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Genetics of membranous nephropathy

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Abstract

An HLA-DR3 association with membranous nephropathy was described in 1979 and additional evidence for a genetic component to membranous nephropathy was suggested in 1984 in reports of familial membranous nephropathy (1,2). In 2009, a pathogenic autoantibody was identified against the phospholipase A_2 receptor 1.

Here, we discuss the genetic studies that have proven the association of human leucocyte antigen class II and phospholipase A_2 receptor 1 variants and disease in membranous nephropathy. The common variants in phospholipase A_2 receptor 1 form a haplotype which is associated with disease incidence. The combination of the variants in both genes significantly increases the risk of disease by 78.5 fold (3). There are important genetic ethnic differences in membranous nephropathy. Disease outcome is difficult to predict and attempts to correlate the genetic association to outcome have so far not been helpful in a reproducible manner. The role of genetic variants may not only extend beyond risk of disease development, but can also help understand the underlying molecular biology of the phospholipase A_2 receptor 1 and its resultant pathogenicity. The genetic variants identified thus far have an association with disease and could therefore become useful biomarkers to stratify disease risk, as well as possibly identifying novel drug targets in the near future.

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Introduction

Membranous nephropathy (MN) is a kidney specific autoimmune disease with an incidence of ten per million per year (4). It is the leading cause of nephrotic syndrome in European adults and progresses to end-stage renal disease (ESRD) in 30-40% of cases (5). Unlike many other autoimmune disorders, males are more often affected. Approximately 25% of patients have a secondary form of MN, which is diagnosed when an alternative identifiable underlying clinical condition is present. For example systemic lupus erythematosus, malignancy, medication or viral infections (5). The remaining 75% of patients have no apparent cause and are termed 'primary' or idiopathic membranous nephropathy (IMN) (6). IMN is caused by *in situ* binding of circulating antibodies to a podocytic antigen. The phospholipase A_2 receptor 1 and thrombospondin type-1 domain-containing 7a are the major target antigens involved in the pathogenesis of IMN (7,8). Sub-epithelial immunoglobulin rich deposits demonstrated by electron microscopy are pathognomonic in MN (9), constituting a definitive phenotype. While IMN does not show simple Mendelian inheritance, the role of underlying genetic factors has been confirmed in recent studies.

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Discovery of autoantigens

The first autoantigen described in a rare case of antenatal MN was neutral endopeptidase (NEP), in 2002 (10). The gene encoding NEP is *metallomembrane endopeptidase*. Truncated mutations were discovered in maternal DNA so the mother did not express NEP protein. When foetal NEP (paternal protein) was encountered during pregnancy, anti-NEP antibodies developed (with no consequence to the mother) which crossed the placenta to cause neonatal MN (10,11).

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The discovery of circulating antibodies to the autoantigen phospholipase A_2 receptor 1 (PLA_2R1) revolutionised our understanding of IMN as an autoimmune disease (7). With Western blots and mass spectrometry the antibody was detected in serum from 26 out of 37 patients (70%) (7). This has been confirmed in subsequent studies and proven to be specific to IMN and implicated in disease progression and outcome (12,13).

Most recently, combined immunologic and proteomic approaches identified thrombospondin type-1 domain-containing 7A (THSD7A) as another target autoantigen in MN (8). THSD7A antibodies are found in approximately 2-3% of MN patients. THSD7A like PLA_2R1 is a heavily glycosylated, multi-domain transmembrane receptor located on the podocyte membrane. THSD7A resembles some of the PLA_2R1 immunological characteristics and autoantibody findings correlate with glomerular staining of the antigen. It is not understood why autoantibodies develop however, in some THSD7A associated cases the development of antibodies may be linked to malignant tumours (14,15). Interestingly, dual positivity to both PLA_2R1 and THSD7A is extremely rare with only 2 cases identified on biopsy staining (16).

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Familial clustering of Membranous Nephropathy

Whilst all available data points towards a strong genetic component, IMN appears not to be inherited in a simple Mendelian fashion. In 1984 the first case of identical twins developing IMN was published (17), and to date sixteen families have been reported to have familial IMN (3,18,17,19–24), suggesting strong genetic contribution. However, several sets of monozygotic twins with IMN had different phenotypes with a different age of onset and progression of disease (17,22). This suggests an environmental contribution to disease, which is not yet well established.

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There is a strong male preponderance in IMN (25) unlike other autoimmune diseases (26). An X-linked recessive pattern of inheritance was suggested based on the clustering of disease between non-identical brothers (17–19,21). Autosomal inheritance was also apparent in other families with male-to-male transmission (20,24) and affected members of both genders (18,23). Further support for the theory of an underlying genetic mechanism was provided by two brothers with a rare syndromic form of IMN (19). These brothers had both IMN and deafness but no linked HLA alleles (19). To date the involvement of antibodies against phospholipase A_2 receptor 1 ($aPLA_2R1ab$) in cases of familial MN are unknown.

Two studies of paediatric primary MN report much lower positivity for PLA_2R1 staining of immune complexes on biopsy at 6% and 45% compared to adult studies at 70-80% (27,28). As yet the genetic background to paediatric MN has not been confirmed.

Concept of genome-wide association studies and confirmation of genetic association

The biggest breakthrough in the contribution of genetic factors in IMN so far was with three genome-wide association studies (GWAS) published in 2011 (3). GWAS works with the hypothesis that the phenotype is associated with variations in a subset of several genes. These variations will be demarked by haplotypes / alleles that display frequency differences in the cases and controls. GWAS examine all chromosomes and its simplest form compares allele frequencies of given variations in cases to allele frequencies of controls (basic allele test). GWAS most often use common single nucleotide polymorphisms (SNP), which are defined by an allele frequency in a given population of > 5%. The tenet is that any given disease, as long as there is no

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heterogeneity, will show a difference in the frequency of genetic variation within disease-associated genomic regions in comparison to unaffected controls. Thus, SNPs utilized as genetic markers, identify a chromosomal location of interest associated with disease. If the phenotype is clearly described and unique then GWAS can be powerful for discovery of associated alleles even with few cases (29,30). The first ever GWAS published in macular degeneration utilised 96 cases and 50 controls only (29). Of all SNPs genotyped 105,980 were analysed and an intronic and common variant in the *complement factor H* gene that increased the likelihood of macular degeneration by a factor of 7.4 was discovered (29). This is contrary to the opinion (misconception) often presented in public that GWAS always need thousands or tens of thousands of samples to be able to identify genetic causes. When a phenotype is complex (i.e. hypertension, kidney failure), then indeed many more samples are needed to be able to identify regions of interest, i.e. associated alleles.

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Genome-wide association studies in membranous nephropathy

The GWAS published in 2011 investigated European populations with renal biopsy proven IMN (3). Three independent GWAS were performed, using 75 French European cases, 146 Dutch European cases and 335 British European cases. Despite the small number of cases even in the smallest cohort (French), a significant association in 3 SNPs in an *HLA-DQA1* allele on chromosome 6 was found. The 146 Dutch cases demonstrated a significant allelic association of 191 SNPs in *HLA-DQA1*. Additionally, 6 SNPs located within the *PLA2R1* gene on chromosome 2 were associated with IMN, the strongest being SNP rs4664308. Finally, the British study found a significant association with 144 SNPs in the *HLA-DQA1* allele and 2 SNPs in the *PLA2R1* allele. Combining then the three cohorts in a meta-analysis with a total case population of 556 further strengthened the association of IMN with

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20 SNPs in *HLA-DQA1* and 13 SNPs in *PLA2R1*. The effect size of the risk SNPs was examined, even in a heterozygous state of the risk allele the odds ratio was increased in both *HLA-DQA1* and *PLA2R1*. The strongest association was with the *HLA-DQA1* region, (the most significantly associated SNP being rs2187668) (3). In a homozygous state of the *HLA-DQA1* risk allele the odds ratio of IMN was 20.2 (3). The odds ratio in a homozygous state for *PLA2R1* was 4.2 (3). Combining these two risk alleles further increased the risk of IMN to an odds ratio of 78.5 (3). This association was very robust for such a modest cohort (31), which is unusual for a GWAS (18). Also, no association was found with immunoglobulin G chains that were previously identified with a candidate gene approach on chromosome 14 (32,33).

Imputation

The SNP coverage of these initial GWAS is low compared to the coverage available with more modern technology, particularly of the HLA alleles (34,35). To further assess the strength of the SNP associations that were found in the British study an imputation study was performed (36). Imputation is a method to increase the statistical power of association studies and potentially identify additional associated alleles (37,38). This technique is based on knowledge about short stretches of shared haplotypes to provide information and predict untyped alleles (39). Imputation takes advantage of haplotype composition to match known SNPs to other SNPs that are in linkage disequilibrium with one another. In this way, it was possible to impute and examine 8.9 million SNPs in the British cohort. The strongest signals remained in *HLA-DQA1* and *PLA2R1*, and no additional loci were found as independent risk factors. The *PLA2R1* signal was somewhat weaker and *HLA-DQA1* somewhat stronger than originally described, with homozygous risk alleles at both loci the

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combined odds ratio was greater at 79.4 (36). In addition, imputation of classical HLA alleles was performed, with the DRB1*0301-DQA1*0501-DQB1*0201 haplotype showing the strongest association but providing little information beyond the lead SNP in HLA-DQA1. Sub-group analyses were undertaken and there was no significant gender specific genetic difference and no additional loci were found on the X chromosome (36), which may have been unexpected given the unusual strong male preponderance in IMN, but statistical power for these analyses was limited. The HLA region was analysed in much more detail and this demonstrated a several hundred kilobase pair linkage disequilibrium around *HLA-DQA1* as well as other HLA class II genes (36).

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M-type phospholipase A_2 receptor 1

To investigate whether specific variants within the *PLA2R1* gene are causing this previously mentioned strong genetic association, sequencing of the 30 *PLA2R1* coding exons was performed. This was also an ethnically homogenous group, all 95 affected patients were white Europeans and only 45% had circulating aPLA_2R1ab (40). All exons and splice sites of *PLA2R1* were sequenced by Sanger sequencing and all observed variants including rare variants (minor allele frequency <1%) were analysed. To our initial surprise, no rare genetic variants causing a conformational change in PLA_2R1 structure were found. Of the variants found 6 were common and 3 in splice sites (exon-intron boundaries). One of these non-synonymous (causing amino acid alteration) common variants (i.e. M292V) encodes an amino acid located within CTLD1 but this is far removed from the immunodominant epitope in the N-terminal cys-rich domain and unlikely to have a contributory role in the pathogenesis of IMN (40,41). One reason for the lack of exonic, i.e. coding, differences may be that the true causal variant(s) lie(s) in the regulatory, i.e.

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intergenic or intronic regions of the gene. For this to be examined, sequencing of the whole genomic region would need to be done. A second reason for the lack of significant results was that only 45% of the cohort had detectable $aPLA_2R1ab$. The remaining patients were $aPLA_2R1ab$ negative, and we now know that the association is strongest in $aPLA_2R1ab$ positive patients.

It is therefore most interesting to note that despite IMN being a rare disease the variants found in *PLA2R1* were common. An explanation for this would be that the common variants recognised together create a rare haplotype (40). Additionally, an interaction between the *PLA2R1* variants and the *HLA-DQA1* haplotype in individuals predisposed to developing IMN might be infrequent in causing autoimmunity and may therefore account for the rarity of disease and suggest a mechanism for how IMN develops (42). Genotyping of hundreds of thousands of individuals will provide an answer to whether there is a unique genetic fingerprint of individuals developing IMN and what proportion of individuals having this genetic fingerprint actually present with IMN (i.e. show penetrance).

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Antibody and gene interplay

The presence of circulating antibodies against PLA_2R1 and THSD7A is variable between patients and throughout the different stages of disease (43). During active nephrosis and disease these levels tend to be high and remission is predated by reducing antibody titres (43). Serologically antibody negative MN patients may have glomerular PLA_2R1 positivity (12). The underlying pathological mechanism in tissue or serological PLA_2R1 positivity is the same and they represent a spectrum of the same disease. A hypothesis is these patients have the same genetic *PLA2R1* risk variants yet are demonstrating incomplete penetrance of disease manifestation. Studies were undertaken to elucidate the association of genetic variants and circulating

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antibodies as the antibody titres have been associated with severity of disease and long term outcome (13).

PLA2R1 risk alleles are positively correlated with positivity of the pathogenic $aPLA_2R1ab$ (44). When patients were divided into low- or high-risk *PLA2R1* genotypes, only 4% of those with the low-risk genotype had detectable $aPLA_2R1ab$ compared to 76% of those with the high-risk genotype (44). This association was further strengthened for the detection of $aPLA_2R1ab$ after combination with the low- or high-risk *HLA-DQA1* genotypes with 0% versus 73% respectively (44). A larger study compared glomerular PLA_2R1 antibody staining (positivity) to negative patients and found the *PLA2R1* association only in patients with PLA_2R1 positivity. In PLA_2R1 negative patients compared to controls there was no association with *PLA2R1* SNPs (45).

This is relevant as increased $aPLA_2R1ab$ correlates with clinical progression of disease; with higher titres associated with ESRD at five years and lower rates of spontaneous remission (13). In an Indian cohort, however, there was no significant association between $aPLA_2R1ab$ status and *PLA2R1* SNPs. Instead there was an association of the *HLA-DQA1* risk allele with $aPLA_2R1ab$ positivity (46). This was subsequently replicated in a European cohort and the presence of the risk alleles in either a heterozygous or homozygous state in *HLA-DQA1* and *-DQB1* was significantly associated with higher circulating $aPLA_2R1ab$ (13). Neither the SNPs in intron 1 or exon 5 in *HLA-DQA1* alone had an effect on $aPLA_2R1ab$ titres (13). Two recent Chinese studies demonstrated the strong HLA association with $aPLA_2R1ab$ positivity (47,48). One had an association with *HLA-DRB1* and the other *HLA-DRB3* both of which share a haplotype so may represent a common mechanism in Chinese patients (47–49). The risk alleles in *PLA2R1* are said to be present in patients with systemic lupus erythematosus (SMN) albeit with

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lower odds ratios (50) and aPLA$_2$R1ab are occasionally found in patients with SMN (51).

Ethnic differences

Our findings from the first IMN GWAS (3) have been replicated in other studies, however different techniques have been used. These studies use a candidate gene approach whereby a specific variant alone is genotyped (52). These SNPs are chosen as the candidate gene based on prior knowledge about PLA$_2$R1 or previously described SNPs (52,53). This is a major limitation of the candidate gene approach; they can only confirm or refute an association with a variant and cannot detect new associations (52,53). Another limitation is findings are often not replicated in subsequent independent studies rendering the results potentially unreliable (52,53). Table 1 provides a summary of genotyping studies to date in MN.

In MN, the first study utilising the candidate gene approach was a small Spanish cohort of 89 patients, where only a single SNP in both the *HLA-DQA1* and *PLA2R1* genes was investigated (54). This study too found the same association in both alleles in their cohort, with an added effect of homozygous risk alleles in both genes increasing the odds ratio of IMN to 7.3 (54). As these studies were performed in European populations it was of interest to investigate if these associations held true in other ethnicities.

In a cohort of 114 Indian patients the same risk alleles were identified as by Stanescu *et al.* (46). The strongest association was with the homozygous genotype in the *HLA-DQA1* SNP rs2187668. Three SNPs were associated within *PLA2R1*, one of which was the same SNP described in the GWAS (3), rs4664308 with the AA risk genotype (46). The risk of IMN was increased by

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58.4 with all four risk alleles in *HLA-DQA1* and *PLA2R1* (46). This is a strong association with a small sample size.

The only study undertaken in African-Americans so far examined 243 African-American and 467 European cases of IMN (45). Targeted sequencing of candidate genes using conventional polymerase chain reaction was performed, with genotyping of 6 *PLA2R1* SNPs and a single SNP in the *HLA-DQA1* region (45). Further, they differentiated between patients who had PLA_2R1 positivity on renal biopsy (using immunofluorescence) (115 African-American cases) and those who did not (128 African-American cases) (45). No association was found in African-Americans with the *HLA-DQA1* SNP rs2187668, suggesting that this SNP is tagging the causal variant(s) in individuals of European and East Asian ancestry but not in African Americans. In the European sub-group analysis however, the strong association was present with *HLA-DQA1* (45). Further the *PLA2R1* signal was associated with glomerular PLA_2R1 positivity in the African-American cohort but not in PLA_2R1 negative patients (45). The strength of this association was lower than that found in Europeans, with the strongest association in Europeans with detectable PLA_2R1 (45).

Chinese patients demonstrated a similar association with *PLA2R1* risk alleles increasing the risk of IMN but without any effect on outcomes and response to treatment (55). Liu *et al.* analysed 2 SNPs in 129 Chinese IMN patients (55). The risk allele increased the rates of IMN (55). There was no difference in the different genotypes relating to progression to ESRD, though the patient numbers were too small to identify such a difference. A heterozygous state for the risk allele in the exonic *PLA2R1* region conferred a lower success rate of achieving remission (55). A larger study including 1112 Chinese patients with IMN genotyped 3 SNPs in *PLA2R1* and 3 SNPs in *HLA* genes and found that both were associated with IMN (44). Interestingly, in the Chinese population

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the association with *HLA-DQA1* was lower than in Europeans, and there was no association with HLA Class II alleles apart from *HLA-DOB* or *-DQB2* (44). A study of 261 IMN Chinese patients has linked *HLA-DRB1*1501* most significantly with IMN (47). After correction for *HLA-DRB1*0301*, the *HLA-DQA1* association was diminished as these two loci are in strong linkage disequilibrium with one another (47). The additive effect of homozygous risk alleles in *HLA-DQA1* and *PLA2R1* increased the odds ratio of IMN to 11.13 which is considerably lower than that found in the European studies (3,44). However, with the newly discovered association of *HLA-DRB1* and *PLA2R1* the odds ratio is considerably higher at 32.4 in the Chinese population (47). Another Chinese study in patients phenotyped by PLA_2R1 positivity demonstrated a stronger association with *HLA-DRB3*0202* and *HLA-DRB1*1501* with odds ratios of 24.9 and 17.7 respectively *(48)*. Both studies have identified the same allele in *HLA-DRB1*1501* which may truly represent the causative allele in Chinese patients. Difficulties arise with analysis of such data as the allele frequencies vary between ethnic groups (56). The Chinese are genetically heterogeneous and within a control population there were different minor allele frequencies in *HLA-DQA1* and *PLA2R1* dependent on their geographical location (56).

A study of 4 SNPs in *PLA2R1* in 199 Korean patients also confirmed an association of disease with rs35771982 and rs3828323 (different to the Stanescu *et al.* SNPs (3,57). Patients with SMN had the same genotype as controls (57).

Finally, a Japanese study performed genotyping of 15 SNPs in the *PLA2R1* gene and 6 HLA genes - *A, B, C, DRB1, DQB1 and DPB1* (58). The discovery sample had 53 patients, and the replication study 130 (58). After corrections for multiple testing and correlation in the replication study 4 SNPs in *PLA2R1* were associated with IMN, 2 of which were intronic (58). None of the class I

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HLA genes (*A, B or C)* were significantly associated with IMN, however *HLA-DRB1*15:01* was the most strongly associated with an odds ratio of 2.85 followed by *HLA-DQB1*, odds ratio 2.6. These odd ratios increased in the replication study and then subsequently in the combined analysis to 3.09 and 3.1 respectively (58). Interactions between the *HLA* and *PLA2R1* homozygous risk alleles further increased the risk of developing IMN, with the largest odds ratio of 17.53 in the *HLA-DRB1*15:01 – DQB1*06:02* and rs2715928 *PLA2R1* combination. Whilst these interactions are statistically significant they are still considerably lower than the strength of interactions found in the European GWAS (3). The differences may be due to sample size differences or because *HLA-DQA1*, which is a larger contributor to the cumulative risk in the European study, was not genotyped in this Japanese study, or because of differences in linkage disequilibrium with the causal variant across different ethnic groups.

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Functional effect of genes

The underlying genetic risk alleles that have been identified to date are different between individual studies but universally there is an association of IMN with the human genes encoding leucocyte class II antigens and *PLA_2R1*. Functional studies to ascertain how these genetic variants increase the risk for disease development are required. It is also possible that the previously identified risk alleles do not affect disease onset but instead disease severity (42).

It is unclear how the genetic risk alleles of class II *HLA* (e.g. *DQA1*) and *PLA2R* are translated through the pathophysiological disease mechanism, but antigen presentation to T cells to initiate T cell help for aPLA_2R1ab production is one possibility. These risk alleles encode protein receptors which interact

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during antigen presentation to stimulate T cells. In this situation, PLA_2R1 protein, processed in macrophage/dendritic cells is displayed on the cell surface as PLA_2R1 peptides bound to the class II receptor (DQA1) groove. The genetics of DQA1 will shape the amino acid structure of its receptor groove thus defining and restricting the possible 15mer peptide sequences available from PLA_2R1 that will fit the groove. The genetics of PLA_2R1 may control the possible enzyme fragmentation pattern of PLA_2R1 by:

a) change in amino acid either creating or destroying an enzyme cut site
b) change in splice sites controlling the protein species available for fragmentation
c) level of transcript leading to higher levels of peptide

As yet these T cell peptides (the PLA_2R1 peptides presented on DQA1) have not been described experimentally but studies are in progress. A recent study has predicted possible T cell epitopes in PLA_2R1 and attempted to model the interaction with known class II risk alleles (47). It is important to emphasise that *DQA1* may not be the causal allele, particularly in non-European ethnicities (47,48).

To elucidate the HLA causal alleles further larger multi-ethnic GWAS combined with larger-scale HLA sequencing and fine-mapping studies are necessary. It is vital to do this before modelling their functional effects however, it would be useful to have transcriptomic and proteomic studies to ascertain if *PLA2R* expression is modified and if this is due to an increase or decrease in transcriptional or post-transcriptional events.

Remission status

A comparison of 23 spontaneously remitting to 55 non-remitting IMN patients found no difference in genetic variants in *HLA-DQA1* or *PLA2R1* (54). In

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contrast, Liu et *al.* reported an association between lower rates of remission after treatment and the *PLA2R1* SNPs rs6757188 (CT genotype) and rs35771982 (CG genotype) (55).

Response to treatment

Patients with the risk genotypes in *HLA-DQA1* and *PLA2R1* respond to immunosuppression, though the odds ratio is low at only 0.12 (54). The total number of patients assessed was small with 27 responders and 28 non-responders (54). After adjustment for baseline proteinuria the predictive value of risk genotype increased (54). Analysis of 2 different *PLA2R1* SNPs revealed no difference between the outcomes of patients treated either conservatively or with immunosuppression (55).

Decline in renal function

The high risk alleles (AA genotype) in *HLA-DQA1,* despite being strongly associated with IMN, are potentially protective against declining renal function (54). High risk genotype patients had a longer time to doubling of their serum creatinine of 16.3 years compared to 13 years, though this was a small subgroup of only 83 patients (54). No association was found in the 8 Japanese patients that had a 50% increase in their serum creatinine with *HLA-DRB1* and *-DQB1* over an 11 year period, nor with patient survival (58). The association with *PLA2R1* risk alleles and declining renal function has been investigated in different ethnicities and no association was found (54,57). In addition there was no association with ESRD or death (55). As yet there has been no conclusive evidence associating genetic variants to remission status, response to treatment or a decline in renal function. These factors are difficult to determine as studies are often done in retrospective

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cohorts where confounders such as immunosuppression or disease severity have a significant effect on the outcomes. Decline in renal function is multifactorial and is related to blood pressure control, severity of proteinuria, renal function at disease onset, age and gender amongst others. These factors themselves are likely to be independent risk factors which is why studies to date have not been significant. It may be argued that these factors are caused or influenced by genetics thereby further complicating the potential genetic risk profile with IMN.

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Response to treatment

There has been an exponential increase in our understanding of IMN since 2009, when PLA_2R1 was identified as the most significant pathogenic autoantigen in IMN. IMN therefore may occur when three independent risk factors combine: unique polymorphisms in *PLA2R1*, the *HLA-DQA1* region and environmental factors. There are ethnic specific differences in these alleles and the potential that risk alleles may contribute in predicting disease outcomes. The complex pathomechanisms of disease development highlight some of the potential problems in analysing and predicting the risk for disease progression. The genetic variants may alter the expression or function of the target antigens and enable autoantibody formation. While no rare variants (i.e. mutations) were found in the coding region of *PLA2R1* the role of intronic variants needs to be investigated given their large regulatory role. As shown before, non-coding SNPs (i.e. intergenic or intronic genetic variations) are associated with ESRD (59) and other autoimmune conditions (60).

Whole genome sequencing is becoming more affordable and faster and may help illuminate the true role of intergenic and intronic genetic variants in IMN. The genomic studies could be augmented with epigenomic, transcriptomic and proteomic studies to ascertain the functional effect of gene variants. The

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regulatory regions that control autoantibody production such as transcription factors or micro RNA could be altered by the identified risk SNPs in a mechanism analogous to psoriasis (31). If upstream and downstream regulatory region variants were found these would be potential therapeutic drug targets, possibly preventing the deleterious effects of current immunotherapy. Given the large odds ratio with joint homozygosity, genotyping could be utilised to stratify disease risk and outcomes. The utility of genetic profiling in IMN could prove to be vital for non-invasive screening or risk stratification (18,31). The tools (aPLA$_2$R1ab) available to us are of assistance but by understanding the genetics we may be able to explain why the autoantibodies develop in the first instance (18). Current studies have been limited by small sample size and so there may be a lack of appreciation of potential other associations. Expanding the horizons further, there may even be a role for ascertaining epidemiologic risk for IMN with risk alleles and seeing if people in the general population have a genetic predisposition to disease (18). There may be an indirect interaction between genetics and disease, such as molecular mimicry whereby a microbe or environmental antigen resembles a *PLA2R1* variant and causes autoimmunity in patients carrying the *HLA-DQA1* risk alleles (42). The reported homology of part of the major epitope sequence in PLA$_2$R1 with a clostridial carbopeptidase enzyme illustrates how antibodies raised during infection may potentially cross react with an autoantigen (41). Normal control populations without IMN but with the risk alleles will be the most useful in identifying the triggers or environmental factors that contribute to eventual disease acquisition which may further our understanding of this complex genetically predisposed disease.

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Conflict of interest statement

None of the authors has a conflict of interest; the results presented in this paper have not been published previously in whole or part.

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			IMN						Controls	
Study authors	SNP	Ethnicity	(n)	Serum ab positivity (n)	Glomerular PLA2R1 positivity (n)	Allele frequency	Odds ratio	p-value	(n)	Allele frequency
Liu *et al.* (2010)		Taiwanese Chinese	129	unknown	unknown				106	
	PLA2R1 - rs6757188					67.80%	1.18	0.4		64.20%
	PLA2R1 - rs35771982					84.10%	1.9	0.005		73.60%
Kim *et al.* (2010)		Korean	199	unknown	unknown				356	
	PLA2R1 - rs35771982					73.60%	2.6	<0.001		68.90%
	PLA2R1 - rs3828323					73.90%	1.35	0.09		71%
Stanescu *et al.* (2011)										
French study		French European	75	unknown	unknown				157	
	HLA-DQA1 rs2187668					31.30%	4.48	1.80E-09		9.20%
	PLA2R1 rs4664308					23.30%	1.87	5.10E-03		36.30%
Dutch study		Dutch European	146	unknown	unknown				1832	
	HLA-DQA1 rs2187668					37%	3.76	5.60E-27		13.50%
	PLA2R1 rs4664308					26%	2.27	1.00E-09		44.40%
British study		British European	335	unknown	unknown				349	
	HLA-DQA1 rs2187668					41.90%	5.33	5.20E-36		11.90%
	PLA2R1 rs4664308					25.30%	2.1	2.10E-10		41.60%
Joint study		European	556	unknown	unknown				2338	
	HLA-DQA1 rs2187668					39.20%	4.32	8.00E-93		13%
	PLA2R1 rs4664308					25.20%	2.28	8.60E-29		43.40%
Lv *et al.* (2013)		Chinese Han	1112	36 of 71 patients (subgroup)	unknown				1020	
	PLA2R1 - rs35771982					15.50%	2.36	1.90E-30		30.10%
	PLA2R1 - rs3749117					15.60%	2.32	2.23E-29		30%
	PLA2R1 - rs4664308					84.50%	2.35	4.17E-30		70%
	HLA-DQA1 - rs2187668					12.10%	2.42	1.11E-14		5.40%
Saeed *et al.* (2014)										
	HLA-DQA1 - rs2187668	Caucasian		only ab positive analysed -->	280	44%	3.03	1.30E-33	337	20%
		African		only ab positive analysed -->	115	20%	2.17	9.84E-07	218	10%
		All		only ab positive analysed -->	530	35%	2.27	1.39E-10	556	16%
	PLA2R1 - rs35771982	Caucasian	813	only ab positive analysed -->	280	26%	1.98	1.44E-14	337	49%
		African	466	only ab positive analysed -->	115	7%	1.74	0.03	218	17%
		All	1512	only ab positive analysed -->	530	21%	1.53	1.39E-10	556	36%
Bullich *et al.* (2014)		Spanish European	89	unknown	unknown				286	
	HLA-DQA1 - rs2187668					29%	3.7	<0.001		14%
	PLA2R1 - rs4664308					26%	2	0.05		36%
Ramachandran *et al.* (2015)		South Asian - Indian	114	76	86					

For Peer Review

			94	either	either				95	
	HLA-DQA1 - rs2187668		114			39.50%	4.73	<0.0001	95	12.20%
				only ab positive analysed -->	94		5.36	<0.0001	95	
	PLA2R1 - rs3749119			only ab positive analysed -->	94	85.20%	unknown	9.40E-05	95	69%
	PLA2R1 - rs35771982			only ab positive analysed -->	94		3.17	<0.0001	95	
	PLA2R1 - rs4664308			only ab positive analysed -->	94		3.1	0.0003	95	
Thiri *et al.* (2016)		Japanese								
Discovery analysis			53	unknown	unknown				419	
	PLA2R1 - rs1511223					83%	2.24	3.08E-03		68.60%
	PLA2R1 - rs35771982					82.10%	3.58	2.99E-07		56.10%
	PLA2R1 - rs2203053					52.90%	1.58	6.17E-03		41.50%
	PLA2R1 - rs10196882					28.80%	2.25	4.41E-03		15.30%
	PLA2R1 - rs16844706					43.70%	1.55	8.27E-03		33.40%
	PLA2R1 - rs877635					49.10%	3.07	1.10E-06		23.90%
	PLA2R1 - rs2715928					67.30%	2.12	5.84E-04		49.40%
	PLA2R1 - rs16844715					72.10%	3.12	6.21E-07		45.30%
	PLA2R1 - rs3749119					15.70%	4.02	7.02E-08		57.20%
	HLA-A*3303					3.80%	0.39	3.00E-02		9.10%
	HLA-B*0702					0.90%	0.13	6.33E-03		6.80%
	HLA-B*3501					14.20%	1.9	3.00E-02		8.00%
	HLA-B*4403					2.80%	0.33	2.00E-02		8.10%
	HLA-Cw*0102					8.50%	0.47	1.00E-02		16.60%
	HLA-Cw*0704					4.70%	5.89	5.79E-03		0.80%
	HLA-Cw*1403					2.80%	0.33	2.00E-02		8.20%
	HLA-DRB1*0101					1.90%		0.02		6.80%
	HLA-DRB1*0405					6.60%		0.01		14.60%
	HLA-DRB1*1302					2.80%		0.03		7.80%
	HLA-DRB1*1501					19.80%		7.72E-05		8.00%
	HLA-DRB1*1602					2.80%		0.01		0.20%
	HLA-DQB1*0401					6.60%		0.01		14.60%
	HLA-DQB1*0501					2.80%		0.03		7.50%
	HLA-DQB1*0602					17.90%		5.12E-04		7.80%
	HLA-DQB1*0604					2.80%		0.03		7.50%
	HLA-DQB1*0401					1.90%		0.04		6.10%
Replication analysis		Japanese	130	unknown	unknown				386	
	PLA2R1 - rs1511223					79.30%	1.57	1.57E-01		70.90%
	PLA2R1 - rs35771982					78.70%	2.57	1.88E-08		59.10%
	PLA2R1 - rs10196882					20.80%	1.41	ns		15.70%
	PLA2R1 - rs877635					27.20%	1.03	ns		26.50%
	PLA2R1 - rs2715928					71.70%	2.36	4.56E-07		51.70%
	PLA2R1 - rs16844715					66.10%	2.23	5.16E-07		46.70%
	PLA2R1 - rs3749119					79.20%	2.61	1.63E-08		59.30%
	HLA-DRB1*1501					20.20%	3.36	4.97E-08		7%
	HLA-DQB1*0602					19.80%	3.56	7.20E-09		6.50%

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Le *et al.* (2017)										
Discovery analysis		Chinese - Nanjing region	99	Dual positivity all	(single positivity excluded)				100	
	HLA-DRB1*1501					81.80%	16.93	2.75E-15		21%
	HLA-DRB3*0202					60.60%	3.96	5.73E-06		28%
Replication analysis		Chinese - Nanjing region	293	Dual positivity all	(single positivity excluded)				285	
	HLA-DRB1*1501						8.32	3.44E-28		
	HLA-DRB3*0202						7.72	2.28E-27		
Combined analysis		Chinese - Nanjing region	392	Dual positivity all	(single positivity excluded)				385	
	HLA-DRB1*1501					72.20%	24.9	2.30E-35		21%
	HLA-DRB3*0202					69.90%	17.7	8.00E-29		26.50%
Cui *et al.* (2017)										
	HLA-DRB1*1501	Chinese Han	261	66.3% positive	Not checked	37.55%	4.65	<0.001	599	14.69%
	HLA-DRB1*0301					12.07%	3.96	<0.001		3.84%

Table 1: Summary of genotyping studies of the *HLA* region and *PLA2R1* in IMN arranged by date of publication.

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Genetics of membranous nephropathy

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Abstract

An HLA-DR3 association with membranous nephropathy was described in 1979 and additional evidence for a genetic component to membranous nephropathy was suggested in 1984 in reports of familial membranous nephropathy (1,2). In 2009, a pathogenic autoantibody was identified against the phospholipase A_2 receptor 1.

Here, we discuss the genetic studies that have proven the association of human leucocyte antigen class II and phospholipase A_2 receptor 1 variants and disease in membranous nephropathy. The common variants in phospholipase A_2 receptor 1 form a haplotype which is associated with disease incidence. The combination of the variants in both genes significantly increases the risk of disease by 78.5 fold (3). There are important genetic ethnic differences in membranous nephropathy. Disease outcome is difficult to predict and attempts to correlate the genetic association to outcome have so far not been helpful in a reproducible manner. The role of genetic variants may not only extend beyond risk of disease development, but can also help understand the underlying molecular biology of the phospholipase A_2 receptor 1 and its resultant pathogenicity. The genetic variants identified thus far have an association with disease and could therefore become useful biomarkers to stratify disease risk, as well as possibly identifying novel drug targets in the near future.

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Introduction

Membranous nephropathy (MN) is a kidney specific autoimmune disease with an incidence of ten per million per year (4). It is the leading cause of nephrotic syndrome in European adults and progresses to end-stage renal disease (ESRD) in 30-40% of cases (5). Unlike many other autoimmune disorders, males are more often affected. Approximately 25% of patients have a secondary form of MN, which is diagnosed when an alternative identifiable underlying clinical condition is present. For example systemic lupus erythematosus, malignancy, medication or viral infections (5). The remaining 75% of patients have no apparent cause and are termed 'primary' or idiopathic membranous nephropathy (IMN) (6). IMN is caused by *in situ* binding of circulating antibodies to a podocytic antigen. The phospholipase A_2 receptor 1 and thrombospondin type-1 domain-containing 7a are the major target antigens involved in the pathogenesis of IMN (7,8). Sub-epithelial immunoglobulin rich deposits demonstrated by electron microscopy are pathognomonic in MN (9)(7), constituting a definitive phenotype. While IMN does not show simple Mendelian inheritance, the role of underlying genetic factors has been confirmed in recent studies.

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Discovery of autoantigens

The first autoantigen described in a rare case of antenatal MN was neutral endopeptidase (NEP), in 2002 (10)(46). The gene encoding NEP is *metallomembrane endopeptidase*. Truncated mutations were discovered in maternal DNA so the mother did not express NEP protein. When foetal NEP (paternal protein) was encountered during pregnancy, anti-NEP antibodies developed (with no consequence to the mother) which crossed the placenta to cause neonatal MN (10,11)(46,47).

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The discovery of circulating antibodies to the autoantigen phospholipase A_2 receptor 1 (PLA_2R1) revolutionised our understanding of IMN as an autoimmune disease (7)(48). With Western blots and mass spectrometry the antibody was detected in serum from 26 out of 37 patients (70%) (7)(48). This has been confirmed in subsequent studies and proven to be specific to IMN and implicated in disease progression and outcome (12,13)(25,49).

Most recently, combined immunologic and proteomic approaches identified thrombospondin type-1 domain-containing 7A (THSD7A) as another target autoantigen in MN (8)(50). THSD7A antibodies are found in approximately 2-3% of MN patients. THSD7A like PLA_2R1 is a heavily glycosylated, multi-domain transmembrane receptor located on the podocyte membrane. THSD7A resembles some of the PLA_2R1 immunological characteristics and autoantibody findings correlate with glomerular staining of the antigen. It is not understood why autoantibodies develop however, in some THSD7A associated cases the development of antibodies may be linked to malignant tumours (14,15)(51,52). Interestingly, dual positivity to both PLA_2R1 and THSD7A is extremely rare with only 2 cases identified on biopsy staining (16)(53).

Familial clustering of Membranous Nephropathy

Whilst all available data points towards a strong genetic component, IMN appears not to be inherited in a simple Mendelian fashion. In 1984 the first case of identical twins developing IMN was published (17), and to date sixteen families have been reported to have familial IMN (3,18,17,19–24), suggesting strong genetic contribution. However, several sets of monozygotic twins with IMN had different phenotypes with a different age of onset and

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progression of disease (17,22). This suggests an environmental contribution to disease, which is not yet well established.

There is a strong male preponderance in IMN (25) unlike other autoimmune diseases (26). An X-linked recessive pattern of inheritance was suggested based on the clustering of disease between non-identical brothers (17–19,21). Autosomal inheritance was also apparent in other families with male-to-male transmission (20,24) and affected members of both genders (18,23). Further support for the theory of an underlying genetic mechanism was provided by two brothers with a rare syndromic form of IMN (19). These brothers had both IMN and deafness but no linked HLA alleles (19). To date the involvement of antibodies against phospholipase A_2 receptor 1 ($aPLA_2R1ab$) in cases of familial MN are unknown.

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Two studies of paediatric primary MN report much lower positivity for PLA_2R1 staining of immune complexes on biopsy at 6% and 45% compared to adult studies at 70-80% (27,28). As yet the genetic background to paediatric MN has not been confirmed.

Concept of genome-wide association studies and confirmation of genetic association

The biggest breakthrough in the contribution of genetic factors in IMN so far was with three genome-wide association studies (GWAS) published in 2011 (3). GWAS works with the hypothesis that the phenotype is associated with variations in a subset of several genes. These variations will be demarked by haplotypes / alleles that display frequency differences in the cases and controls. GWAS examine all chromosomes and its simplest form compares allele frequencies of given variations in cases to allele frequencies of controls (basic allele test). GWAS most often use common single nucleotide

polymorphisms (SNP), which are defined by an allele frequency in a given population of > 5%. The tenet is that any given disease, as long as there is no heterogeneity, will show a difference in the frequency of genetic variation within disease-associated genomic regions in comparison to unaffected controls. Thus, SNPs utilized as genetic markers, identify a chromosomal location of interest associated with disease. If the phenotype is clearly described and unique then GWAS can be powerful for discovery of associated alleles even with few cases (29,30)~~(8,9)~~. The first ever GWAS published in macular degeneration utilised 96 cases and 50 controls only (29)~~(8)~~. Of all SNPs genotyped 105,980 were analysed and an intronic and common variant in the *complement factor H* gene that increased the likelihood of macular degeneration by a factor of 7.4 was discovered (29)~~(8)~~. This is contrary to the opinion (misconception) often presented in public that GWAS always need thousands or tens of thousands of samples to be able to identify genetic causes. When a phenotype is complex (i.e. hypertension, kidney failure), then indeed many more samples are needed to be able to identify regions of interest, i.e. associated alleles.

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Genome-wide association studies in membranous nephropathy

The GWAS published in 2011 investigated European populations with renal biopsy proven IMN (3). Three independent GWAS were performed, using 75 French European cases, 146 Dutch European cases and 335 British European cases. Despite the small number of cases even in the smallest cohort (French), a significant association in 3 SNPs in an *HLA-DQA1* allele on chromosome 6 was found. The 146 Dutch cases demonstrated a significant allelic association of 191 SNPs in *HLA-DQA1*. Additionally, 6 SNPs located within the *PLA2R1* gene on chromosome 2 were associated with IMN, the strongest being SNP rs4664308. Finally, the British study found a significant association with 144 SNPs in the *HLA-DQA1* allele and 2 SNPs in the

PLA2R1 allele. Combining then the three cohorts in a meta-analysis with a total case population of 556 further strengthened the association of IMN with 20 SNPs in *HLA-DQA1* and 13 SNPs in *PLA2R1*. The effect size of the risk SNPs was examined, even in a heterozygous state of the risk allele the odds ratio was increased in both *HLA-DQA1* and *PLA2R1*. The strongest association was with the *HLA-DQA1* region, (the most significantly associated SNP being rs2187668) (3). In a homozygous state of the *HLA-DQA1* risk allele the odds ratio of IMN was 20.2 (3). The odds ratio in a homozygous state for ~~and in~~ *PLA2R1* was 4.2 (3). Combining these two risk alleles further increased the risk of IMN to an odds ratio of 78.5 (3). This association was very robust for such a modest cohort (31)~~(10)~~, which is unusual for a GWAS (18)~~(11)~~. Also, no association was found with immunoglobulin G chains that were previously identified with a candidate gene approach on chromosome 14 (32,33)~~(12,13)~~.

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Imputation

The SNP coverage of these initial GWAS is low compared to the coverage available with more modern technology, particularly of the HLA alleles (34,35)~~(14,15)~~. To further assess the strength of the SNP associations that were found in the British study an imputation study was performed (36)~~(16)~~. Imputation is a method to increase the statistical power of association studies and potentially identify additional associated alleles (37,38)~~(17,18)~~. This technique is based on knowledge about short stretches of shared haplotypes to provide information and predict untyped alleles (39)~~(19)~~. Imputation takes advantage of haplotype composition to match known SNPs to other SNPs that are in linkage disequilibrium with one another. In this way, it was possible to impute and examine 8.9 million SNPs in the British cohort. The strongest signals remained in *HLA-DQA1* and *PLA2R1*, and no additional loci were

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found as independent risk factors. The *PLA2R1* signal was somewhat weaker and *HLA-DQA1* somewhat stronger than originally described, with homozygous risk alleles at both loci the combined odds ratio was greater at 79.4 (36)~~(16)~~. In addition, imputation of classical HLA alleles was performed, with the DRB1*0301-DQA1*0501-DQB1*0201 haplotype showing the strongest association but providing little information beyond the lead SNP in HLA-DQA1. Sub-group analyses were undertaken and there was no significant gender specific genetic difference and no additional loci were found on the X chromosome (36)~~(16)~~, which may have been unexpected given the unusual strong male preponderance in IMN, but statistical power for these analyses was limited. The HLA region was analysed in much more detail and this demonstrated a several hundred kilobase pair linkage disequilibrium around *HLA-DQA1* as well as other HLA class II genes (36)~~(16)~~.

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M-type phospholipase A_2 receptor 1

To investigate whether specific variants within the *PLA2R1* gene are causing this previously mentioned strong genetic association, sequencing of the 30 *PLA2R1* coding exons was performed. This was also an ethnically homogenous group, all 95 affected patients were white Europeans and only 45% had circulating ~~antibodies against phospholipase A_2 receptor 1 (~~aPLA$_2$R1ab~~)~~ (40)~~(20)~~. All exons and splice sites of *PLA2R1* were sequenced by Sanger sequencing and all observed variants including rare variants (minor allele frequency <1%) were analysed. To our initial surprise, no rare genetic variants causing a conformational change in PLA$_2$R1 structure were found. Of the variants found 6 were common and 3 in splice sites (exon-intron boundaries). One of these non-synonymous (causing amino acid alteration) common variants (i.e. M292V) encodes an amino acid located within CTLD1 but this is far removed from the immunodominant epitope in the N-terminal

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cys-rich domain and unlikely to have a contributory role in the pathogenesis of IMN (40,41)(20,21). One reason for the lack of exonic, i.e. coding, differences may be that the true causal variant(s) lie(s) in the regulatory, i.e. intergenic or intronic regions of the gene. For this to be examined, sequencing of the whole genomic region would need to be done. A second reason for the lack of significant results was that only 45% of the cohort had detectable $aPLA_2R1ab$. The remaining patients were $aPLA_2R1ab$ negative, and we now know that the association is strongest in $aPLA_2R1ab$ positive patients.

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It is therefore most interesting to note that despite IMN being a rare disease the variants found in *PLA2R1* were common. An explanation for this would be that the common variants recognised together create a rare haplotype (40)(20). Additionally, an interaction between the *PLA2R1* variants and the *HLA-DQA1* haplotype in individuals predisposed to developing IMN might be infrequent in causing autoimmunity and may therefore account for the rarity of disease and suggest a mechanism for how IMN develops (42)(22). Genotyping of hundreds of thousands of individuals will provide an answer to whether there is a unique genetic fingerprint of individuals developing IMN and what proportion of individuals having this genetic fingerprint actually present with IMN (i.e. show penetrance).

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Antibody and gene interplay

The presence of circulating antibodies against PLA_2R1 and THSD7A is variable between patients and throughout the different stages of disease (43). During active nephrosis and disease these levels tend to be high and remission is predated by reducing antibody titres (43). Serologically antibody negative MN patients may have glomerular PLA_2R1 positivity (12). The underlying pathological mechanism in tissue or serological PLA_2R1 positivity

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is the same and they represent a spectrum of the same disease. A hypothesis is these patients have the same genetic *PLA2R1* risk variants yet are demonstrating incomplete penetrance of disease manifestation. Studies were undertaken to elucidate the association of genetic variants and circulating antibodies as the antibody titres have been associated with severity of disease and long term outcome (13).

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PLA2R1 risk alleles are positively correlated with positivity of the pathogenic aPLA_2R1ab (44)(23). When patients were divided into low- or high-risk *PLA2R1* genotypes, only 4% of those with the low-risk genotype had detectable aPLA_2R1ab compared to 76% of those with the high-risk genotype (44)(23). This association was further strengthened for the detection of aPLA_2R1ab after combination with the low- or high-risk *HLA-DQA1* genotypes with 0% versus 73% respectively (44)(23). A larger study compared aPLA_2R1ab glomerular PLA_2R1 antibody staining (positivity)e to negative patients and found the *PLA2R1* association only in patients with aPLA_2R1ab positivity. In aPLA_2R1ab negative patients compared to controls there was no association with *PLA2R1* SNPs (45)(24).

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This is relevant as increased aPLA_2R1ab correlates with clinical progression of disease; with higher titres associated with ESRD at five years and lower rates of spontaneous remission (13)(25). In an Indian cohort, however, there was no significant association between aPLA_2R1ab status and *PLA2R1* SNPs. Instead there was an association of the *HLA-DQA1* risk allele with aPLA_2R1ab positivity (46)(26). This was subsequently replicated in a European cohort and the presence of the risk alleles in either a heterozygous or homozygous state in *HLA-DQA1* and *-DQB1* was significantly associated with higher circulating aPLA_2R1ab (13)(25). Neither the SNPs in intron 1 or exon 5 in *HLA-DQA1* alone had an effect on aPLA_2R1ab titres (13)(25). A Two recent Chinese study studies demonstrated the strong HLA association

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with ~~-~~aPLA$_2$R1ab positivity (47,48). One had an association with *HLA-~~DQB1~~ DRB1* and the other *HLA-DRB3* both of which share a haplotype so may represent a common mechanism in Chinese patients (47–49)~~having a strong association with aPLA$_2$R1ab positivity (27)~~. The risk alleles in *PLA2R1* are said to be present in patients with systemic lupus erythematosus (SMN) albeit with lower odds ratios (50)~~(28)~~ and aPLA$_2$R1ab are occasionally found in patients with SMN (51)~~(29)~~.

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Ethnic differences

Our findings from the first IMN GWAS (3) have been replicated in other studies, however different techniques have been used. These studies use~~d~~ a candidate gene approach whereby a specific variant alone is genotyped (52). These SNPs are chosen as the candidate gene based on prior knowledge about PLA$_2$R1 or previously described SNPs (52,53). This is a major limitation of the candidate gene approach; they can only confirm or refute an association with a variant and cannot detect new associations (52,53). Another limitation is findings are often not replicated in subsequent independent studies rendering the results potentially unreliable (52,53). Table 1 provides a summary of genotyping studies to date in MN.

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In MN, t~~T~~he first ~~of~~ study utilising the candidate gene approach ~~so~~ was ~~in~~ a small Spanish cohort of 89 patients, where only a single SNP in both the *HLA-DQA1* and *PLA2R1* genes was investigated (54)~~(30)~~. This study too found the same association in both alleles in their cohort, with an added effect of homozygous risk alleles in both genes increasing the odds ratio of IMN to 7.3 (54)~~(30)~~. As these studies were performed in European populations it was of interest to investigate if these associations held true in other ethnicities.

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In a cohort of 114 Indian patients the same risk alleles were identified as by Stanescu *et al.* (46)(26). The strongest association was with the homozygous genotype in the *HLA-DQA1* SNP rs2187668. Three SNPs were associated within *PLA2R1*, one of which was the same SNP described in the GWAS (3), rs4664308 with the AA risk genotype (46)(26). The risk of IMN was increased by 58.4 with all four risk alleles in *HLA-DQA1* and *PLA2R1* (46)(26). This is a strong association with a small sample size.

The only study undertaken in African-Americans so far examined 243 African-American and 467 European cases of IMN (45)(24). Targeted sequencing of candidate genes using conventional polymerase chain reaction was performed, with genotyping of 6 *PLA2R1* SNPs and a single SNP in the *HLA-DQA1* region (45)(24). Further, they differentiated between patients who had PLA$_2$R1 positivity on renal biopsy (using immunofluorescence) detectable antibodies (aPLA$_2$R1ab) (115 African-American cases) and those who did not (128 African-American cases) (45)(24). No association was found in African-Americans with the *HLA-DQA1* SNP rs2187668, suggesting that this SNP is tagging the causal variant(s) in individuals of European and East Asian ancestry but not in African Americans. In the European sub-group analysis however, the strong association was present with *HLA-DQA1* (45)(24). Further the *PLA2R1* signal was associated with glomerular aPLA$_2$R1ab positivitye patients in the African-American cohort but not in aPLA$_2$R1ab negative patients (45)(24). The strength of this association was lower than that found in Europeans, with the strongest association in Europeans with detectable aPLA$_2$R1ab (45)(24).

Chinese patients demonstrated a similar association with *PLA2R1* risk alleles increasing the risk of IMN but without any effect on outcomes and response to treatment (55)(31). Liu *et al.* analysed 2 SNPs in 129 Chinese IMN patients (55)(31). The risk allele increased the rates of IMN (55)(31). There was no

difference in the different genotypes relating to progression to ESRD, though the patient numbers were too small to identify such a difference. A heterozygous state for the risk allele in the exonic *PLA2R1* region conferred a lower success rate of achieving remission (55)(31). A larger study including 1112 Chinese patients with IMN genotyped 3 SNPs in *PLA2R1* and 3 SNPs in *HLA* genes and found that both were associated with IMN (44)(23). Interestingly, in the Chinese population the association with *HLA-DQA1* was lower than in Europeans, and there was no association with HLA Class II alleles apart from *HLA-DOB* or *-DQB2* (44)(23). A study of 261 IMN Chinese patients has linked *HLA-DRB1*1501* most significantly with IMN (47)(27). After correction for *HLA-DRB1*0301*, the *HLA-DQA1* association was diminished as these two loci are in strong linkage disequilibrium with one another (47)(27). The additive effect of homozygous risk alleles in *HLA-DQA1* and *PLA2R1* increased the odds ratio of IMN to 11.13 which is considerably lower than that found in the European studies (3,44)(3,23). However, with the newly discovered association of *HLA-DRB1* and *PLA2R1* the odds ratio is considerably higher at 32.4 in the Chinese population (47)(27). Another Chinese study in patients phenotyped by PLA_2R1 positivity demonstrated a stronger association with *HLA-DRB3*0202* and *HLA-DRB1*1501* with odds ratios of 24.9 and 17.7 respectively *(48)*. Both studies have identified the same allele in *HLA-DRB1*1501* which may truly represent the causative allele in Chinese patients. Difficulties arise with analysis of such data as the allele frequencies vary between ethnic groups (56)(32). The Chinese are genetically heterogeneous and within a control population there were different minor allele frequencies in *HLA-DQA1* and *PLA2R1* dependent on their geographical location (56)(32).

A study of 4 SNPs in *PLA2R1* in 199 Korean patients also confirmed an association of disease with rs35771982 and rs3828323 (different to the

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Stanescu *et al.* SNPs (3,57)~~(3,33)~~. Patients with SMN had the same genotype as controls (57)~~(33)~~.

Finally, a Japanese study performed genotyping of 15 SNPs in the *PLA2R1* gene and 6 HLA genes - *A, B, C, DRB1, DQB1 and DPB1* (58)~~(34)~~. The discovery sample had 53 patients, and the replication study 130 (58)~~(34)~~. After corrections for multiple testing and correlation in the replication study 4 SNPs in *PLA2R1* were associated with IMN, 2 of which were intronic (58)~~(34)~~. None of the class I HLA genes (*A, B or C)* were significantly associated with IMN, however *HLA-DRB1*15:01* was the most strongly associated with an odds ratio of 2.85 followed by *HLA-DQB1*, odds ratio 2.6. These odd ratios increased in the replication study and then subsequently in the combined analysis to 3.09 and 3.1 respectively (58)~~(34)~~. Interactions between the *HLA* and *PLA2R1* homozygous risk alleles further increased the risk of developing IMN, with the largest odds ratio of 17.53 in the *HLA-DRB1*15:01 – DQB1*06:02* and rs2715928 *PLA2R1* combination. Whilst these interactions are statistically significant they are still considerably lower than the strength of interactions found in the European GWAS (3). The differences may be due to sample size differences or because *HLA-DQA1*, which is a larger contributor to the cumulative risk in the European study, was not genotyped in this Japanese study, or because of differences in linkage disequilibrium with the causal variant across different ethnic groups.

Functional effect of genes

The underlying genetic risk alleles that have been identified to date are different between individual studies but universally there is an association of IMN with the human genes encoding leucocyte class II antigens and *PLA_2R1*. Functional studies to ascertain how these genetic variants increase the risk

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for disease development are required. It is also possible that the previously identified risk alleles do not affect disease onset but instead disease severity (42)(22).

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It is unclear how the genetic risk alleles of class II *HLA* (e.g. *DQA1*) and *PLA2R* are translated through the pathophysiological disease mechanism, but antigen presentation to T cells to initiate T cell help for aPLA$_2$R1ab production is one possibility. These risk alleles encode protein receptors which interact during antigen presentation to stimulate T cells. In this situation, PLA$_2$R1 protein, processed in macrophage/dendritic cells is displayed on the cell surface as PLA$_2$R1 peptides bound to the class II receptor (DQA1) groove. The genetics of DQA1 will shape the amino acid structure of its receptor groove thus defining and restricting the possible 15mer peptide sequences available from PLA$_2$R1 that will fit the groove. The genetics of PLA$_2$R1 may control the possible enzyme fragmentation pattern of PLA$_2$R1 by:

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a) change in amino acid either creating or destroying an enzyme cut site
b) change in splice sites controlling the protein species available for fragmentation
c) level of transcript leading to higher levels of peptide

As yet these T cell peptides (the PLA$_2$R1 peptides presented on DQA1) have not been described experimentally but studies are in progress. A recent study has predicted possible T cell epitopes in PLA$_2$R1 and attempted to model the interaction with known class II risk alleles (47)(27). It is important to emphasise that *DQA1* may not be the causal allele, particularly in non-European ethnicities (47,48).

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To elucidate the HLA causal alleles further larger multi-ethnic GWAS combined with larger-scale HLA sequencing and fine-mapping studies are necessary. It is vital to do this before modelling their functional effects

however, ~~I~~it would be useful to have ~~further~~ transcriptomic and proteomic studies to ascertain if *PLA2R* expression is modified and if this is due to an increase or decrease in transcriptional or post-transcriptional events.

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Remission status

A comparison of 23 spontaneously remitting to 55 non-remitting IMN patients found no difference in genetic variants in *HLA-DQA1* or *PLA2R1* (54)~~(30)~~. In contrast, Liu et *al.* reported an association between lower rates of remission after treatment and the *PLA2R1* SNPs rs6757188 (CT genotype) and rs35771982 (CG genotype) (55)~~(31)~~.

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Response to treatment

Patients with the risk genotypes in *HLA-DQA1* and *PLA2R1* respond to immunosuppression, though the odds ratio is low at only 0.12 (54)~~(30)~~. The total number of patients assessed was small with 27 responders and 28 non-responders (54)~~(30)~~. After adjustment for baseline proteinuria the predictive value of risk genotype increased (54)~~(30)~~. Analysis of 2 different *PLA2R1* SNPs revealed no difference between the outcomes of patients treated either conservatively or with immunosuppression (55)~~(31)~~.

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Decline in renal function

The high risk alleles (AA genotype) in *HLA-DQA1,* despite being strongly associated with IMN, are potentially protective against declining renal function (54)~~(30)~~. High risk genotype patients had a longer time to doubling of their serum creatinine of 16.3 years compared to 13 years, though this was a small subgroup of only 83 patients (54)~~(30)~~. No association was found in the 8

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Japanese patients that had a 50% increase in their serum creatinine with *HLA-DRB1* and *-DQB1* over an 11 year period, nor with patient survival (58)~~(34)~~. The association with *PLA2R1* risk alleles and declining renal function has been investigated in different ethnicities and no association was found (54,57)~~(30,33)~~. In addition there was no association with ESRD or death (55)~~(31)~~.

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As yet there has been no conclusive evidence associating genetic variants to remission status, response to treatment or a decline in renal function. These factors are difficult to determine as studies are often done in retrospective cohorts where confounders such as immunosuppression or disease severity have a significant effect on the outcomes. Decline in renal function is multifactorial and is related to blood pressure control, severity of proteinuria, renal function at disease onset, age and gender amongst others. These factors themselves are likely to be independent risk factors which is why studies to date have not been significant. It may be argued that these factors are caused or influenced by genetics thereby further complicating the potential genetic risk profile with IMN.

~~**Familial clustering of Membranous Nephropathy**~~

~~Whilst all available data points towards a strong genetic component, IMN appears not to be inherited in a simple Mendelian fashion which is supported by the discovery of at least two genetic risk loci so far. In 1984 the first case of identical twins developing IMN was published (35), and to date sixteen families have been reported to have familial IMN (3,11,35-41), suggesting strong genetic contribution. However, several sets of monozygotic twins with IMN had different phenotypes with a different age of onset and progression of~~

disease (35,39). This suggests an environmental contribution to disease, which is not yet well established.

There is a strong male preponderance in IMN (42) unlike other autoimmune diseases (43). An X-linked recessive pattern of inheritance was suggested based on the clustering of disease between non-identical brothers (11,35,36,38). Autosomal inheritance was also apparent in other families with male-to-male transmission (37,41) and affected members of both genders (11,40). Further support for the theory of an underlying genetic mechanism was provided by two brothers with a rare syndromic form of IMN (36). These brothers had both IMN and deafness but no linked HLA alleles (36). To date the involvement of aPLA_2R1ab in cases of familial MN in unknown.

Two studies of paediatric primary MN report much lower positivity for PLA_2R1 staining of immune complexes on biopsy at 6% and 45% compared to adult studies at 70-80% (44,45). As yet the genetic background to paediatric MN has not been confirmed.

Discovery of autoantigens

The first autoantigen described in a rare case of antenatal MN was neutral endopeptidase (NEP), in 2002 (46). The gene encoding NEP is *metallomembrane endopeptidase*. Truncated mutations were discovered in maternal DNA so the mother did not express NEP protein. When foetal NEP (paternal protein) was encountered during pregnancy, anti-NEP antibodies developed (with no consequence to the mother) which crossed the placenta to cause neonatal MN (46,47).

The discovery of circulating antibodies to the autoantigen PLA_2R1 revolutionised our understanding of IMN as an autoimmune disease (48). With

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~~Western blots and mass spectrometry the antibody was detected in serum from 26 out of 37 patients (70%) (48). This has been confirmed in subsequent studies and proven to be specific to IMN and implicated in disease progression and outcome (25,49).~~

~~Most recently, combined immunologic and proteomic approaches identified thrombospondin type-1 domain-containing 7A (THSD7A) as another target autoantigen in MN (50). THSD7A antibodies are found in approximately 2-3% of MN patients. THSD7A like PLA_2R1 is a heavily glycosylated, multi-domain transmembrane receptor located on the podocyte membrane. THSD7A resembles some of the PLA_2R1 immunological characteristics and autoantibody findings correlate with glomerular staining of the antigen. It is not understood why autoantibodies develop however, in some THSD7A associated cases the development of antibodies may be linked to malignant tumours (51,52). Interestingly, dual positivity to both PLA2R1 and THSD7A is extremely rare with only 2 cases identified on biopsy staining (53).~~

Conclusion

There has been an exponential increase in our understanding of IMN since 2009, when PLA_2R1 was identified as the most significant pathogenic autoantigen in IMN. IMN therefore may occur when three independent risk factors combine: unique polymorphisms in *PLA2R1*, the *HLA-DQA1* region and environmental factors. There are ethnic specific differences in these alleles and the potential that risk alleles may contribute in predicting disease outcomes. The complex pathomechanisms of disease development highlight some of the potential problems in analysing and predicting the risk for disease progression. The genetic variants may alter the expression or function of the target antigens and enable autoantibody formation. While no rare variants (i.e.

mutations) were found in the coding region of *PLA2R1* the role of intronic variants needs to be investigated given their large regulatory role. As shown before, non-coding SNPs (i.e. intergenic or intronic genetic variations) are associated with ESRD (59)(54) and other autoimmune conditions (60)(55).

Whole genome sequencing is becoming more affordable and faster and may help illuminate the true role of intergenic and intronic genetic variants in IMN. The genomic studies could be augmented with epigenomic, transcriptomic and proteomic studies to ascertain the functional effect of gene variants. The regulatory regions that control autoantibody production such as transcription factors or micro RNA could be altered by the identified risk SNPs in a mechanism analogous to psoriasis (31)(10). If upstream and downstream regulatory region variants were found these would be potential therapeutic drug targets, possibly preventing the deleterious effects of current immunotherapy. Given the large odds ratio with joint homozygosity, genotyping could be utilised to stratify disease risk and outcomes. The utility of genetic profiling in IMN could prove to be vital for non-invasive screening or risk stratification (18,31)(10,11). The tools (aPLA_2R1ab) available to us are of assistance but by understanding the genetics we may be able to explain why the autoantibodies develop in the first instance (18)(11). Current studies have been limited by small sample size and so there may be a lack of appreciation of potential other associations. Expanding the horizons further, there may even be a role for ascertaining epidemiologic risk for IMN with risk alleles and seeing if people in the general population have a genetic predisposition to disease (18)(11). There may be an indirect interaction between genetics and disease, such as molecular mimicry whereby a microbe or environmental antigen resembles a *PLA2R1* variant and causes autoimmunity in patients carrying the *HLA-DQA1* risk alleles (42)(22). The reported homology of part of the major epitope sequence in PLA_2R1 with a clostridial carbopeptidase

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enzyme illustrates how antibodies raised during infection may potentially cross react with an autoantigen (41)~~(21)~~. Normal control populations without IMN but with the risk alleles will be the most useful in identifying the triggers or environmental factors that contribute to eventual disease acquisition which may further our understanding of this complex genetically predisposed disease.

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Conflict of interest statement

None of the authors has a conflict of interest; the results presented in this paper have not been published previously in whole or part.

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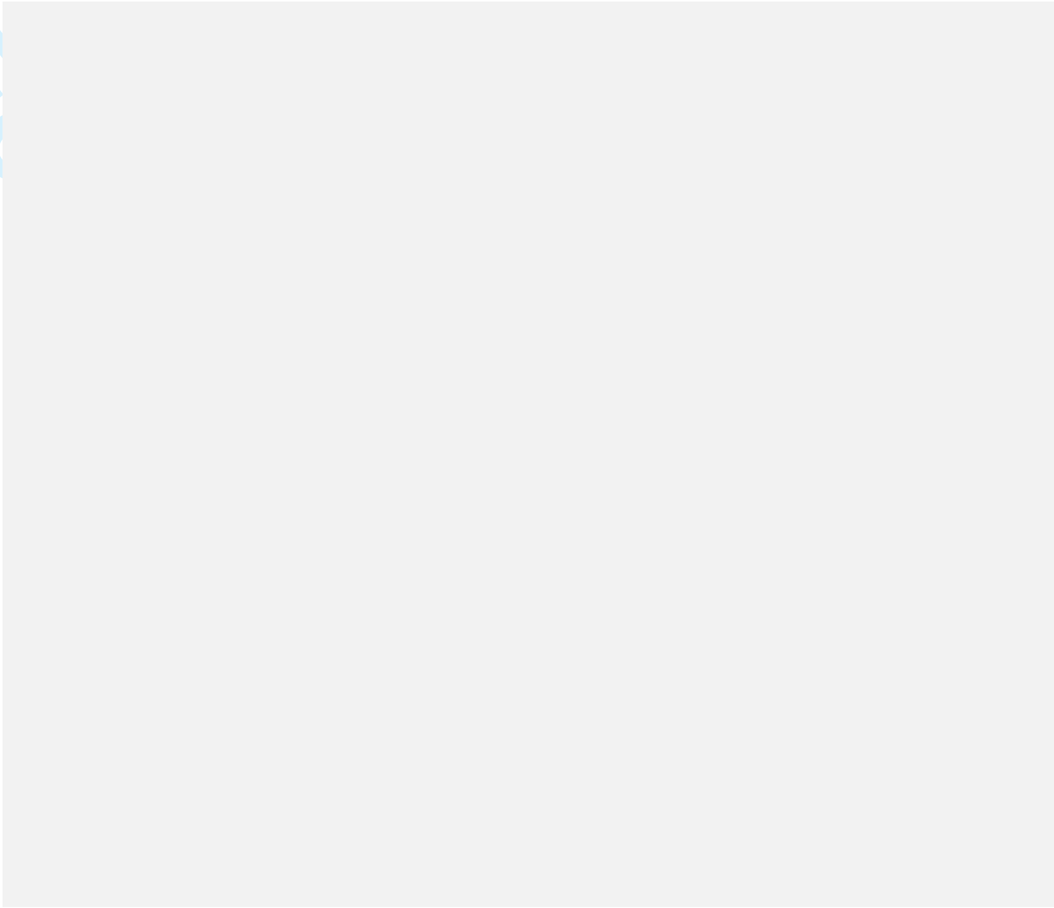

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18th August 2017

Dear Professor Fouque,

We thank you for your kind consideration of our manuscript and the detailed and expert reviews that you have obtained.

We have revised the manuscript and edited it to reflect and incorporate all the changes suggested. Please find below the point by point response to the criticisms that were raised by the reviewers. We are very grateful for your repeated consideration of our manuscript for publication.

Kind regards and best wishes,

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Reviewer 1

1. *Although the focus of this review is on the genetics of membranous nephropathy, I feel that the 'Discovery of autoantigens' section (pp. 14-15) would be better placed at the start of the manuscript, so that the reader has a better idea of how the PLA2R1 genetic locus fits in to the overall understanding of the disease. At the very least, the original articles reporting the identification of the two autoantigens, PLA2R and THSD7A, should be cited in the Introduction where they are first mentioned.*

Thank you, these are very helpful comments and the manuscript has been revised to change the order of the paragraphs. The discovery of autoantigens now comes after the introduction. Further the references have been added to the introduction as you correctly mention where we first introduced the concepts of the antigens and antibodies.

2. *I would also like to see a better discussion about the variable presence of circulating antibodies, perhaps as an introductory paragraph in the 'Antibodies and gene interplay' section (p. 7). In some ways, the phenotype of seropositivity for anti-PLA2R could be considered in genetic terms as one of incomplete penetrance, since individuals may not always exhibit clinical disease or circulating autoantibodies, despite having PLA2R-associated MN as defined by prior episodes of seropositivity, or the presence of PLA2R tissue positivity on biopsy. The relationship between genetics, the presence of circulating antibodies, clinical parameters such as proteinuria, and longer-term outcomes is not immediately obvious in this review, and should be clarified as above.*

We have added in an introductory paragraph in the 'Antibody and gene interplay' section as suggested. The relationship between genetics and circulating antibodies is certainly known but the clinical parameters are more associated with the antibody levels which we also discuss within the same section paragraph 3. For the association of the genetics and clinical parameters please see the 'functional effect of genes' section now on page 14.

3. *The sections describing the associations between genetic variants and 'Remission status' (p. 12), 'Response to treatment' (p. 13), and 'Decline in renal function' (p. 13) leave out any mention of other factors (spontaneous remission, immunosuppressive treatment, duration and severity of proteinuria, etc) that may have more important effects on these outcomes than do the genetic variants. Such a discussion might explain to the reader why these associations with the genetic variants are not significant.*

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We have adjusted the manuscript to include a new paragraph stating some of the other factors that may act independently to affect the outcomes of disease in MN, see page 16 under the 'decline in renal function' heading.

4. *Please rephrase the sentence on p. 5: 'In a homozygous state of the HLA-DQA1 risk allele the odds ratio of IMN was 20.2 and in PLA2R1 4.2.' It is not immediately clearly that the latter odds ratio refers to the risk of IMN in those individuals homozygous for the PLA2R1 risk allele.*

Apologies for this confusion, we have adjusted the manuscript to clarify this point further that it is indeed also a homozygous state of the PLA2R1 allele.

5. *In the 'Ethnic differences' section (p. 8), in keeping with trying to explain basic concepts for those readers uninitiated in the techniques of genetic analysis, I would suggest better explaining the candidate gene approach. In these earlier studies, the investigators focused on SNPs in/near the PLA2R1 gene because of its recent identification of the protein product PLA2R as an autoantigen, or focused on the same SNPs in PLA2R1 or HLA-DQA1 that had been identified in the Stanescu et al. 2011 NEJM paper. The limitations of the candidate gene approach for discovering new associations should be explained, and that they are rather confirmatory (or not) in these different ethnic and geographic cohorts.*

We have integrated and expanded on the candidate gene approach as suggested to reflect and demonstrate this point to readers, thank you for this helpful suggestion.

6. *In the same section (p. 9), please check reference 24 (Saeed et al.) to confirm whether those authors stratified their patients based on autoantibody positivity for anti-PLA2R, or rather tissue positivity for the PLA2R antigen (the reference is not available to me online).*

Saeed et al. stratified their patients by PLA2R immunofluorescence on renal biopsy. The manuscript has been edited to reflect this and highlight this valid point on tissue positivity compared to serological positivity.

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Reviewer 2

7. *To improve the flow of the manuscript, I would suggest moving the sections on the familial clustering and auto-antigen detection to the front of the manuscript, after introduction but before discussing GWAS findings and replication studies. It is important to describe familial clustering before describing various genetic approaches.*

Thank you for this suggestion which is like reviewer 1 with regards to the ordering of the paragraphs. We have edited the manuscript to reflect this.

8. It may be helpful to tabulate the results of all replication studies of PLA2R1 and HLA associations published to date, including sample sizes, ethnicities, allelic frequencies and effect estimates for these two loci.

We have added table 1 with the relevant summary of all the studies done to date as requested.

9. *There is some discussion of the DQA1 binding groove (in the "Functional Effects" section), however, recent sequencing studies suggest that HLA-DQA1 may not be the causal gene. I would recommend discussing the results of these two studies in more depth, especially the association with DRB1*15:01 in Chinese (Cui et al. JASN 2017; Le at al. JASN 2017). I would emphasize again that larger-scale sequencing and fine-mapping studies to define causal alleles within the HLA region are still needed in Europeans before one can model their functional effects.*

We have edited the manuscript in the ethical differences section starting page 13 to include more in depth information about these studies. Further in the functional effects section we have generalised the statements to reflect the differences in class II HLA types in different ethnicities.

10. Page 6: "(after imputation) the PLA2R signal was somewhat weaker". I am not clear why the imputation would weaken an association signal that originates from a genotyped SNP. Presumably the same genotyped SNP that gives the original signal should have very similar association signal after imputation (this is a very minor point, but would be nice to clarify).

Imputation compares more SNPs than the original GWAS so when applying the Bonferroni correction it is possible that previous associations become weaker than they previously were. In addition, and here most relevant, the associations and their significances are relating to a different number of controls which can indeed change significance levels.