University of Szeged, Albert Szent-Györgyi Medical School
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IMMUNOPATHOLOGICAL AND GENETIC OBSERVATIONS IN MEMBRANOUS NEPHROPATHY

PhD Thesis

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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, IF: 2,7**

Dóra Bajcsi, Zoltán Maróti (first co-aurthors), Emőke Endreffy, Péter Légrády, György Ábrahám, Béla Iványi: The Presence of Risk and Protective HLA-DQ Haplotype Combinations and PLA2R1 Risk SNP in Hungarian Patients with Membranous Nephropathy. Int J Mol Sci. 2025, 26(17): 8621. doi: 10.3390/ijms26178621. **D/Q rank: Q1, IF (2024): 4,9**

Introduction

Membranous nephropathy (MN) is a rare immune-complex mediated glomerular disease, it is the main cause of nephrotic syndrome in Caucasian adults. MN is classified clinically as primary MN (pMN) or secondary MN (sMN). 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). The predominant anti-PLA2R antibodies are of the IgG4 subclass, but other subclasses, such as

IgG1 and IgG3, are present in lower amounts. Other MN subgroups were described, including thrombospondin type-1 domain containing protein 7A (THSD7A) and neural epidermal growth-factor-like 1 protein (NELL1)-associated MN. The clinical classification of MN is under the way to be replaced by an antigen-based classification.

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.

The demonstration of circulating autoantibodies against PLA2R has become the standard process in the differentiation of pMN from sMN.

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. 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, two studies from China, and one from Japan have covered the topic of IgG subtype distribution within the immune deposits of MN in native kidney samples.

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.

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. 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.

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. 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

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.

Aims

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.

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.

Patients and methods of the immunopathological study

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. MN was assigned clinically to either pMN (n=63; 72.4%, age: 55.8±14.3 years or sMN (n= 24; 27.6%, age: 52.8±14.7 years).

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) 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 and PLA2R antigen. The staining intensity was graded semiquantitatively on a scale of 0 to 3 + . 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 stages were also determined.

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.

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. A p value <0.05 was treated as statistically significant.

Patients and methods of the genetic study

Patients of the genetic study: A total of 67 MN patients with MN (27 males, 40 females) were analyzed. Our clinical evaluation placed 52 patients (77.6 %, age: 50.1±14.7 years) in the pMN group, and 15 patients (22.4 %, age: 46.8±8.6 years) in the sMN group.

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.

Genetic evaluation: A blood sample was taken from the patients with MN and from 77 age-matched, normotensive, clinically healthy persons. 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.

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 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 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. In order 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:

$$\text{score}_{\text{HLA type}} = \text{freq}(\text{pMN\_AA}) + 2 \times \text{freq}(\text{pMN\_AG}) - 2 \times \text{freq}(\text{control\_AA}) - \text{freq}(\text{control\_AG})$$

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.

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).

Results of the immunopathological study

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).

S**ensitivity and specificity values of anti-PLA2R serological and PLA2R and IgG1-4 subclass immunohistological staining findings:** For pMN, the sensitivity of anti-PLA2R seropositivity was 61.1%, and the specificity was 90.0%; and similar values for PLA2R staining were 81.0%, and 66.7%, respectively. The sensitivity and specificity scores of IgG

subtypes analysis were: IgG4-dominance 52.2% and 91.7%, IgG4-dominance/IgG3-IgG4-codominance 76.2% and 87.5%, IgG4-dominance/IgG1-IgG4-codominance 64.2% and 75%, and IgG4-dominance/codominance with any IgG subclass 92.1% and 70.8%, respectively.

IgG1-4 subclass immunohistological staining findings in pMN and sMN: Regarding the distribution of IgG subclasses in pMN, 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.

Correlation among the different immunoserological and immunohistological in pMN and sMN: The analysis of correlation among anti-PLA2R seropositivity, PLA2R antigen staining, and patterns of IgG subclass distribution 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.

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. There was no significant difference between the prevalence of IgG4-dominance and IgG4-dominance/codominance among the stages ($p=0.950$, $p=0.849$).

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. Among IgG4-dominant pMN cases, C1q negativity was significantly more frequent than C1q positivity.

Results of the genetic study

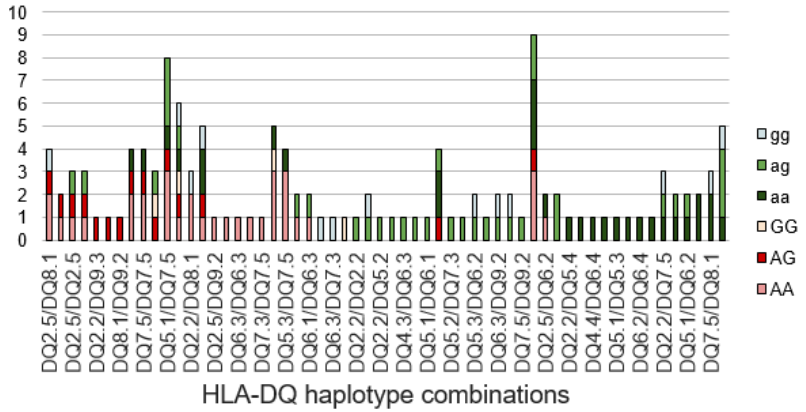
PLA2R immunostaining, serum anti-PLA2R antibody level: Among patients with pMN, PLA2R immunostaining positivity was observed in 24 patients (80%). The immunostaining was negative in 6 patients (20%). Regarding the anti-PLA2R antibody serology testing, 13 (76.5%) patients had positive and 4 patients (23.5%) had negative results. In patients with sMN, the PLA2R immunostaining and anti-PLA2R immunoserology findings were negative.

PLA2R Immunostaining, Anti-PLA2R Antibody Serology for Risk PLA2R1 rs4664308 Alleles: 24 patients were homozygotes for risk PLA2R1 rs4664308 alleles (AA). 19 patients had either PLA2R immunostaining positivity or serum anti-PLA2R antibody positivity, and clinically they were classified as having pMN. 5 patients had neither PLA2R immunostaining positivity nor serum anti-PLA2R antibody positivity; 2 of these 5 patients had clinically diagnosed sMN. 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 3 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.

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, we decided to count the risk and nonrisk alleles for both HLA-DQ 2.5 and the PLA2R1 risk SNP in the different groups. 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.

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 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 the 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).



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

Discussion

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. However, the intensification of diagnostic procedures for looking for a secondary origin may vary in different nephrology units.

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 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. These values are slightly different from those in the present study.

In 2016, investigators from the USA and China 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. 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. While we cannot provide a reasonable explanation for the discordant observations, it appears that pMN is a predominantly IgG4-related disorder right from the start in Hungarian patients. 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.

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.

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.

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, 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).

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, which appear to agree with the published results. 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, it may actually be a general risk trigger of autoimmune response to various epitopes.

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. 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.

Conclusion and novel findings

- With a histological evaluation, PLA2R staining should 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.

- 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.