-associated MN [9,10]. The clinical classification of MN is under the way to be replaced by an antigen-based classification [11].

2.2. Membranous nephropathy – immunohistology, immunoserology

With sMN, it has an underlying disease association, such as systemic lupus erythematosus (SLE), infection, drug exposure or malignancy, and it is usually resolved once the cause is eliminated. Certain biopsy features are not typical for pMN, such as deposits positive for IgA, IgM, and C1q as in class V lupus nephritis, or the dominant IgG subclass is not IgG4, or the distribution of deposits is subglobal as in some cases with malignancy-associated MN [10,12]. The absence of glomerular IgG4 and PLA2R [13], and glomerular leukocytosis [14] suggest malignancy-associated MN. In addition, enhanced glomerular staining of THSD7A [15] or NELL1 [10] may be observed in a fraction of patients with malignancy-associated MN.

The demonstration of circulating autoantibodies against PLA2R has become the standard process in the differentiation of pMN from sMN [16]. Nevertheless, some patients with apparent sMN might be positive for PLA2R antibodies, typically those with hepatitis B virus or C virus infection or sarcoidosis or malignancy [17]. It is not quite clear whether these patients have true sMN or the PLA2R-associated MN and the secondary disease is coincidental; and therefore, caution is required when trying to establish the clinical diagnosis of pMN solely on basis of PLA2R seropositivity, especially in patients with risk factors of neoplasia [18].

In the renal biopsy material of Ohio State University Medical Center (USA), early (stage 1) pMN tended to be IgG1-dominant and PLA2R-negative, and later stages (stages 2 to 4) tended to be IgG4-dominant and PLA2R-positive [12]. The change in IgG subclass dominance during disease progression was interpreted by the authors as a subclass switch in antibody response. IgG subclass staining is optional in the evaluation protocol of kidney biopsies, to the best of our knowledge only the Ohio State University Medical Center publications [12,19], two studies from China [20,21], and one from Japan [22] have covered the topic of IgG subtype distribution within the immune deposits of MN in native kidney samples.

2.3. Genetic background of membranous nephropathy

The genetic background of pMN is currently not entirely clear. Numerous experiments have been performed to link the susceptibility of pMN to different human leukocyte antigene (HLA) class II gene loci coding proteins essential for the presentation of antigens to T-cells. A significant increase in the HLA-DQA1*0501, DQB1*0301, and DQB1*0601 alleles was noted

in Japanese patients with pMN, while the frequency of DQB1*0501 was significantly low [23]. The role of DRB1*0301, DQA1*0501, DQB1*0201 alleles, and therefore HLA-DQ 2.5 serotype (DQA1*0501, DQB1*0201/0202) was also suggested in a British and Greek cohort [24]. In 2011, a multicenter genome-wide association study of single nucleotide polymorphisms (GWAS SNP) identified two SNPs (rs2187668—HLA-DQA1, rs4664308—PLA2R1) strongly associated with pMN in a large cohort of French, Dutch, and British patients [25]. The SNP rs4664308 is localized in the intronic region of the PLA2R1 gene. The authors came up with a model consisting of a trigger (the immune system), a bullet (PLA2R autoantibodies), and a target (glomerular antigen): SNP rs4664308 of the PLA2R1 allele is believed to cause an altered protein conformation, which is presented to the HLA class II receptor and it initiates an autoimmune reaction and in turn, pMN [25].

The role of these two SNPs was also confirmed in a large cohort of non-Caucasian pMN (n = 1112) and control patients (n = 1020) as well [26]. In a subset of Chinese patients (n = 71), the authors showed that out of the patients carrying both risk SNPs, 73% had circulating anti-PLA2R antibodies in the serum and 75% expressed PLA2R in glomeruli. They found the low expression of anti-PLA2R antibodies in controls and in patients with hepatitis B-induced sMN; and circulating anti-PLA2R antibodies displayed a high correlation with PLA2R expression. They also identified two SNPs that induced a protein alteration in PLA2R1 (rs3749117, M292V and rs35771982, H300D) that correlated ($r2>0.80$) with the PLA2R1 risk locus rs4664308 [9]. These studies suggest that these functional SNPs in tight linkage with the previously identified intronic PLA2R1 SNPs are the likely cause of pMN [25,26].

Several studies pin-pointed risk alleles in commonly inherited HLA alleles: DRB1*1501, DRB1*0301, and DRB3*0202 in the Chinese population [27,28], and DRB1*1501 and DQB1*0602 in the Japanese population [29]. In 2020, in a GWAS study of 3782 pMN cases and 9038 controls of East Asian and European descent, three classical risk alleles were also described: DRB1*1501 in East Asians, DQA1*0501 in Europeans (OR = 2.88, $p = 5.7 \times 10^{-93}$), and DRB1*0301 in both ethnicities [30]. Two previously unreported loci, NFKB1 (nuclear factor kappa B1; rs230540) and IRF4 (interferon regulatory factor 4; rs9405192), were also discovered. This association at the NFKB1 locus has become the focus for ascertaining the role of the NFKB pathway in pMN [30].

At the deoxyribonucleic acid (DNA) level, the HLA-DQA and DQB genes are tightly linked, and in most cases specific HLA-DQA and DQB alleles (serotypes) are inherited together on the same haplotype. However, at the protein level, the HLA-DQA and DQB proteins from

the various alleles can freely combine to form the HLA class II protein dimer. Hence, the different allele combinations may result in multiple HLA dimer proteins. The HLA class II antigen-presenting groove is determined by a 3-dimensional conformation of the particular HLA alpha and beta chains of the protein dimer. This is why the potential serotype combinations existing in an individual can only be assessed by the evaluation of the two HLA-DQA/DQB haplotypes.

3. Aims

3.1. Aims of the immunopathological study

The diagnostic performance of PLA2R and IgG subclass staining of kidney biopsies relative to anti-PLA2R seropositivity in the differentiation of pMN and sMN was examined. Besides PLA2R staining - which has a lower specificity than anti-PLA2R antibody serology – there is insufficient knowledge to decide which IgG1-4 subtype immunohistological patterns (IgG4-dominance, IgG4-dominance/IgG1-IgG4-codominance or IgG4-dominance/IgG4-codominance with any IgG subtype) could be used to distinguish between pMN and sMN. Here, we summarize our experiences acquired from the analysis of IgG subclass staining and PLA2R antigen staining in the diagnostic workup of MN in Hungarian patients.

3.2. Aims of the genetic study

We wanted to see whether there is a difference between allele counts of HLA-DQ 2.5 haplotype and PLA2R1 risk SNP (rs4664308) in Hungarian patients with pMN and sMN, and in the controls. We also used the tightly linked HLA-DQA1 and DQB1 loci to identify risk and protective haplotype combinations in pMN based on the homozygous or heterozygous state of PLA2R1 risk SNP. We also examined the presence of PLA2R1 risk SNP relative to PLA2R histological expression and anti-PLA2R antibody serological results.

4. Patients and methods

4.1. Patients and methods of the immunopathological study

4.1.1. Patients of the immunopathological study

Between 2011 and 2022, the evaluation of the renal biopsy samples of 87 adults led to the diagnosis of MN. The patients resided in the south-eastern part of Hungary. 84 were Caucasians, and 3 were Romas. The patients' demographic and clinical parameters are shown in Table 1. A complete panel for autoimmune serology, hepatitis and human immunodeficiency virus (HIV) serology was performed to exclude SLE, other systemic autoimmune diseases and virus-associated MN. Malignancy was screened in each case *via* chest X-ray, abdominal ultrasound, gynecologyical/urological examinations (including a prostate specific antigen test), mammography, a fecal occult bleeding test and panendoscopic evaluation of the gastrointestinal tract in individuals above 40 years. After a thorough review of the patient's medical history, drug-induced MN was also excluded. MN was assigned clinically to either pMN (n=63; 72.4%) or sMN (n= 24; 27.6%) (Table 3). The median follow-up time was 56 months (range: 1 to 276 months). In the pMN group, 5 patients died for different reasons 1 to 8 months after the diagnosis of MN; systemic autoimmune disease or malignancy did not emerge.

Table 1. Clinical and laboratory data of patients with MN

Clinical data		**pMN (n=63)**	**sMN (n=24)**	**P-value**
	Age (years)	55.8±14.3	52.8±14.7	0.388
	Gender M/F	30/33	6/8	0.087
	BMI (kg/m^2)	31.5±6,3	25.2±4.5	<0.001
	SBP (mmHg)	131.4±13.7	124.6±14.8	0.046
	DBP (mmHg)	82.6±8.5	78.8±11.4	0.095
	HR (beat/min)	78.0±10.9	74.0±8.9	0.112
Laboratory data				
	Serum creatinine (µmol/l)	104.4±63.3	98.0±92.9	0.714
	CKD-G1 (number)	23	15	0.030
	CKD-G2 (number)	16	5	0.658
	CKD-G3 (number)	18	2	0.050
	CKD-G4 (number)	5	0	0.316
	CKD-G5 (number)	1	2	0.183
	eGFR-EPI (CKD G2-5) (ml/min./1.73 m^2)	54.3±20.8	50.8±12.1	0.441
	serum albumin (g/l)	28.9±7.2	30.0±9.5	0.563
	serum cholesterol (mmol/l)	8.1± 2.8	7.3± 3.2	0.256
	serum trigliceride (mmol/l)	2.8± 2.7	2.1± 1.1	0.222
	proteinuria (g/day)	10.1± 6.0	5.2± 3.7	0.003
	nephrotic syndrome (number, %)	40 (63.5%)	10 (41.7%)	0.089
	nephrotic proteinuria without nephrotic syndrome (number, %)	19 (30.2%)	5 (20.8%)	0.435
	non-nephrotic proteinuria/ nephritic syndrome (number, %)	4 (6.3%)	9 (37.5%)	<0.001

Values are in mean ± standard deviation (SD) where necessary
(CKD: chronic kidney disease)

Table 2. Protocol used to assign patients to primary membranous nephropathy

1. Exclusion of underlying autoimmune disorder (clinical signs, laboratory parameters, autoimmune serological tests)
2. Exclusion of viral hepatitis (HbsAg, anti-HCV)
3. Exclusion of malignancy (laboratory parameters, chest X-ray, abdominal and pelvic ultrasound, gynecological and urological examinations, fecal occult blood test; in patients over 40 years of age: gastroscopy, colonoscopy)
4. Exploration of medications potentially causing drug-induced MN (NSAIDs, gold, penicillinamin)
5. No manifestation of autoimmune disorders or malignancy within two years after the renal biopsy evaluation

(MN: membranous nephropathy, HbsAg: hepatitis B surface antigen, HCV: hepatitis C virus, NSAIDs: nonsteroidal antiinflammatory drugs)

Table 3. Number of pMN and sMN cases; causes of sMN

pMN (number, %)	63 (72.4%)
sMN (number, %)	24 (27.6%)
Lupus nephritis	16
Lupus-like nephritis*	1
HCV infection + SLE	1
SLE + rheumatoid arthritis + Sjögren syndrome	1
Ulcerative colitis	1
HBV infection + Graves-Basedow disease	1
Non-differentiated collagenosis, crescentic IgA glomerulonephritis (probably IgA-vasculitis) and MN	1
Malignancy**	2

*Renal limited disorder with "full house" positivity but without serological positivity and extrarenal manifestations of SLE.

**Malignancy: squamous cell carcinoma of the oral cavity in a male patient, and acute T-cell leukemia in a female patient.

(pMN: primary membranous nephropathy, sMN: secondary membranous nephropathy, HCV: hepatitis C virus, SLE: systemic lupus erythematosus, HBV: hepatitis B virus, MN: membranous nephropathy)

4.1.2. Evaluation of kidney biopsies

Samples obtained *via* an ultrasound-guided percutaneous kidney biopsy procedure were processed by standard techniques for light microscopy, direct immunofluorescence (IF) on frozen sections with fluorescein isothiocyanate (FITC)-conjugated antibodies against IgG, IgA, IgM, C3, C1q, kappa, lambda, and fibrinogen (Dako, Denmark; 1:20 dilution for IgG, and 1:10 dilution for the others), and electron microscopy. If granular peripheral IgG staining indicating MN was observed on IF, the case was further investigated with FITC-conjugated antibodies to IgG1-4 (The Binding Site, UK; 1:10 dilution) and PLA2R antigen (indirect IF method, Sigma-

Aldrich, Switzerland; primary antibody dilution 1:10). The staining intensity was assessed and photographed at 10x objective lens magnification and graded semiquantitatively on a scale of 0 to 3 + (Figure 1). IgG4-dominance was stated if the intensity score was higher by at least 1 level than that of the other IgG subclasses, and codominance was established if the intensity scores were similar among IgG subclasses. The electron microscopical reading of the stages were as follows: Stage 1: subepithelial deposits without basement membrane reaction; Stage 2: subepithelial deposits with basement membrane spikes between deposits; Stage 3: the spikes enclose the deposits and form a layer between the deposits and the podocytes; and Stage 4: electron-lucent areas in deposits become incorporated into the markedly and irregularly thickened GBM.

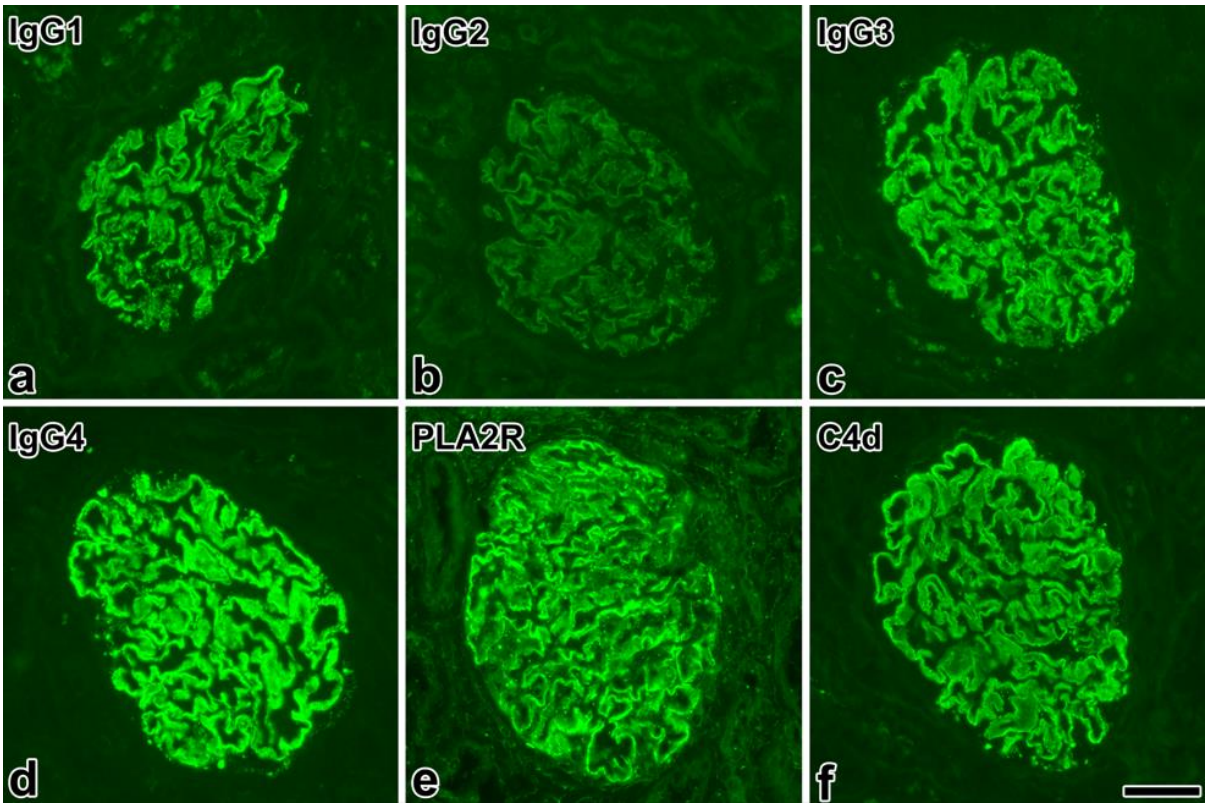

Figure 1. Direct immunofluorescence (IF) on frozen sections with FITC-conjugated antibodies against IgG1-4 subtypes, PLA2R and C4d

(FITC: fluorescein isothiocyanate, PLA2R: phospholipase A2 receptor)

4.1.3. Anti-PLA2R immunoserology

Blood serum samples were collected in 74 patients (54 pMN, 20 sMN) at the time of diagnosis (n=69) or in the case of a relapse (n=5). An indirect IF semiquantitative assay (Euroimmun US) was used to detect circulating anti-PLA2R antibodies. Serum samples were diluted at 1:10, and those found to be positive at 1:10 dilution were further diluted and evaluated. The results of the test given in this paper appear as either positive or negative.

4.1.4. Statistical analysis of the immunopathological study

Continuous variables with a normal distribution were presented as the mean ± standard deviation and were compared using Student's t-test. Categorical variables were described in percentage terms and the association between groups was analyzed using the Fisher exact test. A non-parametric Spearman rank correlation analysis was carried out to investigate the relationship among anti-PLA2R seropositivity, PLA2R antigen staining and patterns of IgG subclass variables. Also, the median follow-up time and its interquartile range were computed. A p value <0.05 was treated as statistically significant.

4.2. Patients and methods of the genetic study

4.2.1. Patients of the genetic study

A total of 67 MN patients with MN (27 males, 40 females) were analyzed. 22 patients were enrolled prospectively, and 55 patients were enrolled retrospectively. The demographic data of patients and controls are shown in Table 4. Our clinical evaluation (see Table 5) placed 52 patients (77.6 %) in the pMN group, and 15 patients (22.4 %) in the sMN group. Figure 2 provides a detailed overview of the patient inclusion process. The most common cause of sMN here was lupus nephritis (Table 5).

Table 4. Demographic data of patients with membranous nephropathy and control persons

	Membranous nephropathy	**Controls**
Number of cases	68 Caucasians	77 Caucasians
Age at the time of genetic examinations (years)	50.1±14.7	46.8±8.6
Age at the time of kidney biopsy (years)	46.0±15.6	NA
Female/Male (%)	56/44	65/35

NA: not applicable

Table 5. Types of sMN cases

Types of sMN	**Number of cases**
Systemic autoimmune diseases	
Membranous lupus nephritis	8
Mixed tissue connective disease	1
Rheumatic arthritis (+ in 1 case: gold exposure)	2
Organ-specific autoimmune diseases	
Hashimoto thyroiditis	1
Autoimmune hepatitis	1
Viral Hepatitis	
Hepatitis B (+ Graves-Basedow disease)	1
Hepatitis C (+ lupus nephritis)	1

(sMN: secondary membranous nephropathy)

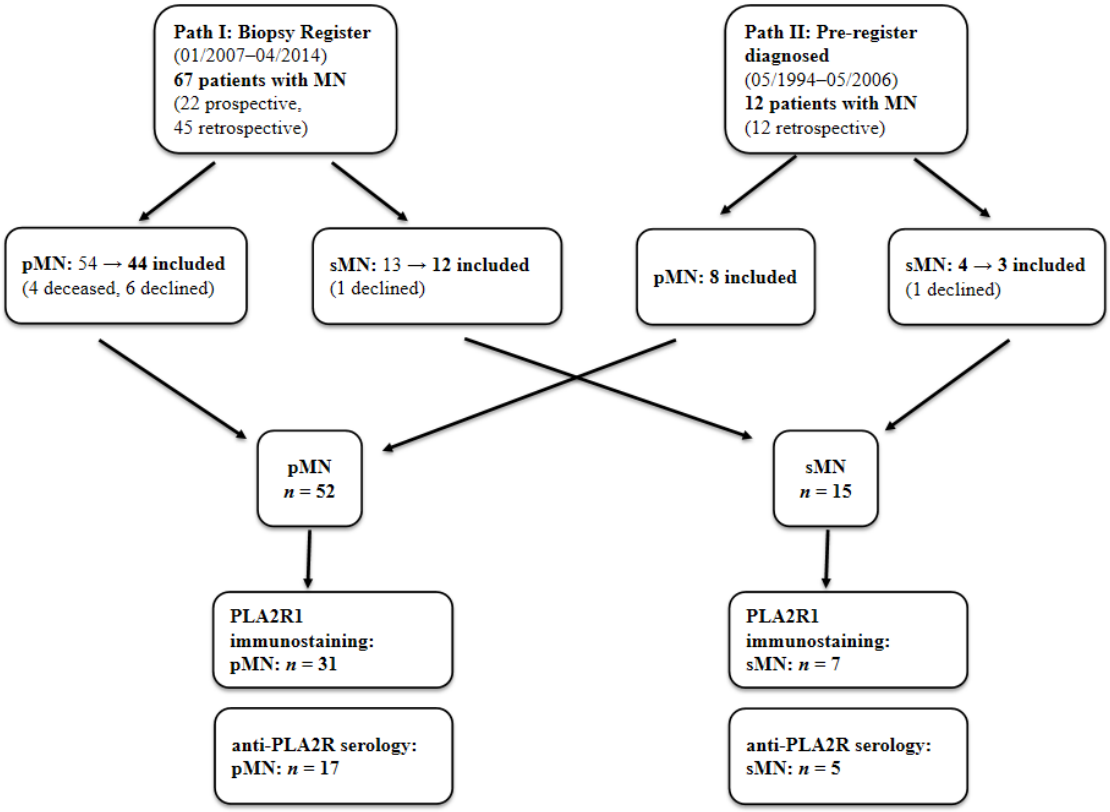

Figure 2. Patient inclusion flow chart

MN: membranous nephropathy, pMN: primary membranous nephropathy, sMN: secondary membranous nephropathy

4.2.2. Histological evaluation and anti-PLA2R serology of the genetic study population

Glomerular PLA2R immunostaining or anti-PLA2R antibody serology was evaluated in 38 patients (31 pMN, 7 sMN); read as negative or positive.

4.2.3. Genetic evaluation

A blood sample was taken from the patients with MN and from 77 age-matched, normotensive, clinically healthy persons (Table 4). The persons were enrolled from blood donors of the Regional Blood Bank of Szeged. Real-time polymerase chain reaction (PCR) with a melting curve analysis was used to genotype the rs4664308 SNP in the PLA2R1 gene. HLA-

DQA1 and the tightly linked HLA-DQB1 were determined using Inno Lipa diagnostic kits, and the HLA-DQ serotypes were derived from the haplotype results [25].

4.2.4. Scoring of the risk property of HLA-DQ serotype combinations in pMN

Literature findings suggest that the PLA2R1 risk allele (rs4664308, A) gives rise to the illegitimate expression of PLA2R protein [26] in the kidney. The homozygous GG alleles (rs4664308) do not cause the expression of PLA2R pathogenic epitopes; in the case of the AG heterozygotes, only a single allele expresses PLA2R; and in the case of the AA homozygous risk allele combination, there is double illegitimate PLA2R expression. In a patient with histologically confirmed MN, a heterozygous PLA2R1 risk allele carries an elevated risk of the HLA-DQ haplotype combination since MN develops even in the case of lower PLA2R expression from a single allele. Likewise, the presence of homozygous risk alleles in a control patient suggests a protective HLA-DQ haplotype combination, since even with potentially higher illegitimate PLA2R expression, the patient does not develop MN. With this biological assumption, we created a risk scoring system for the observed HLA-DQ serotype combinations. We counted the number of homozygous, heterozygous PLA2R1 rs4664308 risk alleles and the homozygous reference alleles in MN and the control patients. To take into account the number of individuals in the different groups, we decided to normalize the count data using allele frequencies based on the sample size.

We marked the allele combinations of the control persons as ‘aa’ (homozygous risk alleles), ‘ag’ (heterozygous risk alleles), and ‘gg’ (homozygous reference alleles). The patients with pMN were assigned in the same way with capital letters (AA: homozygotes to PLA2R1 risk allele; AG: heterozygotes; GG: not carrying the PLA2R1 risk allele). With our biological assumption, we calculated a risk score. For each HLA-DQ serotype, we calculated the frequency of PLA2R1 risk alleles in the pMN and control individuals carrying the particular HLA serotype using the following formula:

$$\text{risk score HLA haplotype combination} =$$

$$\text{freq (pMN\_AA)} + 2 \times \text{freq (pMN\_AG)} - 2 \times \text{freq (control\_AA)} - \text{freq (control\_AG)}$$

In the case of a neutral effect, we do not expect differences in the allele frequencies of the PLA2R1 risk alleles, and the score should be close to zero. A negative score means a

protective effect, while a positive score means an increased risk of the HLA-DQ haplotype combination developing PLA2R antigen-based MN.

4.2.5. Statistical analysis of the genetic study

Fisher's exact test of the raw count data was used to see whether there was any significant difference in the total number of PLA2R1 risk SNP and HLA-DQ 2.5 haplotype in the groups of pMN, sMN, and control group. If there was any significant difference, as a post-test Fisher's exact test was used to ascertain whether there was any significant difference in the count for pMN-controls, pMN-sMN, and sMN-controls. Because multiple assumptions were tested, the p threshold was set to 0.05/3 (p = 0.017—the Bonferoni correction).

5. Results

5.1. Results of the immunopathological study

5.1.1. Anti-PLA2R seropositivity, PLA2R antigen staining positivity

Among patients assigned to pMN, 61.1% displayed anti-PLA2R seropositivity and 81.0% displayed PLA2R antigen staining positivity. 4 patients with PLA2R-negative pMN were tested for anti-thrombospondin 7A-seropositivity, with a negative result (note: this test at our institution has been available since 2020).

In 3 patients out of the 24 placed in the sMN group (Table 3), the causal link between the glomerulopathy and the underlying disease was not entirely obvious. The first patient was a 57-year-old woman in whom T-cell acute lymphoblastic leukemia (T-ALL) and concurrent MN were diagnosed. The case was published recently elsewhere [31]. In brief, the proteinuria displayed a close relationship with the presence, chemotherapy-induced remission, and then relapse of T-ALL, thus it met the clinical criteria of malignancy-associated MN proposed by P. Ronco [32]. The pathological phenotype was characterized by mild and segmental positivity for glomerular PLA2R antigen, an incomplete global distribution of IgG1-IgG4 codominant deposits, stage 1-2 glomerulopathy, and more than 8 endocapillary leukocytes/glomerular profile. The immunostainings for glomerular THSD7A and NELL1 expression were negative.

An indirect immunofluorescence assay for autoantibodies against PLA2R, THSD7A and neutral endopeptidase revealed a low anti-PLA2R titer. Although the leukemic lymphoblasts expressed CD10 (neutral endopeptidase) immunohistochemically, no change was observed in the CD10 expression of podocytes. It appeared that the T-ALL-associated MN was mediated by a strong autoimmune response for a hidden tumor antigen, and a weak one for PLA2R. The patient died six weeks after the renal biopsy procedure [31]. The second patient was a 66-year-old man, and he had PLA2R antigen-positive, IgG4-dominant, stage 3 MN, exhibiting glomerular leukocytosis; and anti-PLA2R antibodies were demonstrated in high titer. The nephrotic syndrome did not respond to glucocorticoid plus cyclophosphamide therapy. Metastatic squamous cell carcinoma of the oral cavity was diagnosed 12 months after the diagnosis of MN, and the patient passed away shortly thereafter. Glomerular leukocytosis was treated in retrospect as the feature of malignancy-associated MN [14], since thrombi in veins and/or glomeruli, dilation and congestion of glomerular and peritubular capillaries, disproportionate interstitial edema and/or focal interstitial hemorrhages suggestive of renal vein thrombosis were not observed in the biopsy specimen, and the ultrasound evaluation of the kidneys before and after the biopsy procedure did not raise the suspicion of renal vein thrombosis. A computed tomography angiography of kidneys, however, was not performed during the clinical evaluation of the patient. The third patient, a 53-year-old man with non-differentiated collagenosis (asymmetric oligoarthritis, gastrointestinal and other extrarenal symptoms, 0.73 g/day proteinuria, microhematuria, decreased renal function, raised inflammatory markers, negativity for ANCAs, positivity for anti-nuclear antibodies, anti-SSA antibodies, and anti-Jo1 antibodies, and mild positivity for rheumatoid factor-IgA) showed the simultaneous presence of stage 4 MN and crescentic IgA glomerulonephritis (probably IgA-vasculitis). The MN displayed IgG4-dominance and mildly intense positivity for PLA2R antigen.

5.1.2. Sensitivity and specificity values of anti-PLA2R serological and PLA2R and IgG1-4 subclass immunohistological staining findings

For pMN, the sensitivity and specificity values for anti-PLA2R seropositivity, PLA2R antigen staining and patterns of IgG subclass distribution are shown in Table 6. As can be seen, the specificity of PLA2R seropositivity, IgG4-dominance, and IgG4-dominance/IgG3-IgG4-codominance was high, around 90%. Among the sensitivity values, IgG4-

dominance/codominance with any other IgG subtype displayed the best sensitivity (92.1%), followed by PLA2R staining (81.0%), and IgG4-dominance/IgG3-IgG4-codominance (76.2%).

Table 6. Sensitivity and specificity values of serological and immunohistological examinations for the detection of pMN

	Sensitivity (%)	**Specificity** (%)
Anti-PLA2R seropositivity	61.1	90.0
PLA2R antigen staining	81.0	66.7
IgG4-dominance	52.2	91.7
IgG4-dominance/codominance with any other IgG subtype	92.1	70.8
IgG4-dominance/IgG1-IgG4-codominance	64.2	75.0
IgG4-dominance/IgG3-IgG4 codominance	76.2	87.5

5.1.3. IgG1-4 subclass immunohistological staining findings in pMN and sMN

Regarding the distribution of IgG subclasses in pMN (Figure 3), IgG4-dominance was the predominant pattern (55.5%). The second most frequent pattern was IgG3/IgG4-codominance (20.6%), and the third was IgG1/IgG4-codominance (14.2%). In contrast, IgG1-dominance, followed by IgG3-dominance characterized sMN. In sMN, IgG4-dominance was rarely encountered, observed in the case of MN and concurrent crescentic IgA glomerulonephritis, and in the case of MN and squamous cell carcinoma of the tonsils. Of interest, PLA2R antigen staining positivity was detected in 3 out of the 5 non-lupus sMN cases.

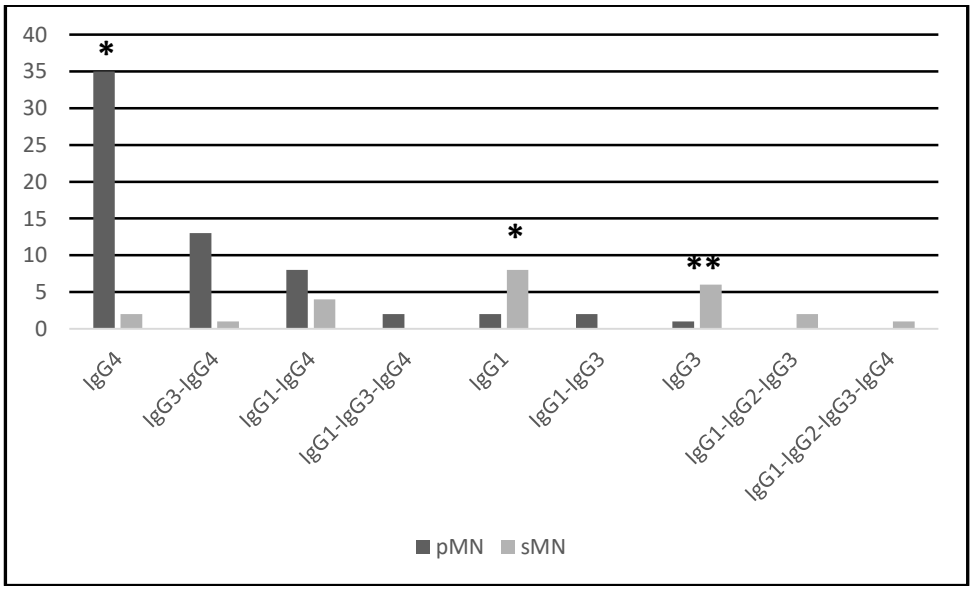

Figure 3. IgG subclass dominance/codominance in pMN and sMN

There is a significant difference in the prevalence of the IgG subtype dominance/codominance between cohorts of pMN and sMN (*p<0.001, **p=0.002, respectively)

5.1.4. Correlation among the different immunoserological and immunohistological patterns in pMN and sMN

The analysis of correlation among anti-PLA2R seropositivity, PLA2R antigen staining, and patterns of IgG subclass distribution (Table 7) found that almost all the parameters had a significant correlation with each other; that is, anti-PLA2R seropositivity had an excellent correlation with PLA2R antigen staining, IgG4-dominance/codominance, and IgG4-dominance/IgG3-IgG4-codominance, respectively. Anti-PLA2R seropositivity and PLA2R antigen staining were concordantly positive in 29 and concordantly negative in 7 out of the 54 pMN cases. Anti-PLA2R seropositivity was accompanied by negative PLA2R staining in 4 patients; and conversely, positive PLA2R staining was accompanied by a negative anti-PLA2R antibody test result in 14 patients out of the 54 pMN cases. Most of the latter cases were classified electron microscopically as early MN because stage 1 was observed in 8 samples, and in 2 cases with stage 4 disease fresh subepithelial deposits were noted, indicating a relapse in the formation of immune deposits. There were 5 cases with positive PLA2R antigen staining that did not display IgG4-dominance/codominance, and there were 12 cases in which IgG4-dominance/codominance was accompanied by negative PLA2R staining.

Table 7. Correlation between the immunohistological parameters and anti-PLA2R seropositivity

Examinations		p value of correlation
Anti-PLA2R seropositivity	PLA2R antigen staining	<**0.001**
Anti-PLA2R seropositivity	IgG4-dominance	0.057 (NS)
Anti-PLA2R seropositivity	IgG4-dominance/codominance (with any other IgG subtype)	**<0.001**
Anti-PLA2R seropositivity	IgG4-dominance/IgG1-IgG4-codominance	0.108 (NS)
Anti-PLA2R seropositivity	IgG4-dominance/IgG3-IgG4-codominance	**<0.001**
PLA2R antigen staining	IgG4-dominance	0.005
PLA2R antigen staining	IgG4-dominance/codominance (with any other IgG subtype)	0.011
PLA2R antigen staining	IgG4-dominance/IgG1-IgG4-codominance	0.007
PLA2R antigen staining	IgG4-dominance/IgG3-IgG4-dominance	0.005

Correlations were analyzed in 74 cases, NS: not significant

5.1.5. IgG subclass dominance/codominance in different stages of pMN

Regarding the distribution pattern of IgG subclasses in different stages of pMN, IgG4-dominance was observed in all the stages of pMN (Figure 4). There was no significant difference between the prevalence of IgG4-dominance and IgG4-dominance/codominance among the stages (p=0.950, p=0.849).

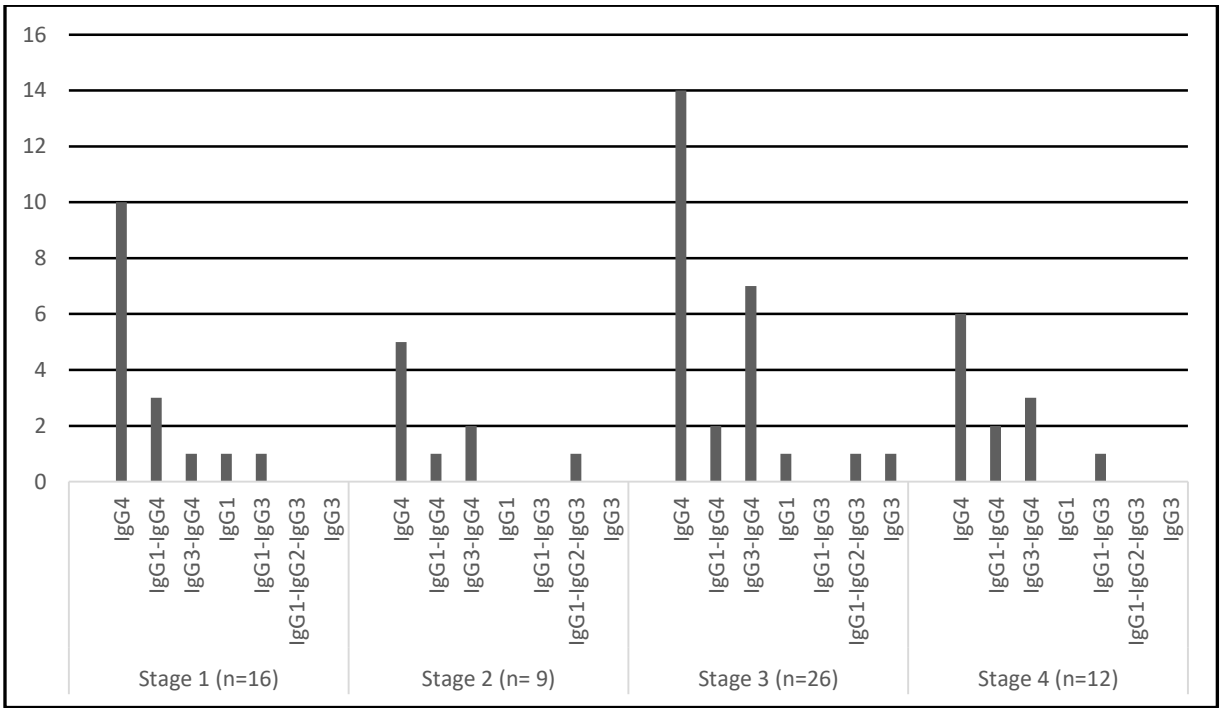

Figure 4. IgG subclass dominance/codominance in different stages of pMN

5.1.6. C1q positivity in pMN and sMN cases in the different IgG subtype dominance/codominance groups

C1q positivity was significantly more frequent in sMN than pMN (p<0.001). In pMN, C1q positivity was mainly associated with cases of IgG3-IgG4-codominance (Figure 5.). Among IgG4-dominant pMN cases, C1q negativity was significantly more frequent than C1q positivity.

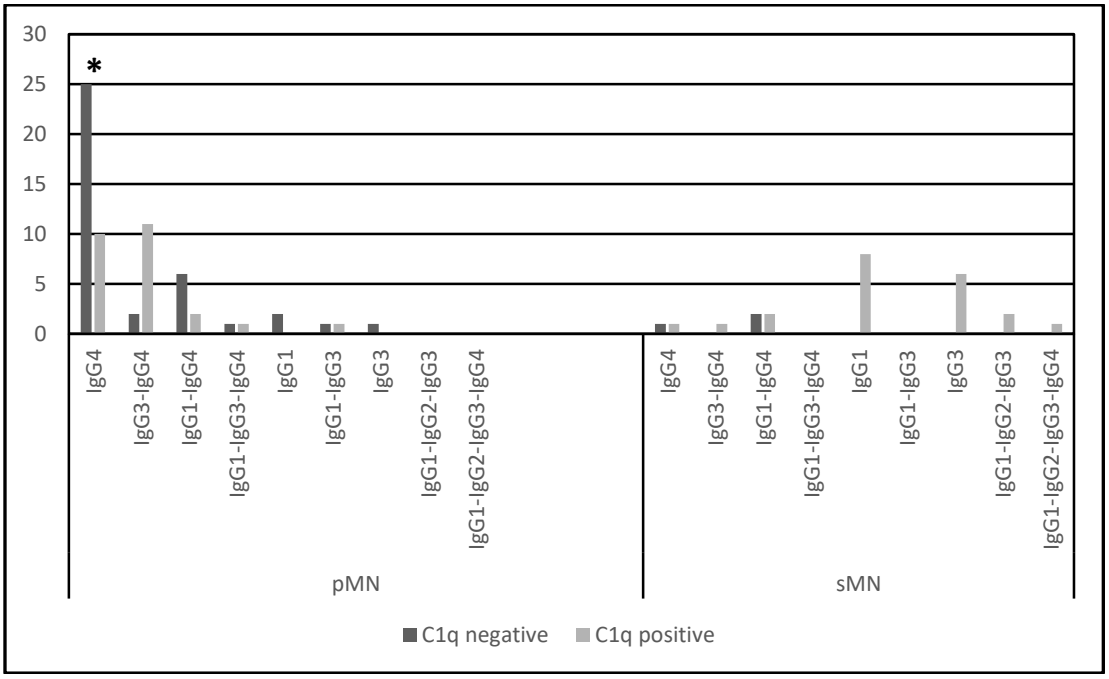

Figure 5. C1q positivity in pMN and sMN cases in the different IgG subtype dominance/codominance groups
Among the IgG4-dominant pMN cases, C1q negativity was more frequent than C1q positivity (*p=0.039).

5.2. Results of the genetic study

5.2.1. PLA2R immunostaining, serum anti-PLA2R antibody level

Among patients with pMN, PLA2R immunostaining positivity was observed in 24 patients (80%). The immunostaining was negative in 6 patients (20%). Regarding the anti-PLA2R antibody serology testing, 13 (76.5%) patients had positive and 4 patients (23.5%) had

negative results. In patients with sMN, the PLA2R immunostaining and anti-PLA2R immunoserology findings were negative.

5.2.2. PLA2R immunostaining, anti-PLA2R antibody serology for risk PLA2R1 rs4664308 alleles

Twenty-four patients were homozygotes for risk PLA2R1 rs4664308 alleles (AA). Nineteen patients had either PLA2R immunostaining positivity or serum anti-PLA2R antibody positivity, and clinically they were classified as having pMN. Five patients had neither PLA2R immunostaining positivity nor serum anti-PLA2R antibody positivity; 2 of these 5 patients had clinically diagnosed sMN (see Figure 6).

From the 7 sMN, 4 were heterozygotes for risk PLA2R1 rs4664308 alleles (AG); they had neither positive PLA2R immunostaining nor anti-PLA2R serology. Of the three patients not carrying PLA2R1 rs4664308 risk alleles (GG), all had no PLA2R immunostaining or anti-PLA2R immunoserology positivity. Two of them were classified as having sMN, and one of them as having pMN (see Figure 6).

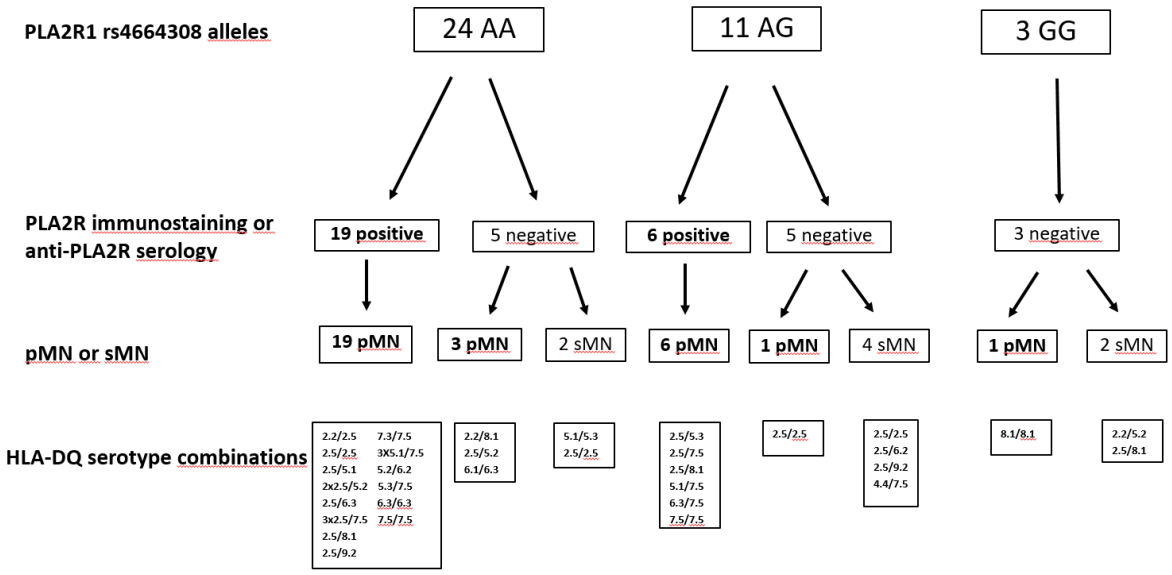

Figure 6. PLA2R immunostaining, anti-PLA2R antibody serology in the case of risk PLA2R1 rs4664308 alleles

(pMN: primary membranous nephropathy, sMN: secondary membranous nephropathy, PLA2R: phospholipase A2 receptor)

5.2.3. Allele counts of PLA2R1 rs4664308 SNP and HLA-DQ 2.5 haplotype

Since both the HLA-DQ 2.5 allele and the PLA2R1 risk SNP were statistically associated with pMN in the literature [24-28], we decided to count the risk and nonrisk alleles for both HLA-DQ 2.5 and the PLA2R1 risk SNP in the different groups. Table 4 gives the allele counts of HLA-DQ2.5 haplotype and PLA2R1 rs4664308 SNP in patients with pMN, sMN, and the control group. The HLA-DQ 2.5 haplotype counts were significantly higher in patients with pMN and sMN than those in the controls; and the difference between the counts of pMN and sMN patients was not significant. The PLA2R1 risk SNP counts were significantly higher in patients with pMN than those in the controls. No significant difference was found between the allele counts of the sMN and control groups. After the Bonferroni correction for multiple hypothesis testing, there was no statistically significant (the modified threshold being 0.016) difference between patients with sMN and pMN (p = 0.034), which was most likely due to the lower number of sMN cases (see Table 8).

Table 8. The allele counts for the HLA-DQ 2.5 allele (top) **and PLA2R1 risk allele (rs4664308) (**bottom).

	Allele counts		
	pMN	**sMN**	**Control**
HLA-DQ 2.5	27+	11++	18
Other HLA-DQ haplotypes	77	19	136

+ pMN is significantly different from the control (p=0.004), but not from sMN (p=0.259)
++ sMN is significantly different from the control (p=0.002)

	Allele counts		
	pMN	**sMN**	**Control**
PLA2R1 risk SNP (rs4664308)	81*	17	94
PLA2R1 non risk SNP	23	13	60

* pMN is significantly different from the control (p=0.005)
The difference between pMN and sMN is within the significance threshold after Bonferroni correction (p=0.034)
The difference between sMN and the control was not significant (p=0.687)
(pMN: primary membranous nephropathy, sMN: secondary membranous nephropathy, PLA2R1: phospholipase A2 receptor, SNP: single-nucleotide polymorphism)

5.2.4. Scoring Procedure Used for Assessing the HLA-DQ Serotype Combinations in pMN

As described in the Methods section, we created a scoring procedure for the HLA-DQ serotype combinations based on the observed absence or presence of heterozygous or homozygous PLA2R1 risk allele(s) and the disease status of the individual. We arranged the observed HLA-DQ serotype combinations in our cohort based on this scoring system (see Figure 7) to assess the risk and protective serotype combinations. Among the combinations, HLA-DQ 2.5 seemed to carry the highest risk of developing pMN (present in both first two combinations of the highest score), HLA-DQ 8.1 seemed to carry a risk (present twice in the combinations of the highest scores). As for protective HLA-DQ serotype combinations, HLA-DQ 7.5 and 6.2 seemed to be protective, being present in the most protective combinations (not found in patients with pMN, but occurring in controls carrying the PLA2R1 risk allele).

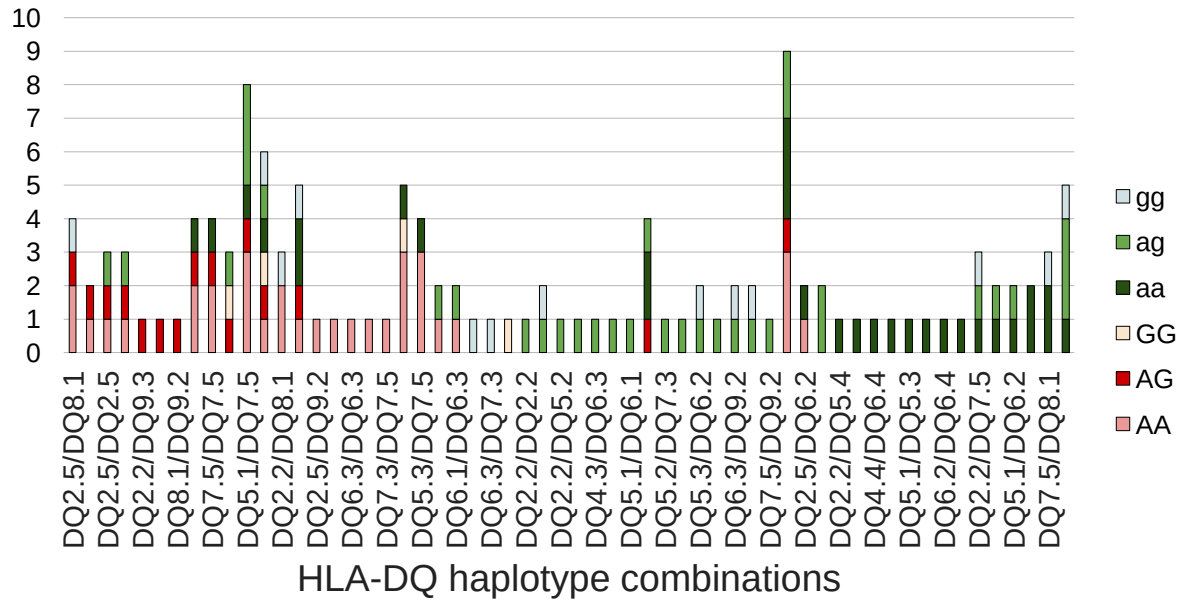

Figure 7. HLA-DQ haplotype combinations in the pMN patients – risk score
(pMN: primary membranous nephropathy)

6. Discussion

6.1. Discussion of the immunopathological study

In the clinical management of patients with MN, it is mandatory to exclude SLE, infections, drugs and malignancy, regardless of whether anti-PLA2R antibodies and/or anti-THSD7A antibodies are absent or present [16]. However, the intensification of diagnostic procedures for looking for a secondary origin may vary in different nephrology units. To look for malignancy, some centers conduct the basic examinations of chest X-ray, abdominal ultrasound, a pelvic/rectal exam for gynecological/urological malignancy, the fecal occult bleeding test, and mammography, while others perform panendoscopy of the gastrointestinal tract. Still it is possible that the malignancy may only be detectable on a PET or CT scan or it cannot be detected at the time of diagnosis of MN, just several months later [33]. Moreover, it is not clear how intensively and how often clinicians should carry out the examinations for sMN. And, if a potentially secondary origin is identified, it may not be easy to determine the causal relationship between the underlying disease and MN; an association of the two disorders may be merely coincidental. The results of PLA2R antigen and IgG subclass distribution in glomerular deposits [17, 34] can provide relevant information alongside clinical examinations in the separation of pMN from sMN. This is discussed below.

The features of pMN presented refer to patients living in an agricultural region of Central-Eastern Europe, and their being almost exclusively Caucasians. As far as we know, no similar study has been published for this part of Europe. The diagnostic performance of PLA2R staining relative to anti-PLA2R seropositivity for the detection of pMN was 81% sensitivity and 66.7% specificity for glomerular PLA2R, and 61.1% sensitivity and 90% specificity for anti-PLA2R seropositivity. In the cohort of 42 pMN patients from France [35] there were 10 patients with positive PLA2R staining and no measurable antibodies in the serum, and 3 patients with high levels of anti-PLA2R antibodies and negative PLA2R glomerular staining (biopsy sensitivity 74%; serum sensitivity 57%). In the study of 22 pMN from Japan, glomerular PLA2R deposits were observed in 64% of cases, and serum anti-PLA2R antibodies in 55% of cases [22]. In the meta analysis of 19 studies involving 1160 patients PLA2R staining positivity had a sensitivity of 78% and specificity of 91% for detecting PMN, while anti-PLA2R seropositivity had a sensitivity of 68% and specificity of 97% for the same set of patients [36]. These values are slightly different from those in the present study. Several factors might

influence the diagnostic accuracy of glomerular PLA2R staining and anti-PLA2R seropositivity for pMN detection, such as differences in the sensitivity of the technique used for the testing of anti-PLA2R antibodies [7,37], the method of immunostaining [38], the test time interval [39] and the dynamics of antibody response during the progression of MN. Anti-PLA2R antibodies may already be present in the non-nephrotic stage of MN [40], and conversely, they may be absent at the onset of the nephrotic stage of the disease [7, 41]. Seronegative pMN with glomerular PLA2R-positivity may turn into seropositive pMN provided the follow-up is sufficiently long [7,41,42]. Taking these factors in account, glomerular PLA2R staining usually has better sensitivity, and anti-PLA2R seropositivity usually has a higher specificity score for pMN detection.

The discordance between the results of anti-PLA2R serology and PLA2R staining may be due to the following: 1. The patient develops a spontaneous serological remission or has an undulating serum level of anti-PLA2R antibody, while the PLA2R antigen is still demonstrable in glomerular immune deposits [39]. 2. According to the hypothesis of the kidney as a sink, the anti-PLA2R antibodies may sometimes appear in the blood after the kidneys' buffering capacity has been exceeded [39,42,43]. This may be so in majority of cases in our series, since among the 14 seronegative cases with glomerular PLA2R positivity, 8 cases were in stage 1 disease, and 2 cases with stage 4 disease had fresh subepithelial deposits. 3. Anti-PLA2R serological positivity with lack of staining on biopsy rarely occurs; as indirect IF has a higher sensitivity but lower specificity in detecting anti-PLA2R antibodies. An ELISA assay could exclude false positive results, but the assay was not available in Hungary during the study period.

In 2016, investigators from the USA [34] and China [20] independently found IgG subclass staining to be a valuable tool in the differentiation of pMN from sMN: IgG4-dominance/codominance characterized pMN in both studies. Our study on Hungarian patients provides similar observations. It remains unclear, however, whether IgG4-dominance or IgG4-dominance/codominance with any other IgG subclass or IgG4-dominance/IgG1-IgG4-codominance has the highest specificity for the detection of pMN. In our cohort, IgG4-dominance performed the best with 91.7% specificity, comparable with the performance of anti-PLA2R seropositivity (90.0%) and much higher than the specificity of glomerular PLA2R positivity (66.7%). The second and the third subclass distribution pattern was IgG3/IgG4-codominance, and IgG1/IgG4-codominance, respectively; the diagnostic performance of IgG4-dominance/IgG3-IgG4-codominance indicated good sensitivity (76.2%) and high specificity (87.5%). In the cohort of 286 pMN cases from Columbus, USA, IgG1 (97%) was the most

frequent subclass, followed by IgG4 (94%), and the staining intensity assessed semiquantitatively was 1.9 for IgG1, and 2.4 for IgG4, resulting in an IgG1/IgG4-codominant or an IgG4-dominant staining pattern [34]. The IgG subclass distribution pattern in our patients significantly differed from that in the Columbus series, because we observed a striking IgG4-dominance in all stages of pMN, followed by IgG4-dominance/IgG3-IgG4 codominance distribution pattern. Consistent with our findings, the IgG4 subclass dominated all the stages of PLA2R-associated MN in patients from Beijing, China [21]. In the series from the Mayo Clinic (USA), analyzing the temporal IgG subtype changes in recurrent idiopathic MN, IgG4 was the dominant IgG subtype, regardless of the time from recurrence or PLA2R-association, and in the majority of recurrent MN the (co)dominant subtype did not change over time [44]. While we cannot provide a reasonable explanation for the discordant observations, it appears that pMN is a predominantly IgG4-related disorder right from the beginning in Hungarian patients. This conclusion may be considered overstated in the absence of repeat renal biopsies or measurements of IgG1 and IgG4 antibodies in patients' serum- Indeed, these results would be of value here, but the clinical situation did not necessitate repeating the renal biopsy procedure and/or a subclass-specific analysis of anti-PLA2R antibodies. Probably for similar reasons, however, none of the publications of Huang et al. from Columbus, USA [12], Dong et al. [20] and Cui et al. [21] from China, and Hayashi et al. [22] from Japan included the results of repeat biopsies or a subclass-specific analysis of anti-PLA2R antibodies while investigating the presence or absence of an IgG subclass switch from IgG1 to IgG4 in pMN. In summary, an IgG subtype analysis and a glomerular PLA2R staining support the differentiation of pMN from sMN, and *IgG4 subclass dominance has the highest specificity score in our cohort.*

In agreement with the IgG subclass distribution pattern of pMN, C1q-positivity was obviously not characteristic for pMN in our cohort, since IgG4 cannot bind C1q, a major precursor in the classical pathway. The lectin pathway might be involved in complement activation in pMN [45], activated for example by aberrantly glycosylated IgG4 [46], because C4d, a product of mannose binding lectin-associated serine proteases is commonly found in pMN (see Figure 1). The alternative pathway does not cleave C4.

Malignancy-associated MN is a challenging subset of MN. Pathologically, an increased number of leukocytes in glomerular capillaries [14], the dominance of IgG1 and IgG2 subclasses in deposits [13, 47], the negativity of deposits for PLA2R and subglobally distributed deposits [10] all suggest that the patient has a malignant tumor, often clinically hidden. Malignancy is usually found within a year of the diagnosis of MN [33], and mostly it is

discovered before or at the time of the diagnosis of renal disease. Solid tumors, such as carcinomas of the lungs, prostate, gastrointestinal tract, breast, kidney, urinary bladder or the skin may have an association with MN [48], but it is difficult to prove a link between the two disorders. In 1999, P. Ronco proposed three criteria for diagnosing malignancy-associated MN: the treatment-induced remission of cancer is followed by the remission of the nephrotic syndrome; the recurrence of the neoplasia is accompanied by a renal relapse; and tumor antigens and antitumor antibodies are detected within the subepithelial deposits [32]. However, the tumor antigen frequently remains undetected during the management of the patients. Although malignancy-associated MN is usually seronegative for anti-PLA2R antibodies [13,18], there are occasionally cases which display a high titer of anti-PLA2R antibodies [49,50]. Therefore, some authors think that the subtype analysis of glomerular IgG, along with the summarized biopsy features of malignancy-associated MN is not sufficient to provide a definite diagnosis of pMN or sMN [15,51]. If malignancy-associated MN is suspected, the glomerular antigen status should be completed with THSD7A and NELL1 examinations [10, 52]. The evaluation of NELL1 axis is, however, not yet widely available, and we also performed it in an international collaboration.

In our series, the clinicopathological features of T-ALL and concurrent MN negative for THSD7A and NELL1, and segmentally and mildly positive for PLA2R indicated true paraneoplastic MN, although the exact pathogenetic link between the leukemia and MN remained unexplored. In the case of a 66-year-old man with PLA2R antigen-positive, IgG4-dominant, stage 3 MN and glomerular leukocytosis, and metastatic squamous cell carcinoma of the oral cavity one year after the diagnosis of MN, the results obtained were not enough to conclude whether the patient had PLA2R-associated pMN with coincidentally discovered malignancy, or the malignancy induced the formation of anti-PLA2R autoantibodies that resulted in MN. Since the proteinuria did not respond to conventional therapy of MN, we felt that the case should be placed in the sMN group. Lastly, we had the case of a 53-year-old man with non-differentiated collagenosis in whom concurrent crescentic IgA glomerulonephritis and stage 4 IgG4-dominant MN with mildly intense positivity of PLA2R antigen were found. Because of the features of non-differentiated collagenosis, the case was included in the sMN group, although it might just represent the coincidence of two primary renal diseases. If we assigned the last two patients erroneously, and they were clear examples of pMN, then *IgG4-dominance was exclusively a feature of pMN in Hungarian patients*.

The KDIGO 2021 Clinical Practice Guideline for the Management of Glomerular Diseases [16] stated that in the case of anti-PLA2R seropositivity, normal eGFR and no immunosuppressive therapy, carrying out a kidney biopsy is not needed for making the clinical diagnosis of MN. If immunosuppressive therapy needs to be administered, a kidney biopsy should be considered. In contrast, the biopsy procedure should be conducted if there is an unusual clinical course or a rapid eGFR decrease is detected or there are serological abnormalities (for instance positive nuclear antibodies) or there is unresponsiveness to immunosuppressive therapy [16]. According to the review of Ronco [3] based on the study of Bobart el al. [53], a biopsy procedure is not recommended if eGFR >60 ml/min/1.73 m2, if anti-PLA2R serology is positive and there is no evidence of a secondary cause or diabetes. Among the serologically evaluated 74 MN patients, 6 patients had anti-PLA2R positivity and normal eGFR (>90 ml/min./m2). However, immunosuppressive treatment was initiated in all of them, so on basis of the KDIGO protocol [16] they were placed in the group where a biopsy should be considered. According to Ronco's recommendation [3], the biopsy confirmation of MN could in retrospect have been replaced by a serology-based diagnostic approach in 10 patients out of the 74 patients, and this indicates that the diagnosis of MN still necessitates the evaluation of the kidney biopsy sample in the majority of patients. The good point of our study was that we compared the statistical value of widely available immunohistological methods in the differentiation of pMN and sMN. Previously there was no direct comparison of the specificity of IgG4-dominance and different IgG4-dominance/codominance patterns and there was little knowledge about the comparison of PLA2R-based diagnostics (serology, immunohistology) and IgG1-4 subtype evaluation. The limitations were that most sMN cases were lupus MN, the number of malignancy-associated MN was low, and the antigen status was not always complete.

6.2. Discussion of the genetic study

MN is an umbrella disease that can be classified clinically into primary and secondary types. Although in both types, autoimmune response and specific HLA types were shown to pose an increased risk of developing the disease, the genetic background of MN is still not fully understood. It was found that the presence of the PLA2R1 (chromosome 2) risk SNP (rs4664308) increased the genetic risk for developing pMN, the odds ratio (OR) in a homozygous state being 4.2. However, the HLA-DQA1 risk SNP (rs2187668) in the homozygous state has an even

bigger risk, with an OR of 20.2. Combining these two risk alleles with the homozygous state, the OR is 78.5 [25].

After sequencing 30 PLA2R1 coding exons in 95 patients with MN, Coenen et al. could not find any rare genetic variants [54]. This suggests that conformational changes in PLA2R probably do not trigger an autoimmune response [3]. One reason for the lack of rare exonic variants might be that the intronic regulatory regions are involved, and post-translational modifications or the increased expression of PLA2R antigens may have a role [3]. Another possibility is that despite pMN being a rare disease, common variants in the PLA2R1 gene, combined with common variants of HLA haplotypes, might create a rare haplotype [3,54].

Primary MN can also develop in the absence of the rs4664308 PLA2R1 risk allele as other PLA2R1 variants (rs35771982, rs3749117, rs6757188, rs35771982, rs3828323, rs3749119, rs1511223, rs2203053, rs10196882, rs16844706, rs877635, rs2715928, rs16844715, rs3749119) were also found to play a role in the pathogenesis [26,29,55-57]. These findings suggest that the intronic rs4664308 risk allele is only tightly linked to the functional variants that cause the illegitimate expression of PLA2R in the kidney.

Among our pMN cases, 76.5% had positive anti-PLA2R antibody serology, and 80% had PLA2R immunostaining positivity. In our pMN cohort, 85% of patients carrying two risk PLA2R1 SNPs (AA) had circulating anti-PLA2R antibody or expressed PLA2R in glomeruli, which is similar that found in Chinese patients [26]. This is why in Hungary, as in other countries, the anti-PLA2R-based pathomechanism is also the main cause of pMN. In sMN cases, these findings were negative, which accords with previous published results [36].

Due to the hypervariability at the MHC II loci, there is a plethora of gene variations (subtypes) of the HLA-DQA1/B1 genes. While these two genes are usually inherited together on tightly linked haplotypes at the DNA level, the DQA1 proteins may freely combine with the DQB1 proteins transcribed from either the cis or trans alleles to form the MHC II dimer protein. This leads to 1 to 4 different HLA-DQ protein dimers/serotypes depending on the two HLA-DQ haplotypes. As the different DQA1 and DQB1 gene variations have slightly different amino acid sequences and protein conformations, the antigen recognition site of the dimer protein is also slightly different based on the actual HLA-DQ serotype. *Some haplotypes may also have risk and protective scores depending on the resulting serotype(s), as different combinations can lead to different 3D conformations with different autoimmune responses to PLA2R.*

Based on the assumption that the pathogenesis of pMN is driven by the autoimmune response of specific HLA-DQ serotypes to the illegitimate expression of PLA2R in the kidney,

we investigated the HLA-DQA1/B1 haplotypes together with the PLA2R1 risk allele (rs4664308) status. We sought to pinpoint the HLA-DQ haplotype combinations that are enriched in MN patients. We also tried to identify those protective HLA-DQ haplotype combinations that are only found in age-matched control patients that carry the PLA2R1 risk alleles without the manifestation of MN.

Since the number of observed haplotype combinations was comparable to the number of sMN cases, we only performed a statistical analysis for the more frequent HLA-DQ 2.5 haplotype allele counts in the three groups (pMN, sMN, controls) that were previously associated with an elevated risk of pMN. We compared patients with pMN and sMN based on HLA-DQ 2.5 haplotype and PLA2R1 risk SNP counts. *We noted a significant difference in the counts of HLA-DQ 2.5 between pMN and controls; however, there was no significant difference between pMN and sMN cases* (see Table 8)*, which means that HLA-DQ 2.5 seems to be a strong "lock" in the pathogenesis of MN independently of the "key" used (the immunogenic epitope).*

The allele counts of the PLA2R1 risk SNP (rs4664308) were significantly higher in the pMN group compared to the controls. The sMN and control groups had similar risk allele frequencies, and the Fisher exact test was not significant ($p = 0.686$). The pMN group had higher risk allele frequencies compared to the sMN groups; however, after the Bonferroni correction, the significance ($p = 0.034$) fell below the adjusted ($p = 0.016$) threshold (see Table 8). As the relative ratio of risk and non-risk alleles in sMN is comparable to the control, we suggest that this is most likely attributable to the low number of sMN cases. These results tell us that in about 70% of cases, pMN is based on an anti-PLA2R antibody-mediated pathomechanism, consistent with findings stated in the literature [26]. These results also suggest that for pMN, the PLA2R1 and the HLA susceptibility factors play a permissive role in the pathogenesis of the disease.

Our findings also confirmed that HLA-DQ 2.5 by itself or combined with some other HLA-DQ haplotypes are overrepresented in patients with pMN compared to controls (see Figure 7), which appear to agree with the published results [24,27,28]. In sMN, the HLA-DQ 2.5 haplotype also appears to carry the highest risk. Recalling that it is a risk haplotype in other autoimmune diseases, such as celiac disease [58,59], it may actually be a general risk trigger of autoimmune response to various epitopes.

MHC II plays a role in antigen presentation; thus, HLA genes play a key role in autoimmune disorders. In the celiac disease HLA-DQ 2.5 heterodimers - encoded by DQA1*0501 and DQB1*0201/0202 alleles both in cis or trans configuration - and DQ8

molecules - encoded by DQB1*03:02 usually in combination with DQA1*03 variant - are known to be genetic susceptibility factors. HLA-DQ 2.5 - being present in more than 90% of celiac patients - is more common than HLA-DQ8 [58,59].

Besides the celiac disease, other autoimmune disorders have similar susceptibility HLA alleles to MN: a significant increase was noted in HLA-DRB1*03:01 and *15:01 alleles in systemic lupus erythematosus and in multiple sclerosis [60-65]. Furthermore, the HLA-DQ 2.5 haplotype is also associated with other autoimmune diseases, such as type 1 diabetes [66,67], autoimmune hepatitis [68,69], and dermatitis herpetiformis [70]; and HLA-DQ 2.5 has been investigated in rheumatoid arthritis [71,72], and systemic lupus erythematosus [73], although the associations are less clearcut.

Due to the complex interactions and large number of potential serotype combinations that may be present in a patient, the risk assessment of the different HLA-DQ serotypes would require a large dataset. The number of cases in our study does not allow the statistical analysis of the risk or protective HLA-DQ isoforms. However, evaluating the different combinations of potential HLA-DQ haplotypes in patients with MN and controls could help to identify the main risk and protective haplotype combinations. With our biological assumption, *we created a risk scoring method to assess the potential role of the HLA-DQ haplotypes. Our scoring system reflects the enrichment or depletion of the PLA2R1 risk alleles observed in the subset of patients with pMN compared to the control persons carrying the particular HLA-DQ haplotype combination.* As described in the Methods section, a positive score indicates an increased risk of developing pMN in the presence of the PLA2R1 risk allele, while a negative score suggests a protective role of the given HLA-DQ haplotype combination. *Our procedure confirmed that the HLA-DQ 2.5 haplotype has the highest risk and suggests that the HLA-DQ 8.1 haplotype most likely carries an increased risk in developing pMN* (see Figure 7). Our approach confirmed the main HLA-DQ haplotype combination already associated with MN and suggested additional risk and potentially protective HLA-DQ haplotypes. Our statistical analysis was limited by the small sample size and the diversity of the HLA-DQ combinations. We think that the same approach could be applied to a bigger cohort to achieve a more precise identification of risk/protective haplotype combinations and proper risk assessment. While our scoring procedure was used for the assessment of pMN risk, a similar approach could be used to analyze the risk and protective HLA-DQ haplotypes in sMN with a suitably large control and sMN dataset, and where HLA-DQ genotype datasets are available.

As autoimmune diseases have a complex pathogenesis, apart from genetic factors, environmental and immunological factors are also essential: environmental triggers such as infections, toxins, stress, and diet can induce the disease onset or progression; furthermore, dysregulation of the immune system - including loss of self-tolerance and abnormal activation of immune cells - contributes to the development of autoimmunity. This multifactorial nature makes autoimmune diseases heterogeneous and often difficult to predict or treat. This is why, in vitro or in vivo studies are needed to assess the functional impact of specific HLA-DQ haplotype combinations on antigen presentation and immune response; and experimental confirmation of the biological relevance of these haplotype combinations would strengthen the conclusions drawn above.

These findings may have some potential clinical utility in the future. The identification of HLA-DQ haplotype combinations associated with increased susceptibility to MN could support risk stratification and early detection in genetically predisposed individuals - even among healthy individuals and among patients having an underlying disease predisposed for sMN - such as SLE. And, as our understanding of the immunogenetic landscape improves, therapies targeting specific immune pathways associated with these haplotypes might be mapped out. In individuals carrying genetic risk factors for autoimmune diseases, the use of certain immunomodulatory interventions (for example, vitamin D) may offer a preventive or modulatory effect.

7. Conclusion

In the retrospective cohort of MN patients from Central-Eastern Europe, anti-PLA2R serology had a lower sensitivity score for pMN than that given in most of the publications; however, the specificity of seropositivity was comparably high, hence it has a definite value in the classification of MN. On reevaluating the biopsy indication of our cases on basis of new non-invasive approaches, the biopsy procedure had to be carried out or it should have been considered in most of the cases. With a histological evaluation, PLA2R staining alone had a lower specificity score than that for serology. Nevertheless, we recommend that it be supplemented with an IgG subtype analysis, since IgG4-dominance, followed by IgG4-dominance/IgG3-IgG4-codominance offers high specificity in differentiating pMN from sMN.

However, malignancy should still be carefully looked for. The possibility of an IgG1 to IgG4 subtype switch during the progression of pMN was not demonstrated in the Hungarian cohort.

The classification of MN into primary and secondary is on the way to being replaced by the antigen detected [11], because the presence of autoantibodies is not always in accord with the clinical definitions of pMN and sMN. In clinical practice, however, the results of anti-PLA2R serology, PLA2R immunohistology, along with the determination of IgG subclass in glomerular immune deposits are widely available, and their results might narrow the range of tests required for evaluating MN patients. As IgG subclass switching during the progression of pMN was not the feature of our cohort, pMN in Hungarian patients is presumed to be an IgG4-related disorder right from the start.

In both pMN and sMN, the main risk haplotype was HLA-DQ 2.5, which seems to be a common "lock" in the pathogenesis of MN, and in the pathogenesis of other autoimmune diseases as well. HLA-DQ 8.1 seems to be protective, HLA-DQ 7.5 and 6.2 seems to be protective in the development of pMN. The presence of PLA2R1 functional SNPs that induce the illegitimate expression of PLA2R in the glomeruli is a triggering factor that is required for anti-PLA2R antibody-associated pMN. In the pathogenesis of MN, HLA-DQ haplotype combinations may only be permissive factors, since HLA-DQ susceptibility factor DQ 2.5 haplotype counts are similar in both pMN and sMN groups, and MN will not occur in the presence of protective HLA-DQ haplotype combinations. In sMN, the HLA-DQ susceptibility factors may be the same or different, depending on the various epitopes. However, as the pathogenic antigens ("keys") of sMN differ (especially in patients with sMN who had lupus nephritis), besides the illegitimate PLA2R expression or other antigens of pMN, there may also be differences in the HLA susceptibility factors.

8. Novel findings

- With a histological evaluation, PLA2R staining supplemented with an IgG subtype analysis offers high specificity in differentiating pMN from sMN.

- As IgG subclass switching during the progression of pMN was not the feature of our cohort, pMN in Hungarian patients is presumed to be an IgG4-related disorder right from the start.

- HLA-DQ 8.1 seems to be a risk haplotype, HLA-DQ 7.5 and 6.2 seems to be protective in the development of pMN.

- Not only in pMN, however also in sMN patients the main risk haplotype was HLA-DQ 2.5, which seems to be a common “lock” in the pathogenesis of MN as in the pathogenesis of other autoimmune diseases as well.

- Not only HLA-DQ haplotypes, however haplotype combinations should be investigated as genetic predisposing factors in pMN, as a haplotype can be risk or protective as well depending on the combined haplotype.

- A novel risk score based on the PLA2R1 SNP allele status was created to determine the risk and protective haplotype combinations.

Funding

This research was supported by TÁMOP 4.2.2.A-11/1/KONV-2012-0035 and the MANET-2012 research grant.

Institutional Review Board Statement

The study was conducted based on the guidelines of the Declaration of Helsinki and approved by the Regional Research Ethics Committee of the University of Szeged (71/2012, 79/2022), the Medical Research Council Scientific and Research Ethics Committee (24294-2/2014/EKU) and the Public Health Department of the Csongrád County Government Office (CSR/039/01284-6/2014).

9. Ackowledgements

I would like to express my gratitude to **Professor Béla Iványi**, my supervisor, for drawing my attention to glomerulonephritis, deepening my interest in the topic, encouraging me to focus on the immunopathological and genetic aspects of membranous nephropathy and guiding me throughout this multidisciplinary research process.

I am deeply grateful to **Professor György Ábrahám** for his continuous mentorship, guidance, and for inspiring me with his holistic approach to internal medicine and nephrology.

Special thanks go to **Zoltán Maróti**, geneticist, for his creative input and the high-quality work he contributed and **Emőke Endreffy** facilitating me to study the genetic aspects of membranous nephropathy.

I would also like to thank **Professors Csaba Lengyel, Tamás Várkonyi, György Ábrahám** (again here), **Tibor Wittmann, and János Lonovics** — the current and former heads of the Department of Internal Medicine — for giving me the opportunity to pursue clinical and research work in their institution and for their continued support. In memory of the late **Professor Sándor Sonkodi**, I would like to express my gratitude for the lasting influence he had on my professional journey.

I am thankful to all my colleagues and past and present working group members — **Péter, Légrády, László Bitó, Alexandra Belovai, Kypros Constantinou, Imola Fejes, Ákos Bogó, Tamás Nagy, Diána Dávid, Amir Reza Manafzadeh, Annamária Letoha, Zoltán Ondrik, Gerda Lencse, Tímea Boros, Domonkos Schulcz, Ágnes Kovács, Petra Majtényi, and Bernadett Nádasdi** — for their collaboration and encouragement over the years. I would also like to thank **my co-authors** and my **previous TDK students**, János Árgyelán and Zsolt Bodó, for their work in data collection.

Special thanks to our assistants, especially to **the late Istvánné Török**, for her continuous assistance, encouragement, and support. I am also grateful to the nursing staff, in particular **Angéla Sipos** for their help.

I would like to thank the nephropathologists, **Éva Kemény, Deján Dobi** and **Sándor Turkevi-Nagy**, as well as the pathology assistants, **Krisztián Daru and Erika Németh** and pathology

photographer**, Mihály Dezső** for their thorough, efficient, and high-quality work. I am also grateful to **Tibor Nyári** for his statistical help in my scientific work.

Finally, I owe the most to **my family** — **my late parents, my sister, Éva, my husband, Zsolt, my sons, Zsolt and Attila** — **and my friends** for their love and unwavering support.

With deep gratitude, I dedicate this dissertation to **the late Istvánné Bajcsi**, my mother, and **the late Istvánné Török**, our assistant, whose constant support and generous assistance have guided me throughout my studies and work.

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