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Phospholipase A2 Receptor and α-enolase in the Immunodiagnosis of Membranous Nephropathy

by

Ying Zhang

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Abstract

M-type phospholipase A_2 receptor (PLA2R) and α-enolase are two recently identified autoantigens in adult idiopathic membranous nephropathy (IMN). To identify their diagnostic value and the potential roles in the pathogenesis of MN, renal biopsies and sera from 23 patients with MN were investigated. In 19 IMN cases, the positivity for glomerular PLA2R and circulating anti-PLA2R antibodies was 47% and 26% respectively, while only 5% glomeruli showed positive α-enolase staining. IgG4 was often codominant with either IgG1 or IgG3 in PLA2R-positive glomeruli which also showed more prevalent and stronger C3 staining as compared with PLA2R-negative glomeruli. A positive correlation between IgG1 with complement C3C expression in glomeruli was observed. Circulating anti-PLA2R autoantibody titers and PLA2R expression in renal biopsies were associated with clinical outcomes (follow up proteinuria). The results indicate that in PLA2R related MN, autoantibody –antigen interaction activates complement C3 directly or indirectly, resulting in podocyte injury and proteinuria.

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Symbol	Definition
ALBIA	Addressable Laser Bead Immunoassay
BSA	Bovine Serum Albumin
EDC	Ethyl-3-(3-Dimethylaminopropyl) Carbodiimide
ESRD	End-Stage Renal Disease
FITC	Fluorescein Isothiocyanate
GBM	Glomerular Basement Membrane
GFP	Green Fluorescent Protein
HEK293	Human Embryonic Kidney 293 cells
HN	Heymann Nephritis
IgG	Immunoglobin G
IIF-CBA	Cell Based Indirect Immunofluorescence Assay
IMN	Idiopathic Membranous Nephropathy
MBL	Mannose-Binding Lectin
MFI	Median Fluorescent Index
MN	Membranous Nephropathy
NEP	Neutral Endopeptidase
NHS	Normal Human Serum
NS	Nephrotic Syndrome
OCT	Optimal Cutting Temperature
PBS	Phosphate Buffered Saline

PLA2R	Phospholipase A2 Receptor
RCC	Renal Cell Carcinoma
SC65	Synaptonemal Complex Protein 65
SMN	Secondary Membranous Nephropathy
SOD2	Superoxide Dismutase 2
TE	Tris-EDTA

Chapter One: **Literature review**

1.1 Membranous nephropathy-Introduction

1.1.1 Definition

Membranous nephropathy (MN) is an immunologically mediated kidney disease, which is the leading cause of nephrotic syndrome (NS) in Caucasian adults, responsible for approximately 20%-30% of NS cases (Simon, Ramee et al. 1994, Maisonneuve, Agodoa et al. 2000). With a peak incidence in the fourth and fifth decades, MN is more prevalent in men than women. Proteinuria, often in the nephrotic range, is the hallmark of MN. Adult MN is most commonly a primary or idiopathic disease without an identified cause (70-80% of the cases) (IMN), but in 20-30% of patients it may also occur secondary to other conditions (SMN) such as neoplasms, infections, systemic lupus erythematosus, and certain drug exposures. Other autoimmune diseases such as rheumatoid arthritis, autoimmune thyroid diseases, and Sjögren's syndrome can all be associated with MN (Kerjaschki 2000, Glassock 2010). MN also occurs in renal allografts as a recurrent MN or develops *de novo* MN. Both of them have a poor prognosis and frequently result in graft loss (Briganti, Russ et al. 2002, Pirson, Ghysen et al. 1985). MN occurs less frequently in young people, accounting for less than 5% of biopsy diagnosed renal disease (Eddy, Symons 2003, Chen, Frank et al. 2007). Most of the pediatric MN cases are associated with other diseases and are hence classified as SMN.

1.1.2 Histological features

Histologically, MN is characterized by subepithelial (early stage) or intramembranous (late stage) immune complex deposits, resulting in a spectrum of changes in the glomerular basement membrane (GBM). Early stages of the disease may show no light microscopic changes, while marked glomerular changes are often evident by light microscopy in later stages of the disease. Irregular outgrowths at the subepithelial side of the glomerular basement membrane around the immune deposits appear in silver stained biopsies as "spikes". Electron microscopic changes reveal amorphous, electron-dense deposits; effacement of the podocyte foot processes, and other signs of podocyte injury. According to Ehrenreich–Churg staging system (Ponticelli 2007), MN is histologically classified as four stages: Stage I MN is defined by small and sparse subepithelial deposits immediately adjacent to the podocyte foot processes. Stage II MN is characterized by global subepithelial deposits separated by projections of newly formed matrix material. In stage III, the intervening projections of GBM envelop the subepithelial deposits such that they become intramembranous deposits. Stage IV reflects a remodeling phase in which the deposits become more electron lucent as they undergo absorption. This histological staging system does not have precise clinical implications, as no significant correlation with clinical presentation (e.g. degree of proteinuria or renal function) or prognosis has been reported (Ponticelli, 2007). Furthermore, "progression" from one stage to another does not necessarily indicate worsening disease but can also be seen with disease resolution (Forland, Spargo 1969). Comparing with IMN, secondary forms, especially membranous lupus nephritis, often have additional mesangial or subendothelial deposits and tubuloreticular inclusions, but not always the case. Immunohistologically, MN is characterized by subepithelial deposition of predominantly

IgG, complement C3 and terminal complement complex C5b-9, also known as membrane attack complex (Ponticelli, 2007).

1.1.3 Clinical prognosis and treatments

The clinical course of IMN is quite variable and unpredictable, with 1/3 of patients undergoing spontaneous remission, 1/3 experiencing sustained symptoms, and 1/3 progressing to the end-stage renal disease (ESRD) (Glassock 2010). Predictors of poor renal outcome are advanced age, male gender, heavy proteinuria, hypertension, glomerulosclerosis, interstitial fibrosis, and renal insufficiency at presentation (Nayer, Asif 2013). Therapeutic strategies to reduce proteinuria, including inhibition of the renin-angiotensin system, are recommended for all patients with MN. Whereas definitive therapy for secondary forms of MN should be targeted at the underlying cause, immunosuppressive therapy tend to be reserved for patients who demonstrate predictors of poor renal outcome (Hogan, Mohan et al. 2014). Nevertheless, treatment strategies are controversial, and response rates are highly variable, likely because of disease heterogeneity (Waldman, Austin 2012). The investigation of the pathogenesis of MN, especially the identification of biomarkers will help define patients who may derive the most benefit from more aggressive immunosuppression or any new treatments.

1.2 Pathogenesis of IMN

The presence of immunoglobulins and complement components in capillary walls support the notion that MN is an immune complex mediated disease. The definition and characterization of the immune deposits have been a main focus of the pathology research for

years. Recent reports suggested that membranous nephropathy most likely is a heterogeneous disease involving multiple antigens and antibodies.

1.2.1 Search for antigens

1.2.1.1 Experimental models

The initial understanding of the MN pathogenic mechanisms is based on the experimental animal studies described by Heymann more than 50 years ago (HEYMANN 1952, HEYMANN, HACKEL et al. 1959). Passive Heymann nephritis (HN) is induced in susceptible rat strains by injection of heterologous antisera from sheep or rabbit immunized with a crude extract of rat proximal tubular antigens referred to as Fx1A. Renal pathology in HN is consistently characterized by the presence of glomerular subepithelial immune deposits, highly similar to human MN. This model has offered a great opportunity to study the structure of immune deposits and the mechanisms involved in their formation. Megalin, a glycoprotein which is synthesized by the glomerular visceral epithelial podocyte cells has been demonstrated as the target antigen of subepithelial IgG (Kerjaschki, Farquhar 1982). Epitope mapping showed that full-blown disease with proteinuria was associated with a specific epitope located in a small glycosylated N-terminal fragment of megalin, as well as with intramolecular epitope spreading (Shah, Tramontano et al. 2007). Complement factors and other ancillary proteins such as receptor associated protein (RAP) and anti-RAP IgG complete the structure of immune deposits (Farquhar 1982, Kerjaschki, Miettinen et al. 1987, Kerjaschki, Ullrich et al. 1992). The presence of IgG directed against megalin triggers the complement cascade culminated by the formation of the C5b-9 complex, a key mediator of podocyte damage in MN (Kerjaschki 2004). Later in the

1980s, Border *et al*. developed an alternative model to Heymann nephritis in rabbit, rats and mice, induced by injection of repeated doses of cationized bovine serum albumin (BSA) as exogenous planted antigen (Border, Ward et al. 1982, Adler, Wang et al. 1983). Recently, BSA has also been identified as a novel antigen that can contribute to the development of MN in young children (Debiec, Lefeu et al. 2011). The animal models showed the heterogeneity of potential podocyte antigens in MN and straightened the concept of 'in situ' formation of immune deposit with circulating antibodies recognizing antigens in the capillary walls. However, HN could not be utilized as a direct model of human MN because megalin was not identified in immune deposits and its involvement in human IMN was never proved (Allegri 1997).

1.2.1.2 Human MN

Over the past few years, great advances have been made toward the understanding of the molecular pathogenic mechanisms of human MN with the identification of several glomerular antigens. Neutral endopeptidase (NEP), a podocyte antigen that can digest biologically active peptides, was first identified as the target antigen deposited in the subepithelial space of glomeruli in a subset of patients with antenatal MN (Debiec, Guigonis et al. 2002), where maternal anti-NEP antibodies that cross the placenta, bind to fetal glomerular podocytes and mediate the renal disease. Phospholipase A2 receptor 1, a member of the mannose-receptor family that is normally expressed on the podocyte membrane was first identified as a major autoantigen in IMN (Beck, Bonegio et al. 2009). Later, a few other podocyte target antigens were reported in IMN (Prunotto, Carnevali et al. 2010, Bruschi, Carnevali et al. 2011) In secondary MN, exogenous antigens including hepatitis B and hepatitis C antigens, tumor

antigens, thyroglobulin and DNA-containing material have been detected in subepithelial immune deposits (Ronco, Debiec 2012).

1.2.1.2.1 PLA2R

In 2009, Beck and colleagues detected autoantibodies directed against a 185-KD protein under non-reducing conditions in about 70% of serum samples from patients with IMN (but not secondary MN) (Beck, Bonegio et al. 2009). This protein was identified as the type-M phospholipase A2 receptor (PLA2R) by mass spectrometry. PLA2R and IgG4 were shown to be co-localized within subepithelial deposits. The authors further showed that IgG eluted from biopsy samples reacted with recombinant PLA2R. Subsequent studies confirmed that antibodies against PLA2R were present in approximately 57%-89% of IMN patients in circulating, with IgG4 being the prevalent isotype (Qin, Beck et al. 2011, Debiec, Ronco 2011, Hoxha, Harendza et al. 2011, Hofstra, Beck et al. 2011). Recent studies have indicated that levels of circulating anti-PLA2R were related to proteinuria, disease activity and clinical outcomes. Hofstra *et al*. assessed anti-PLA2R levels by a western blot immunoassay in 54 serum samples from 18 patients with IMN collected in various stages of clinical disease (Hofstra, Beck et al. 2011). The antibody levels in these patients correlated strongly with both clinical status and proteinuria. Kanigicherla *et al*. measured the anti-PLA2R by ELISA and found that high levels of PLA2R antibodies were linked with active disease and a higher risk of declining renal function during follow-up (Kanigicherla, Gummadova et al. 2013). Hofstra *et al*. also reported that high autoantibody titers were associated with a low likelihood of spontaneous remission (Hofstra, Debiec et al. 2012). Hoxha *et al*. performed a prospective multicenter study of 133 adult patients with primary MN and detectable serum PLA2R antibodies who had not received

immunosuppressive therapy. Their results showed that a decrease of PLA2R antibodies level was associated with a decrease of proteinuria in IMN patients (Hoxha, Thiele et al. 2014). The authors also found that PLA2R antibody levels were significantly higher in patients with active disease than in patients who were in remission. The median time to remission was significantly longer in patients with autoantibody levels above versus below the median. Anti-PLA2R antibodies may also predict relapse rate after immunosuppressive therapy in patients with IMN. In a small series of 26 patients, the relapse rate was 71% in patients with anti- PLA2R positivity at the end of treatment as compared to 21% in whom PLA2R antibodies had disappeared (Debiec, Ronco 2014). Further prospective studies on large cohorts of patients are needed before drawing definitive conclusions for meaningful correlations of autoantibodies and clinical outcomes.

Although most studies assessed the role of PLA2R antibodies in the serum of patients with MN, some PLA2R antigen was also detected in approximately 70-80% of IMN glomeruli as a finely granular staining pattern along glomerular capillary loops (Debiec, Ronco 2011, Svobodova, Honsova et al. 2013). The presence of autoantibody in serum was not always associated with antigens in glomerulus. Svobodova *et al*. found PLA2R glomerular deposits in 10 anti-PLA2R serum-negative patients (Svobodova, Honsova et al. 2013). Debiec *et al.* studied PLA2R immune deposits in renal tissue of 42 IMN patients without evidence of secondary forms (Debiec, Ronco 2011). These patients had blood and tissue samples collected prior to the immunosuppressive therapy. The sensitivity and specificity of serum anti-PLA2R in the context of PLA2R in glomeruli was 57% and 74%, respectively. Taken together, these results suggested that glomerular tissue and serum could stratify different stages of the disease. Combined

assessment of circulating anti-PLA2R antibodies and PLA2R antigen in biopsy specimens might help to better select the patients for appropriate therapy (Debiec, Ronco 2014).

Early recurrence of MN in kidney transplant recipients with circulating anti-PLA2R antibodies supports a pathogenic role for these antibodies, although some patients with high titers of anti-PLA2R antibodies at the time transplantation did not develop recurrent disease (Stahl, Hoxha et al. 2010, Blosser, Ayalon et al. 2012). PLA2R staining was almost always negative in *de novo* MN (Larsen, Walker 2013), suggesting a different mechanism in this unique form of MN. Anti-PLA2R antibodies or antigens were also found in approximately 20% of patients with Secondary MN (Hofstra, Wetzels 2014): In a cohort study by Qin *et al.*, anti-PLA2R antibodies were detected in 1 of 20 patients with membranous lupus nephritis, as well as in a minor number of patients with membranous nephritis secondary to hepatitis B or cancer (Qin, Beck et al. 2011). Svobodova *et al*. detected PLA2R in 3 patients with secondary MN (2 with hepatitis B, and 1 with sarcoidosis) but in none of the 16 patients with lupus (Svobodova, Honsova et al. 2013). Larsen *et al.* investigated 80 SMN and found positive glomeruli PLA2R in 7 Hepatitis C, 3 sarcoidosis and 3 malignancy associated SMN (Larsen, Messias et al. 2013). Anti-PLA2R autoantibodies have been found in children MN as well, but with a relatively lower prevalence (Cossey, Walker et al. 2013).

1.2.1.2.2 Additional autoantigens

Additional podocyte autoantigens including superoxide dismutase 2 (SOD2), aldose reductase and α-enolase (Prunotto, Carnevali et al. 2010, Bruschi, Carnevali et al. 2011) have also been reported as targets in some IMN patients. Unlike PLA2R, these antigens are not expressed in the normal glomerulus but can be induced and routed to the podocyte membrane

under some stress conditions. **SOD2** is a key anti-oxidant mitochondrial enzyme implicated in transformation of superoxide ions into hydrogen peroxide and diatomic oxygen. In kidney, SOD2 is widely expressed in tubular epithelial cells, especially in the cortex, where it plays a central role in preserving the kidney during ischemia/reperfusion events but it has not been reported in normal glomeruli (Son, Kojima et al. 2008). Prunotto *et al*. indicated that SOD2 is neo-expressed in podocytes and in subepithelial immune deposits of patients with IMN (Prunotto, Carnevali et al. 2010). **Aldose reductase,** usually a cytoplasmic enzyme, belongs to the family of aldo-keto reductases involved in catalysis of NADPH dependent reduction of aliphatic and aromatic aldehydes and ketones (Petrash 2004). It converts glucose into sorbitol and is involved in regulation of tissue tonicity and osmolality. Like SOD2, localization of aldose reductase within the normal kidney is limited to tubular epithelial cells of the medulla and it is absent in glomeruli (Terubayashi, Sato et al. 1989). In MN patients aldose reductase is detectable in glomeruli and it co-localizes with IgG4 (Prunotto, Carnevali et al. 2010). **α-enolase** is one of the most abundant proteins in cytosol where it participates in the glycolysis process as a catalyst of the conversion of 2- phosphoglycerate to phosphoenolpiruvate (Merkulova, Dehaupas et al. 2000). In kidney it is particularly expressed in tubular cells. Plasma membrane localization has also been described (Ueta, Nagasawa et al. 2004). Several autoimmune diseases were found to be associated with circulating anti- α-enolase antibodies (Gitlits, Toh et al. 2001). Wakui *et al.* detected circulating antibodies against α-enolase in IMN patients (Wakui, Imai et al. 1999), where interestingly the autoantibodies were IgG1 and IgG3 but not the IgG4 subclass as observed for PLA2R, SOD2 and aldose reductase. However, a recent study by Murtas *et al.* showed that IgG4 autoantibodies directed against α-enolase were detected in 43% of MN patients (Murtas, Bruschi et al. 2012). Recently, Bruschi *et al.* reported that α-enolase neo-

expressed in MN glomeruli and colocalized with IgG4, which strengthens the concept that it is another autoantigen implicated in MN pathogenesis (Bruschi, Carnevali et al. 2011). The exact roles of these additional podocyte antigens in the pathogenesis of MN remain to be established as they are intra-cytoplasm components and have not been detected on the surface of normal podocytes. It seems that these neo-antigens could be targeted by antibodies after podocyte damage has occurred. In disease conditions, antibodies to these cytoplasmic proteins could be pathogenic by perturbing important cellular functions (Ronco, Debiec 2012). Recently, synaptonemal complex protein 65(SC65) and lecithin-cholesterol acetyltransferase (LCAT) (Cavazzini, Magistroni et al. 2012, Takahashi, Hiromura et al. 2013) have also been suggested as candidate autoimmune proteins in MN. As new technologies are applied in the field of tissue micro-dissection and proteomics, more autoantigens are expected to be identified from the immune deposits.

1.2.2 Antibody isotypes in glomeruli deposits

It has been repeatedly shown that IgG4 predominates in the glomerular immune complex in IMN (Imai, Hamai et al. 1997, Ohtani, Wakui et al. 2004, Segawa, Hisano et al. 2010), however, other isotypes were also been reported frequently. Bannister *et al.* found staining for IgG4 in 100% of 10 patients with IMN, but IgG3 stained more intensely (Bannister, Howarth et al. 1983). Haas *et al.* found strongest IgG4 staining in 28 IMN patients, but IgG1 was found in 100%, IgG2 in 79% and IgG3 in 75% of the cases (Haas 1994). In 6 of them, IgG3 staining was approximately equal to IgG4. Noel *et al.* studied 16 IMN and found IgG4 in 81% and IgG1 in 75% of cases. In majority of systemic lupus MN cases, IgG4 subclass was not detected (Noel,

Aucouturier et al. 1988). Ohtani *et al.* compared subclass distribution between 15 patients with IMN and 10 with malignancy-associated MN (Ohtani, Wakui et al. 2004). The glomerular IF intensities of IgG1 and IgG2 were significantly stronger in the malignancy group than in the IMN groups. There was no difference for IgG3 and IgG4 between the two groups. In a more recent study Qu *et al.* found negative IgG4 staining in 7 of 8 malignancy-associated cases (Qu, Liu et al. 2012). The authors suggested that a negative stain for IgG4 in suspected IMN should prompt a search for underlying malignancy.

IgG4 is a unique subclass of IgG. It exists in the lowest concentration of all 4 subclasses (approximately 4% of total IgG) (Aalberse, Stapel et al. 2009). The regulation of IgG4 production requires typical Th2 cytokines (IL-4 or IL-13) for appropriate class switch (Fujita, Meyer et al. 2012). Due to relatively weak inter-chain binding with easy susceptibility to reduction, it is continuously involved in half molecular exchanges *in vivo* known as "Fab arm exchange" (van der Neut Kolfschoten, Schuurman et al. 2007). IgG4 behaves as functionally monovalent, does not cross-link and is reportedly incapable of producing large immune complexes. It does not bind C1q and cannot activate complement via the classical pathway (van der Zee, van Swieten et al. 1986). Huang et al. showed that in early histological stage (stage I), IgG1 was the dominant IgG subclass; in all later stages IgG4 dominated (Huang, Lehman et al. 2013). However, the exact pathophysiology of IgG4 isotype in MN has remained an enigma. urther research is required to investigate the differences in the glomerular deposition of IgG subclasses, especially IgG4, in cases of secondary MN and idiopathic MN and in different clinical phases.

1.2.3 Potential roles of PLA2R antigen, IgG subclasses and complement proteins in MN pathogenesis

In normal kidneys, PLA2R is synthesized by podocytes and expressed on the cell membrane (Lambeau, Lazdunski 1999). The protein comprises a long extracellular domain which consists of a cysteine-rich head and fibronectin type II like repeat domains and eight repeated carbohydrate-recognition domains. It also has short transmembrane and intracellular domains. This receptor has been reported to participate in the regulation of PLA2R biological responses involving cell proliferation, adhesion, production of lipid mediators, and the release of arachidonic acid (Hanasaki 2004). Ontogenetic studies implicate PLA2R as a multifunctional receptor; changes in the expression of which have a major impact on human cell senescence via generation of reactive oxygen species (Bernard, Vindrieux 2014). The signals for cell injury could follow the p-53 pathway, a protein that plays a central role in cellular response, including cell cycle arrest, allowing DNA damage repair or cell death induction. It has also been reported that deficiency of phospholipase A2 receptor exacerbates ovalbumin-induced lung inflammation (Tamaru, Mishina et al. 2013) and increases susceptibility to cardiac rupture after myocardial infarction (Mishina, Watanabe et al. 2014). The precise biologic function of PLA2R within the podocyte is yet to be completely elucidated.

PLA2R can present with at least two configurations: an extended conformation with the N-terminal cysteine-rich domain oriented outwards from the cell surface or a bent confirmation where the N-terminal domain folds back to interact with C-type lectin-like domains at the middle of the structure, thus affecting ligand binding and oligomerization (Ronco, Debiec 2012). The epitope of PLA2R appears to be conformational in nature and dependent on cysteine-rich

sequences. A recent epitope mapping described seven epitopes all located in the extracellular domain of PLA2R (Behnert, Fritzler et al. 2013). Genome-wide association studies have further demonstrated that single nucleotide polymorphisms in the PLA2R gene were strongly associated with the susceptibility of IMN (Stanescu, Arcos-Burgos et al. 2011, Lv, Hou et al. 2013). Stanescu *et al.* revealed significant associations of the 6p21 HLADQA1 and 2q24 PLA2R1 loci with IMN in patients of white ancestry (Stanescu, Arcos-Burgos et al. 2011). The association of these HLADQA1 and PLA2R1 alleles with IMN was also confirmed in Asia (Kim, Chin et al. 2011, Lv, Hou et al. 2013). The study by Liu *et al.* demonstrated that the rs35771982 SNPs would have a more selective expression in Chinese population with MN (Liu, Chen et al. 2010). The frequency of the G allele at rs35771982 and the G/G genotype of this SNP are even associated with a low rate of MN remission. Recently in a Spanish cohort, these HLA-DQA1 and PLA2R polymorphisms predicted IMN response to immunosuppressive agents and disease progression (Bullich, Ballarin et al. 2014). These results show a close relationship between IMN and HLA-DQA1 and PLA2R risk alleles, however, a straightforward "conformeropathy pathogenic role for PLA2R in MN have been excluded by recent studies (Coenen, Hofstra et al. 2013). Ardalan *et al.* recently demonstrated a direct correlation between anti-PLA2R and anti-sPLA2 antibodies in the sera from Iranian patients with IMN (Ardalan, Ghafari et al. 2013), which suggested that PLA2R could also mediate receptor-dependent actions of sPLA2 such as those involved in cell proliferation and inflammation.

Although the conditions that lead to exposure of PLA2R epitope on the surface of podocytes and then to autoreactivity are unknown, data from PLA2R studies suggested that, like Heymann nephritis and alloimmune neonatal MN, autoimmune IMN involves *in situ* formation

of subepithelial deposits resulting from binding of circulating anti-PLA2R autoantibodies to podocyte PLA2R. It is also unclear if anti-PLA2R antibodies initiate the disease or are preceded by some unknown initiating factors. The evidence drawn from experiments using Heymann models of MN indicated that subepithelial immune deposits provoke local complement activation, mainly C5b-9 and induce podocyte injury (Kerjaschki 2004). C5b-9 could activate various mediators, such as phospholipases, protein kinases, cyclooxygenases, transcription factors, and cytokines. These signals influence podocyte cell metabolic pathways, structure and function of key cytoskeletal proteins, expression and localization of nephrin, turnover of extracellular matrix, and DNA integrity (Nangaku, Couser 2005, Nangaku, Shankland et al. 2005, Cunningham, Quigg 2005).

Evidence for complement activation in human MN comes mainly from clinical observations. C3 renal deposits were first detected in 8 of 16 patients with primary MN (Doi, Kanatsu et al. 1984). Patients with glomerular C3 deposits showed more proteinuria than those lacking glomerular C3 deposits, suggesting the association of complement activation with disease severity in human MN. C3C, a short-lived breakdown product of C3, is the predominant form of C3 in deposits (Endo, Fuke et al. 2004). C4d is another molecule of the complement system that more recently has been related to MN. It is the breakdown product of C4 generated during activation of classic complement or lectin pathways. Recently, Val-Bernal *et al.* reported characteristic granular basement membrane deposition of C4d in 100% of IMN (31 cases) and pure class V membranous lupus nephritis (5 cases) (Val-Bernal, Garijo et al. 2011). In addition, membrane attack complex were observed in subepithelial immune deposits in 50% of patients with IMN (Nangaku, Couser 2005).

The complement cascade can be activated by classical, lectin and alternative pathways which all lead to activation of the C3 component by C3 convertases (Sarma, Ward 2011). It is conceivable that the binding of anti-PLA2R antibodies to PLA2R (or other antigen antibody binding) could activate complement. As described earlier, IgG4 has unique properties as compared to other IgG subclasses. It does not activate complement by the classical pathway and behaves mostly as a monovalent, low –affinity immunoglobulin (van der Zee, van Swieten et al. 1986). The concomitant presence of IgG1 in glomerular deposits, especially in early immune deposits, could possibly be responsible for complement activation through the classical pathway. However, most patients with MN have very low or undetectable levels of C1q (the initiating protein in the classical complement cascade) in deposits in contrast to patients with secondary forms of MN (Jennette, Hipp 1985). It suggests that the alternative or lectin pathways might be also involved in complement activation and formation of the C5b-9 complex. Indeed, mannose-binding lectin (MBL) has been identified in the glomeruli of many patients with idiopathic MN (Lhotta, Wurzner et al. 1999). Except for the complement cascade activation, it is also possible that the binding of anti-PLA2R antibodies to PLA2R on podocytes could alter receptor function resulting in podocyte dysfunction directly.

There is no doubt that the complement system has an important role in experimental and human MN. However, the exact role of complement activation in the pathogenesis of MN is not clear. Our knowledge about the association between autoantigens, autoantibodies and the complement proteins is scant. In the current study, I hypothesized that in PLA2R related MN, autoantibodies bind to PLA2R and activate complements directly or indirectly, resulting in podocyte injury and proteinuria.

Chapter Two: **Materials and methods**

2.1 Patients and controls

A database at Calgary Laboratory Service was searched for registered cases of adult MN (18 years or older) that were recorded from 2006 to 2013. Patients with biopsy proven MN were selected according availability of renal biopsy tissues and serum samples). Cases were designated as secondary MN if they had an active disease that was a known secondary etiology of MN including infections, systemic lupus erythematosus and other autoimmune diseases, malignancy, and exposure to certain toxic substances and drugs. Criteria for diagnosis of IMN were as follows: histologically proven MN; negative tests for serum autoantibodies including antinuclear antibody and ANCA, cryoglobulins and viral markers including hepatitis B surface antigen and HIV; absence of any clinical suspicion of secondary MN. Cases without a known workup for determination of secondary MN were excluded from the study.

Patients' serum samples were collected between 2011 and 2013 by Mitogen Advanced Diagnostics Laboratory, the University of Calgary. Renal biopsy paraffin blocks were prepared by the Department of Pathology and Laboratory Medicine, Foothills Medical Centre, Calgary Laboratory Service. Frozen biopsy tissue sections were prepared at the Immunohistochemistry Laboratory, Calgary Laboratory Service after biopsies were performed by a standard protocol.

As controls, renal tissues were obtained from patients with a normal morphological renal biopsy and with unrelated diseases. Normal serum samples were obtained from normal blood donors who had normal urinalysis and serology tests.

This study was approved by the Institutional Research Information Services Solution (IRISS) in the University of Calgary (Ethics ID: REB13-0292). The samples were assigned numbers to render them anonymous.

2.2 Patients' clinical and pathological characteristics

Clinical and laboratory data were reviewed to determine patients' 24h proteinuria and serum creatinine levels at the time of biopsy. Clinicians' hospital notes were reviewed as evidence for the workup of secondary MN.

Patients' biopsy data, including electron microscopy findings, hematoxylin and eosin, PAS, trichrome, and Jones methenamine silver stains were reviewed to confirm the characteristics of immune deposits and to determine the pathological stages of MN. For the ultrastructural staging of MN, Ehrenreich–Churg staging system (Ponticelli, 2007) is followed with slight modification: Stage I, sparse small subepithelial deposits without thickening of the GBM and no spike formation; Stage I-II, segmental spike formation between subepithelial deposits; Stage II, spike formation was diffuse with only a few deposits incorporated into the GBM; Stage II-III, some deposits were already incorporated into the thickened GBM (intramembranous deposits), but there were still numerous subepithelial deposits with spike formation; Stage III, mostly intramembranous immune complex deposits with only partial reabsorption; Stage IV, mostly reabsorbed immune complex deposits with widespread electron lucencies within thickened GBM; Stage III-IV, combination of stage III and stage IV.

2.3 Antibodies

Affinity purified specific rabbit anti- PLA2R antibodies were purchased from Sigma (SIGMA-Aldrich, St Louis, MO, USA). Rabbit monoclonal anti-human α-enolase antibodies were from Abcam (Abcam, Toronto, ON, Canada). Goat Alexa 488 conjugated anti-rabbit Fab IgG, goat Alexa 568 conjugated anti-rabbit Fab IgG and goat Alexa 568 conjugated anti-human Fab IgG from Molecular Probes (Life Technologies**,** Grand Island, NY, USA) were used as secondary antibodies in indirect immunofluorescence assays. Fluorescein isothyocyanate (FITC) conjugated IgG1, IgG3 and IgG4 were purchased from Sigma and were used in direct immunofluorescence assay. FITC conjugated anti-human IgG were from Immuno Concepts (Immuno Concepts, Sacramento, CA, USA). FITC conjugated anti-human C3C antibodies were purchased from Dako (Dako Denmark, Glostrup, Denmark). Phycoerythrin (PE) conjugated goat anti-human IgG was purchased from Jackson ImmunoResearch (Jackson ImmunoResearch, West Grove, PA, USA) and used in the addressable laser bead immunoassays (ALBIA) described below.

2.4 Detection of autoantigens in renal biopsy sections

2.4.1 Indirect immunofluorescence assay for PLA2R

Renal biopsy tissues were routinely fixed in B-plus fixative solution (BBC Biochemical, Seattle, WA, USA) and embedded in paraffin. 4-μm-thick sections were cut from paraffin blocks using a microtome (Leica RM 2235, Leica Mycrosystems, Heerbrog, Germany) and subsequently deparaffinised in xylene, 2x5 minutes, followed by rehydration with 100%, 95%

and 70% ethanol, and distilled water respectively. The tissue sections were pretreated with proteinase K to unmask the antigens (antigen retrieval) followed by indirect immunofluorescence to visualize the PLA2R protein. Briefly, sections were flooded with Proteinase K working solution (0.6 units /ml in TE buffer) and incubated for 30 minutes at 37℃ in a humidified chamber. After cooling to room temperature for 10 minutes, sections were rinsed in PBS-Tween (0.05% Tween 20 in PBS buffer) for 2x2 minutes. After washing, the sections were blocked by incubation in blocking buffer (PBS with 1% goat serum and 1% BSA) for one hour at room temperature. Sections were further incubated with primary antibodies (rabbit anti-PLA2R antibody at 1:500 dilutions) in PBS containing 1% BSA at room temperature for one hour in a moisture chamber. Sections were washed subsequently with PBS-Tween for 3x5 minutes. Secondary antibodies (goat Alex 488-conjugated anti-rabbit IgG antibodies at 1:500 dilutions) were added and incubated for an additional 30 minutes at room temperature in a dark humidify chamber. After rinsing in PBS-Tween for 3x5 minutes, samples were mounted in 10μl aqueous of mounting medium with anti-fading agents (Biomeda, Foster city, CA, US). Images were then observed with a Zeiss fluorescence microscope and pictured by a Zeiss camera. Each specimen was run with positive and negative (normal kidney and secondary antibody only) controls.

2.4.2 Indirect immunofluorescence assay for α-enolase

OCT (Optimal Cutting Temperature: Tissure Tek, Miles, Elkhart, IN) was the embedding medium for biopsy tissue that was snap-frozen in liquid nitrogen. These cryopreserved renal biopsy tissues were cut as 4-μm-thick sections by a cryostat (Leica 1720, Leica Mycrosystems, Heerbrog, Germany) and air dried for 20-40 minutes at room temperature prior to fixation in 3.7% paraformaldehyde in PBS at room temperature for 10 minutes followed by washing twice

in PBS-Tween. After blocking for 2 hours at room temperature in blocking buffer, sections were incubated with rabbit anti-human α-enolase antibodies (1:100 dilutions) at 4 ℃ for overnight. Sections were washed with PBS-Tween for 3x 5 minutes. Secondary antibodies (goat Alex 568-conjugated anti-rabbit IgG antibody at 1:500 dilutions) were added and incubated for an additional 30 minutes at room temperature. Sections were mounted with coverslips and images obtained by fluorescence microscopy as described above.

2.5 Detection of IgG, IgG subclasses (IgG1, IgG3, IgG4) in renal sections

2.5.1 Direct immunofluorescence assay for IgG

Paraffin sections, which were prepared for PLA2R detection, were also incubated with goat Alex 568-conjugated anti-human IgG (1:500 dilutions) for 30 minutes at room temperature as described above. Images were obtained and the co-localization with PLA2R was analysed using Adobe Photoshop.

2.5.2 Direct immunofluorescence assay for IgG subclasses.

OCT embedded frozen tissues were cut into 4-µm-thick sections and air dried for 20-40 minutes at room temperature prior fixation. After 10 minutes fixation in ice cold acetone, slides were washed by pouring PBS working solution into a coplin jar for 10minues. Non-specific binding was blocked by incubation in PBS containing 2% BSA for 20 minutes at room temperature. Sections were then incubated with FITC-conjugated mouse anti-human IgG1or IgG3 or IgG4 (1:50 dilutions) for 45 minutes at room temperature in a light tight humidity chamber. Slides were washed with PBS-Tween for 3x 5 minutes, mounted and observed using

immunofluorescence microscopy. Immunofluorescence staining intensity was semi-quantitatively scored from 0 to 3+ (0 negative, 1+ week staining, 2+ moderate staining, 3+ strong staining).

2.6 Direct immunofluorescence assay for C3C in renal sections

4-µm-thick frozen sections fixed in cold acetone were washed with PBS-Tween and incubated with 1:10 diluted, FITC-conjugated anti-human C3C antibodies for 45 minutes at room temperature. After washing with PBS-Tween and mounting, C3C staining was visualized by immunofluorescence microscopy. Immunofluorescence staining intensity was semi-quantitatively scored as described above.

2.7 Detection of circulating autoantibodies

2.7.1 ALBIA for PLA2R antibodies

2.7.1.1 Preparation of GFP-PLA2R transfected 293 cell lysates

Expression of a GFP-PLA2R construct and protein coupling were done by Dr. Fritzler's lab at the University of Calgary. A PLA2R isoform1 (Accession: Q13018) was constructed and inserted into a GFP vector (Clontech Laboratories Inc., Saint-German-en-Laye, France). HEK293 cells (American Type Culture Collection, Cedarlane, Burlington, ON, Canada) were seeded in culture plates (NuncUpCell Surface 10 cm, Thermo Fisher Scientific, Langenselbold, Germany) and incubated for one day to enhance attachment before transfection with the GFP-PLA2R construct. The HEK293 cells were then incubated for additional 48 hours, harvested from the media on ice and washed with ice cold PBS and then lysed with NETN buffer (150 mM

NaCl, 1 mM EDTA, 50 mM Tris-HCl (pH 7,4), 1% Nonidet P-40/Tergitol, protease inhibitor (Complete Mini, Roche, Indianapolis, IN, USA), phosphatase inhibitor (PhosSTOP, Roche, Indianapolis, IN, USA). Lysates were stored at −80°C overnight and then centrifuged for 15 min at 11.000 rpm at 4 ℃. The supernatant was transferred into a new tube and stored at −80°C.

2.7.1.2 Protein coupling

ALBIA beads (MicroPlex Microspheres (non-magnetic) LC10052, MiriaBio Group, San Francisco, CA, USA) were co-valently coupled to mouse anti-GFP (Abcam) , blocked with normal human serum and stored following the procedure described previously (Behnert, Fritzler et al. 2013). Briefly, 10 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and normal human serum (NHS) were dissolved in 200 μl of Activation Buffer (0.1 M NaH_2PO_4, 5 M NaOH, pH ~ 6.2). A desired volume of beads was pipetted into micro tubes (USA Scientific Inc. Ocala, FL, USA) and centrifuged at 14,000 rpm for 1 min. The supernatant was carefully decanted, the desired amount of activation buffer was added and the beads were resuspended by gentle sonication and vortexing. Diluted EDC and NHS were added and the beads were sonicated and vortexed again followed by a 20 minute-incubation in the dark at room temperature. While the beads were incubating, protein samples were diluted to the optimal concentration in Coupling Buffer (0.14 M NaCl, 0.01 $NaPO_4$, pH ~ 7.2). After incubation, beads were centrifuged at 14,000 rpm for 3 minutes and the supernatant decanted before adding coupling buffer at 2–3 times the original bead volume. The microspheres were again sonicated and vortexed before centrifugation at 14,000 rpm for another 3 minutes. The supernatant was decanted and protein was coupled to microspheres by adding the optimal amount of protein to

the microspheres, which were resuspended again as described above. The beads were then incubated overnight at 4 ℃ on a rotator and then stored at 4 ℃ in the dark until required for use.

2.7.1.3 PLA2R coupled beads preparation

A 200 µl suspension of anti-GFP coupled beads were added to 1 ml of GFP-PLA2R transfected HEK293 cell lysate and incubated on a shaker for 1 hour at room temperature. Beads were then washed twice with 500 µl Wash Buffer (PBS-T, Millipore Corp., Billerica, MA, USA) and once with 500 µl Blocking/Storage Buffer (PBS, 0.1% bovine serum albumin (BSA), 0.02% Tween-20, 0.05% azide, pH 7.4) followed by resuspending them in 200 µl Blocking/Storage Buffer. The PLA2R coupled beads were stored at 4 ℃ until required for the ALBIA.

2.7.1.4 ALBIA using patients' serum samples

2 µl of the resuspended ALBIA beads coupled with PLA2R, 30 µl of HRP sample diluent (INOVA Diagnostics Inc., San Diego, CA, USA) and 10 µl of diluted serum (1:100 in HRP sample diluent) were pipetted into the wells of microtiter plate, covered with foil and incubated on a shaker, 600 rpm, for 1 hour at room temperature. 40 µl of PE conjugated goat anti-human IgG (1:50/HRP sample diluent) was then added and the plate was incubated for an additional 30 minutes at room temperature. The reactivity of individual serum was then analyzed using a Luminex-100 plate reader (Luminex Corp., Austin, TX).

2.7.2 Indirect immunofluorescence cell based assay for anti-PLA2R

To validate the results obtained by ALBIA, MN sera were evaluated for anti-PLA2R antibodies by indirect immunofluorescence with a commercially available test kit, following the manufacturer's instruction (Euroimmun, Luebeck, Germany). Briefly, 30ul of PBS diluted serum

samples was applied to each reaction field of the BIOCHIP slide (coated with PLA2R transfected HEK 293 cells, included in the kit) and incubated for 30 minutes at room temperature. The slide was then rinsed with PBS-Tween and then immersed in a cuvette containing PBS- Tween for at least 5 minutes. 25 µl of FITC labelled anti-human IgG or anti-human IgG4 was applied to each reaction field and incubated for additional 30 minutes at room temperature. After washing in PBS-Tween for 5 minutes, anti-PLA2R total IgG titers or IgG4 titers were evaluated by immunofluorescence microscopy as described earlier. Antibody positivity was defined as positive staining at serum dilutions of 1/10 or higher. Immunofluorescence staining intensity was semi-quantitatively scored from 0 to 3 as described above.

2.7.3 ALBIA for α-enolase antibodies

The full length human α-enolase full length protein coupled ALBIA beads was prepared as described above. 2 µl of the α-enolase coupled ALBIA beads, 30 µl of HRP sample diluent and 10 µl of diluted serum (1:100 dilutions) were pipetted into the wells of microtiter plate, covered and incubated on a shaker, 600 rpm, for 1 hour at room temperature. 40 µl of 1:50 diluted PE conjugated goat anti-human IgG was then added and the plate was incubated for an additional 30 min at room temperature. The reactivity of individual serum was analyzed using a Luminex-100 plate reader.

2.8 Statistical analyses

All statistical analyses were performed with PASW (SPSS) software, version 20 (IBM, Armonk, NY). Differences were considered significant with P value <0.05.

For descriptive statistics, data were presented as mean ±SD or median (range) when appropriate. The correlations between several parameters (anti-PLA2R antibody titers, anti-α-enolase antibody titers, proteinuria and serum creatinine) were analyzed by Pearson correlation. The unpaired t test and Pearson Chi-square test were used for the comparison between groups. ROC curves were performed to compare the ALBIA to the IIF-CBA assay for anti-PLA2R antibody titer detection.

Chapter Three: **Results**

3.1 Clinical and morphological features

Membranous nephropathy (MN) is one of the most common forms of nephrotic syndrome in adults. Since 2006, about 145 cases have been diagnosed in Calgary Laboratory Services. A total of 23 patients with biopsy proven MN were recruited for this study, which had adequate paraffin and frozen renal biopsy tissues, as well as serum samples. This included 19 cases of idiopathic MN (IMN), 3 cases with secondary MN (1 systemic lupus, 1 breast cancer associated and 1 renal cell carcinoma (RCC) associated MN) and one with *de novo* MN in a renal allograft. Within the 19 IMN cases, the male/female ratio was 15:4, predominantly male. The average age was 55.5 years (range 18-82 years) at the time of diagnosis. Based on the renal biopsy data, most of the patients were classified as stage II or II-III with 3 patients in early stage (stage I) and 3 in advanced stage (stage III-IV). The *de novo* MN case was a 62-year-old male with a stage II biopsy. The systemic lupus case was a 36-year-old female with stage I MN. The breast cancer associated MN patient was a 73-year-old female classified as stage III-IV and the RCC associated case was a 59-year-old male in stage II. All MN patients had proteinuria at the time of biopsy and had serum creatinine levels that ranged from 60 mg/dl to 323 mg/dl. The MN patients' clinical and morphological details are shown in Table 1.

3.1.1 PLA2R

3.1.2 PLA2R autoantigen in glomeruli

The important role of PLA2R in the pathogenesis of IMN has been reported by several groups (Beck, Bonegio et al. 2009, Hofstra, Beck et al. 2011). Here, PLA2R expression was

Table 1 Clinical and morphological characteristics of MN patients enrolled in this study

	IMN (n=19)	SMN (n=3)	*De novo* (n=1)
Gender (m:f)	**14:5**	**2:1**	**1:0**
Age (mean ±SD)	**55.5 ±15.4**	**56.0 ±18.7**	**62**
Serum creatinine, mg/dl (mean ±SD)	**138.1 ±97.4**	**121.7 ±68.6**	**166**
Proteinuria, g/24h (mean ±SD)	**6.35 ±4.7**	**6.2 ±3.4**	**2.6**
Morphological Stages			
I	**3**	**1**	**1**
II, II-III	**13**	**1**	
III-IV	**3**	**1**	

investigated by immunofluorescence microscopy with rabbit affinity purified specific anti-PLA2R antibodies followed by goat Alexa 488 conjugated anti-rabbit IgG on paraffin-embedded biopsy samples from each of the MN patients. PLA2R showed very weak staining in normal kidney biopsies (Figure 1A), but in 9 of the 19 IMN patients (47%), PLA2R was detected as a finely granular pattern along glomerular capillary loops (Figure 1 B). In 2 IMN cases PLA2R staining was consistently detected in repeat biopsies. In the remaining 10 (53%) IMN patients and the systemic lupus patient, there was no detectable glomerular PLA2R staining as shown in figure 1 C and D. However, the two cases with malignancy associated secondary MN also demonstrated PLA2R staining (Figure 1 E, F). Consistent with a previous report, the *de novo* MN case showed negative PLA2R staining (Figure 1 G). The staining results were summarized in Table 2.

3.1.3 Anti-PLA2R antibodies in circulation

The diagnostic value of circulating anti-PLA2R antibodies has been reported by several groups (Hu, Wang et al. 2014, Hu, Wang et al. 2014). To analyze the anti-PLA2R antibodies in our patient groups, circulating antibodies against PLA2R were first evaluated by the commercially available cell based indirect immunofluorescence test (IIF-CBA) using FITC conjugated anti-human IgG or IgG4 as secondary antibodies (Figure 2). 6 of 23 sera (26%) from the MN patients showed positive IgG results; 5 were IMN cases and one was RCC associated with MN (Table 3). 5 of the 6 anti-PLA2R positive MN patients also showed positive PLA2R glomerular staining. Interestingly, one IMN patient who had negative PLA2R glomerular deposits staining also showed positive circulating IgG anti-PLA2R antibodies, but circulating IgG4 anti-PLA2R was not detected. By comparison, the RCC patient had positive IgG and IgG4

Figure 1 Expression of PLA2R in glomeruli

Immunofluorescence microscopic analysis of paraffin kidney biopsy specimens show: a very weak expression of PLA2R in normal kidney (A); the granular PLA2R staining along glomerular capillary loops in patients with IMN (B); PLA2R is absence in some IMN cases(C), lupus (D) and *de novo* MN(E); PLA2R positive staining in patients with malignancy associated MN (F and G).

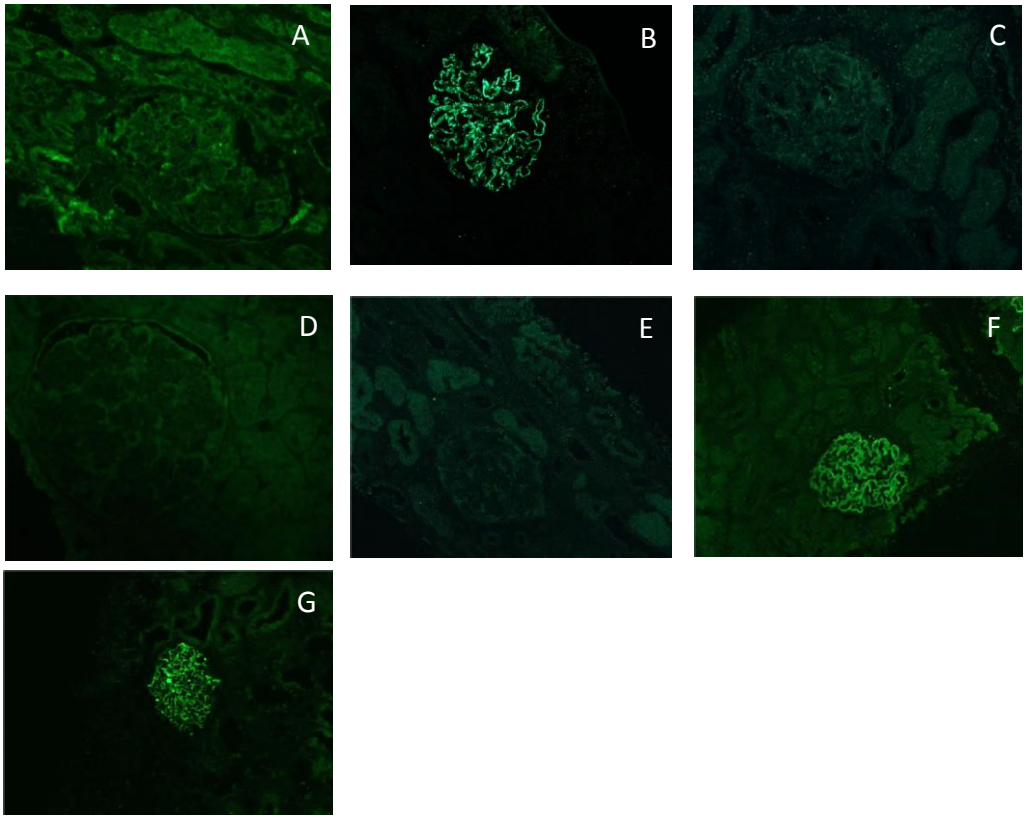
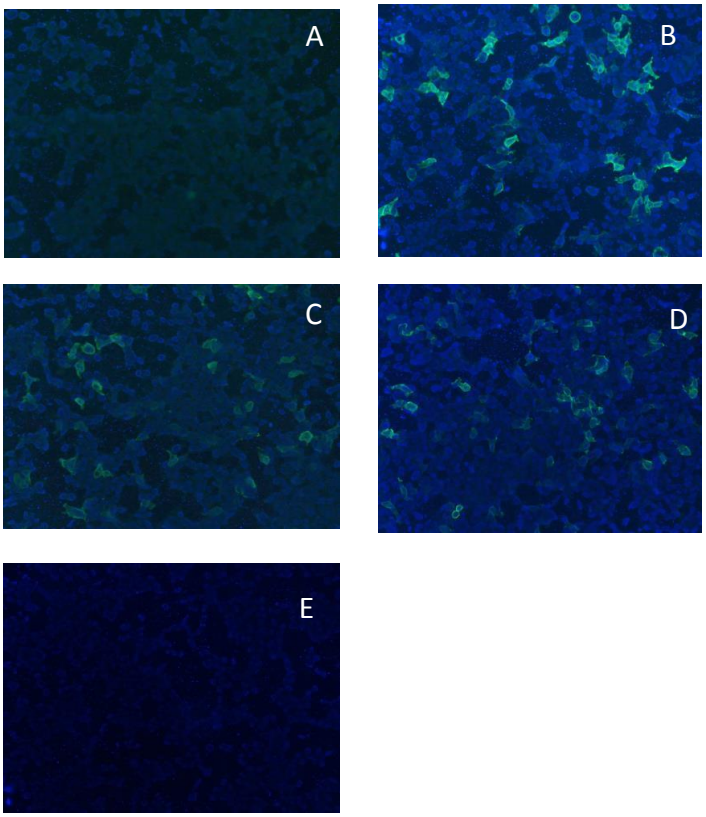


Table 2 Summary of PLA2R expression in glomeruli

	IMN (n=19)	**SMN (n=3)**	***De novo* (n=1)**
PLA2R +	9	1	0
PLA2R −	10	2	1

Figure 2 Anti-PLA2R antibody status detected by IIF-CBA

Circulating anti-PLA2R autoantibodies were evaluated by IIF-CBA using FITC conjugated anti-human IgG or IgG4 as secondary antibodies: IMN with negative anti-PLA2R titer detected by FITC-IgG/IgG4 (A); IMN with positive anti-PLA2R titer detected by FITC-IgG (B); IMN with positive anti-PLA2R titer detected by FITC-IgG4 (C); SMN with positive anti-PLA2R titer detected by FITC-IgG4 (D); Negative anti-PLA2R titer detected by FITC-IgG/IgG4 in *de novo* MN (E). Images are overlapped with Dapi staining



anti-PLA2R autoantibodies. 6 patients were negative for circulating IgG and/or IgG4 anti-PLA2R antibodies even though PLA2R antigen was detected in their renal biopsies (Table 3). In accord with other publications (Beck, Bonegio et al. 2009, Larsen, Walker 2013), both the systemic lupus and the *de novo* MN patients did not have IgG or IgG4 circulating antibodies directed against PLA2R.

Recently, a new addressable laser bead immunoassay (ALBIA) has been developed in Dr. Fritzler's laboratory at the University of Calgary to detect and quantitate circulating anti-PLA2R antibodies (Behnert, Fritzler et al. 2013). In this assay, the full-length human PLA2R construct was cloned into a GFP vector and overexpressed in HEK 293 cells. The expressed protein was indirectly coupled to ALBIA beads and tested with sera from the 23 MN patients. The anti-PLA2R antibody titer was quantitated using a Luminex-100 plate reader (Luminex Corp, Austin, TX). The results showed that the ALBIA had results that were consistent with the commercial IIF-CBA assay: samples with a high titre on IIF-CBA had a high median fluorescent index value (MFI) on the ALBIA assay. ROC analysis was performed to compare the sensitivity and specificity of ALBIA to the IIF-CBA assay (Figure 3). The ALBIA curve covered all the positive cases with the cut off value (450 MFI) calculated from the analysis. The assay classified patients with a sensitivity of 100% and specificity when the IIF-CBA was used as the reference 'gold' standard to define the outcome.

3.2 α-enolase

It has been suggested that more than one podocyte protein may be target autoantigens in the pathogenesis of human MN. α-enolase, as expressed in tubular kidney cells, is one of the most abundant cytosolic proteins. In keeping with these observations, it has been reported that

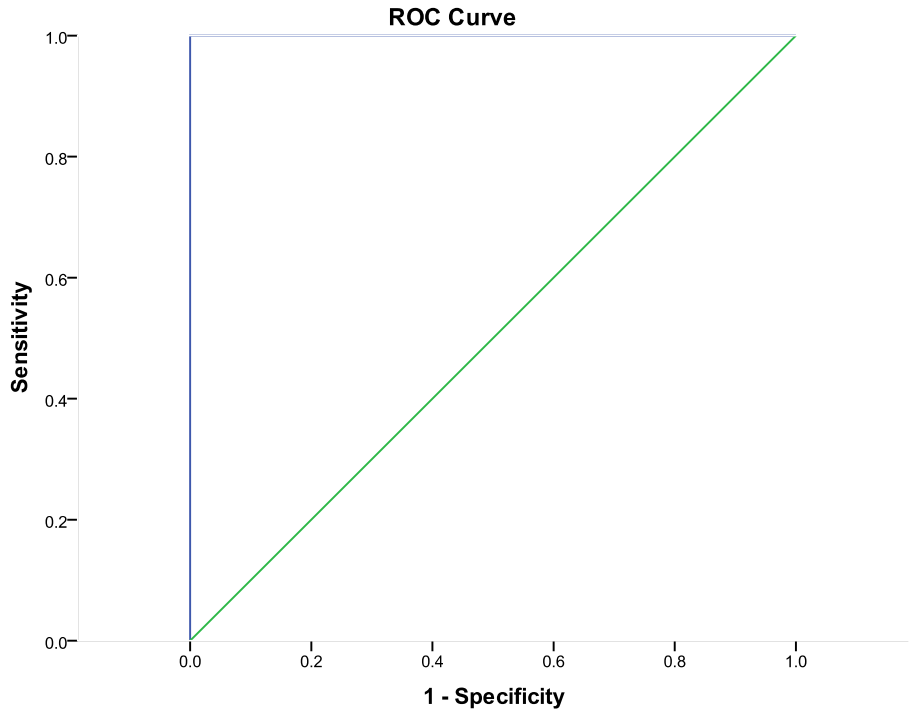
Table 3 Summary of anti-PLA2R antibody status in circulation

	IMN(n=19)		SMN (n=3)	*De novo* (n=1)
	PLA2R+	/PLA2R -		
CBA-IgG				
Anti-PLA2R (+)	4	1	1	0
CBA-IgG4				
Anti-PLA2R (+)	4	0	1	0
ALBIA				
Reading ≥450MFI	4	1	1	0

Figure 3 ALBIA-IgG.

ALBIA readings were analyzed according to antibody titers determined on IIF-CBA (A). Cut off value calculated from the analysis (B).

A



B

Positive if Greater Than or Equal To[a]	Sensitivity	1 - Specificity
29.0000	1.000	1.000
31.5000	1.000	.941
37.2500	1.000	.882
44.0000	1.000	.824
47.2500	1.000	.765
57.2500	1.000	.706
74.0000	1.000	.647
86.7500	1.000	.588
102.7500	1.000	.529
114.0000	1.000	.471
123.7500	1.000	.412
138.0000	1.000	.353
145.2500	1.000	.294
161.2500	1.000	.235
182.5000	1.000	.176
196.7500	1.000	.118
310.7500	1.000	.059
450.0000	1.000	.000
544.0000	.833	.000
1961.0000	.667	.000
3790.5000	.500	.000
4600.0000	.333	.000
5198.5000	.167	.000
5463.0000	.000	.000

MN was associated with circulating anti- α-enolase antibodies and there was neo-expression of α-enolase in glomeruli of MN patients (Prunotto, Carnevali et al. 2010, Bruschi, Carnevali et al. 2011), indicating that indeed α-enolase is another possible autoantigen implicated in the pathogenesis of MN. The present study investigated the expression of α-enolase in glomeruli and its cognate autoantibody titers in sera.

3.2.1 α-enolase in renal sections

a-enolase expression was assessed by immunofluorescence microscopy in frozen sections with rabbit anti-human α-enolase antibodies followed by goat Alexa 568 conjugated anti-rabbit IgG. Only 2 of the 23 cases showed detectable positive glomerular staining with a finely granular pattern that decorated the glomerular capillary loops (Figure 4 A): both are PLA2R antigen positive cases. The remaining 21cases present had either staining localized to renal tubules but negative staining of glomeruli (Figure 4 B).

3.2.2 α-enolase autoantibodies in circulation

To detect the anti- α-enolase antibodies in circulation, ALBIA beads were first covalently coupled to the full-length human α-enolase protein and then incubated with MN or normal sera. The resulting MFI indicated that anti- α-enolase titers were higher in MN cases (mean=345.3) than in normal controls (mean=73.3) (P=0.001) (table 4). 3 of the 19 IMN sera showed very high (>500) MFI including 2 glomeruli α-enolase positive cases and one negative case (Table 5). Unexpectedly, the *de novo* MN also had high anti- α-enolase antibody levels. The anti- α-enolase antibody titers were not associated with serum anti-PLA2R antibodies.

Figure 4 α-enolase in renal sections

a-enolase expression was assessed by immunofluorescence microscopy in frozen sections with rabbit anti-human α-enolase antibodies followed by goat Alexa 568 conjugated anti-rabbit IgG. Positive α-enolase staining (A); Negative α-enolase staining (B) and normal control (C)

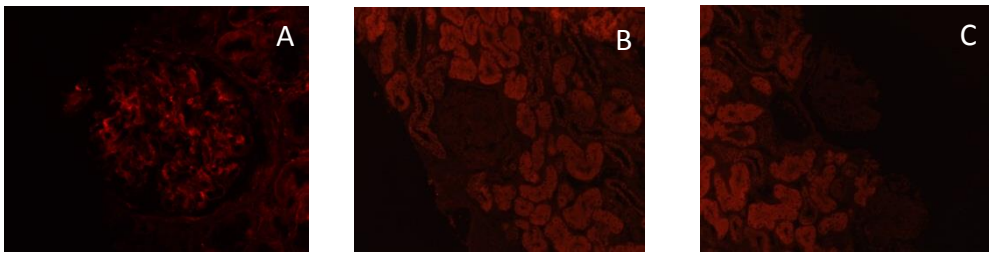


Table 4 Comparing anti-α-enolase autoantibodies in MN patients' sera with normal sera

	MN (n=23)	**Normal Sera (n=25)**	**P**
mean ±SD(MFI)	345.3 ±391.3	73.3 ±152.3	0.001

Table 5 Summary of anti-α-enolase autoantibodies in circulation

	IMN		**SMN**	***De novo***
	aPLA2R+	**aPLA2R-**	**aPLA2R+**	**aPLA2R-**
ALBIA Reading (≥500 MFI)	729 481	1669	1302	507

3.3 Evaluation of IgG and complement proteins in renal sections

3.3.1 IgG and subclasses

Although a number of studies have focussed on IgG1 and IgG3 isotopes, recent breakthrough findings revealed that IgG4 was the most common isotype for both PLA2R and α-enolase antibodies (Murtas, Bruschi et al. 2012, Huang, Lehman et al. 2013). To evaluate the IgG and subclasses distribution in the MN patients, direct immunofluorescence assays were performed with paraffin embedded sections (for IgG) or frozen sections (for IgG1, IgG3 and IgG4). Immunofluorescence staining intensity was semi-quantitatively scored from 0 to 3. All MN cases had IgG positive staining with 20 strong (2-3) and 3 faint staining (0-1) in paraffin sections. The 3 cases with faint IgG staining were also PLA2R and α-enolase antigen, antibody negative cases. The merged images of the PLA2R and IgG staining indicated that PLA2R colocalized with IgG in glomeruli deposits (Figure 5).

IgG subclasses evaluation showed that 12/19 (63%) IMN cases were positive for IgG4 staining, 10/19 (53%) with IgG1 and 11/19 (58%) with IgG3 staining (Table 6). 10/19 (53%) IMN were IgG4 predominant or codominant, 5/19 (23%) with IgG1 predominant or codominant and 3/19 (16%) with IgG3 codominant (Figure 6). It was unexpected that all 3 SMN cases also showed strong IgG4 reactivity. The staining for all the 3 subclasses was negative in *de novo* case.

3.3.2 Complement protein C3

Previous studies have suggested that subepithelial immune complex-induced complement activation plays an important role in the pathogenesis of MN (Ma, Sandor et al. 2013). C3C is a

Figure 5 PLA2R and IgG colocalized in glomerulus

PLA2R detected by rabbit affinity purified specific anti-PLA2R antibodies followed by goat Alexa 488 conjugated anti-rabbit IgG (A); IgG detected by goat Alexa 568 conjugated anti-human IgG (B); merged image showing colocalization (C).

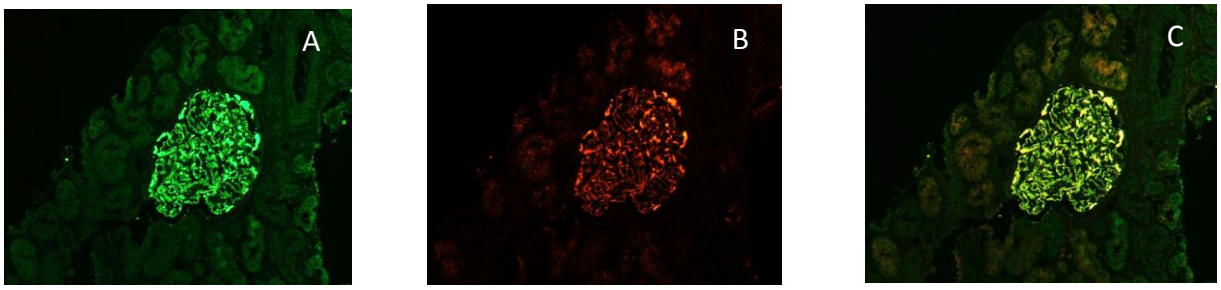
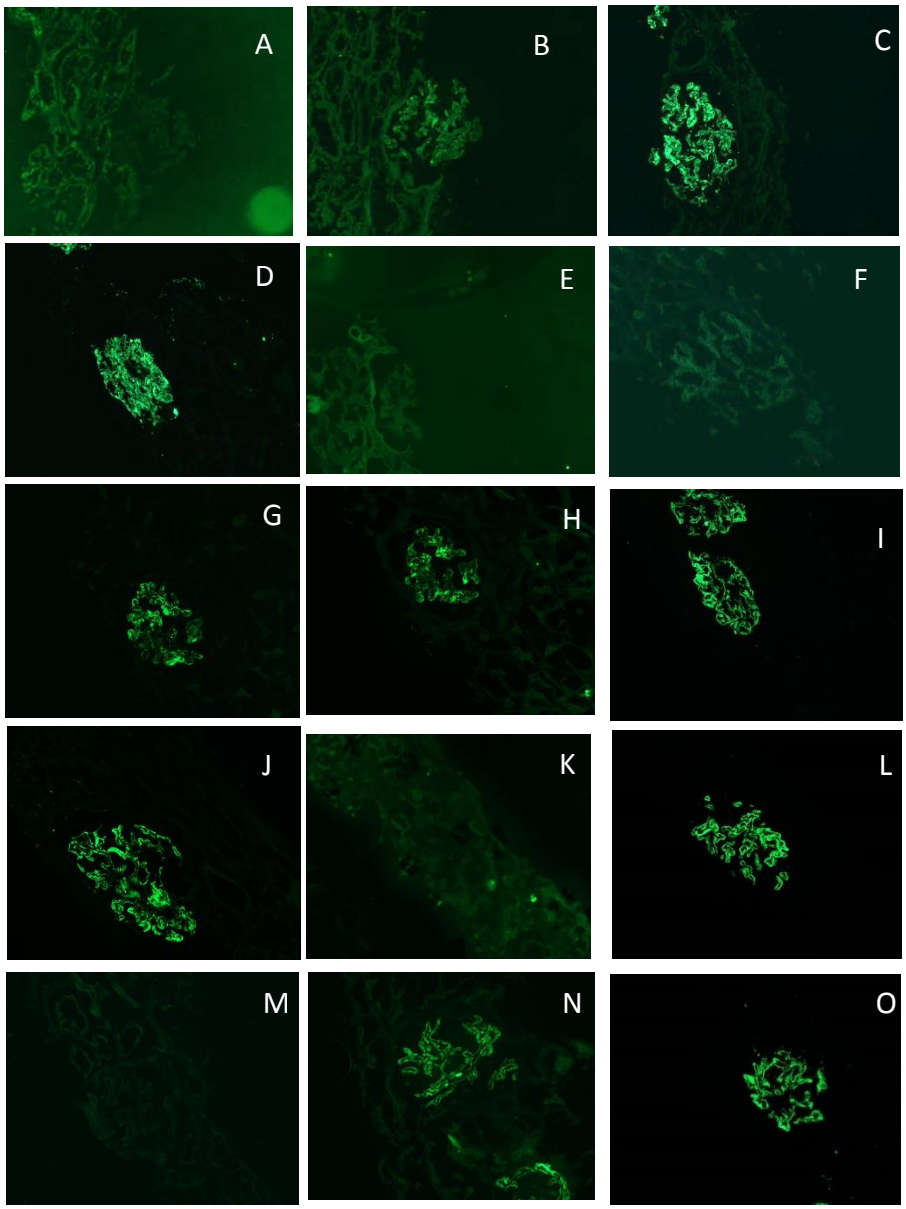


Table 6 Summary of the distribution of IgG subclasses in MN

	IMN (n=19)	**SMN (n=3)**	***De novo*** **(n=1)**
IgG1+ IgG1predominant IgG1+IgG4 codominant	10 3 2	2 1	0
IgG3+ IgG3+IgG4 codominant	11 3	0	0
IgG4+ IgG4 predominant IgG1,3,4 codominant	12 3 2	3 2	0

Figure 6 Distribution of IgG subclasses in glomeruli

A-C: IgG4 predominant case (A:IgG1, B: IgG3, C: IgG4); D-F: IgG1 dominant case (D:IgG1, E: IgG3, F: IgG4); G-I: IgG1, 3, 4 codominant case (G:IgG1, H: IgG3, I: IgG4); J-L: IgG1, 4 codominant case (J:IgG1, K: IgG3, L: IgG4); M-O: IgG 3, 4 codominant case (M:IgG1, N: IgG3, O: IgG4)



short lived breakdown product of C3 and could be a marker of ongoing immune deposit formation. C3C expression was detected in MN renal biopsy frozen sections by direct immunofluorescence assay using FITC-conjugated anti-human C3C antibodies. Immunofluorescence staining intensity was semi-quantitatively scored from 0 to 3. 10/19 (53%) IMN had strong, finely granular C3C staining along the glomerular capillary wall (Table 7, Figure 7 A). 4/19 (21%) IMN showed very faint C3C in glomeruli and the remaining 5/19 (26%) cases were negative for C3C staining (Figure 7 B, C). Interestingly, the 2 malignancy associated MN also had strong C3C staining (Figure 7 D, E) but the systemic lupus case only showed faint staining (Figure 7 F).

3.4 Statistical analysis

3.4.1 Glomeruli PLA2R deposits with baseline characteristics

The 19 IMN patients were divided into 2 groups according to their PLA2R staining status in glomeruli (9 PLA2R positive, 10 PLA2R negative). The baseline characteristics including age, gender ratio, clinical parameters and morphological stages were compared between the two groups (Table 8). Consistent with previous reports, there was no significant difference in relevant clinical parameters including proteinuria, serum creatinine, age and gender ratio ($P>0.05$). There was a trend that the PLA2R positive group had a higher 24h proteinuria, but this did not achieve statistical significance ($P=0.068$). The pathological stages were rather uniformly distributed within the two groups with no significant difference ($P >0.05$).

Figure 7 Assessment of C3C expression in glomeruli

C3C expression in MN renal sections was detected by direct immunofluorescence assay using FITC-conjugated anti-human C3C antibodies. Immunofluorescence staining intensity was semi-quantitatively scored from 0 to 3. IMN case presented strong (2-3) (A), faint (1) (B) or negative (0) (C) C3C staining. For SMN cases, The 2 malignancy associated MN showed strong C3C staining (D, E), and the lupus case only showed faint staining (F).

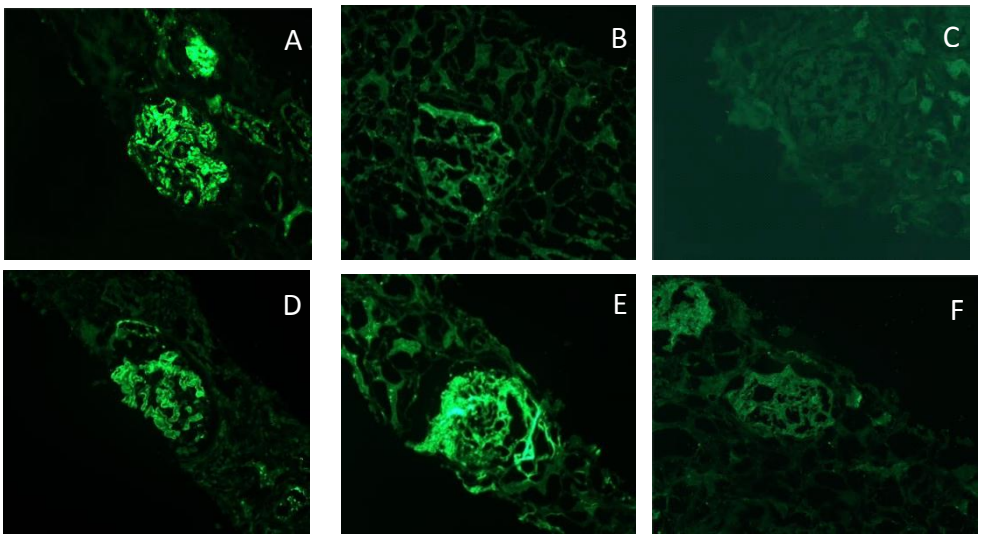


Table 7 Summary of C3C expression in glomeruli

	IMN (n=19)	**SMN (N=3)**	***De novo* (n=1)**
C3C (2-3)	**10**	**2**	**0**
C3C (1)	**4**	**1**	**0**
C3C (0)	**5**	**0**	**1**

Table 8 Patients' baseline characteristics by PLA2R status in glomerular deposits

	PLA2R + **(n=9)**	**PLA2R −** **(n=10)**	**P**
Age (mean ±SD)	53.1±16.2	57.6±15.2	0.572
Gender (m:f)	6:3	8:2	0.510
Pathological stage			
(I, I-II)	1	1	0.352
(II, II-III)	7	6	
(III, III-IV)	1	3	
Proteinuria (mean ±SD) g/24h	6.4±3.0	6.3±6.0	0.068
Serum creatinine (mean ±SD) mg/dl	137.8±120.6	138.3±77.7	0.252

3.4.2 PLA2R with IgG subclasses and C3C in glomeruli

Comparing PLA2R-positive versus negative IMN patients (Table 9), IgG4 was dominant in the PLA2R positive IMNs (8/9), but not in the PLA2R negative MNs (4/10) (P=0.027). IgG1 was also more frequent in PLA2R-positive group (P=0.037). Interestingly, the C3C staining showed a significant difference between the two groups: the PLA2R-positive group displayed stronger C3C staining than negative groups as detected in cryopreserved renal sections (P=0.037). IgG3 did not significantly differ between the two groups (P>0.05).

3.4.3 C3C with IgG subclasses, proteinuria and creatinine

Next, the IMN patients were grouped according to their C3C staining status in glomeruli and analysed its correlation with IgG subclasses as well as 24h proteinuria and creatinine levels (Table 10). Although not reaching statistical significance, the presence of C3C tended to be correlated with IgG1 expression (P=0.089), but not with IgG4 or IgG3. 24h proteinuria and creatinine levels did not significantly differ between the C3C positive and negative groups (P>0.05).

3.4.4 Circulating anti-PLA2R titers with IgG subclasses and C3C

19 IMN patients were also grouped according to their anti-PLA2R circulating antibody levels (aPLA2R-positive and -negative). The IgG subclasses and C3C expression were compared between the two groups (Table 11). Only IgG4 had more frequent expression in

Table 9 IgG subclasses and C3C by PLA2R status in glomerular deposits

	PLA2R positive (n=9)	PLA2R negative (n=10)	*p*
IgG4	1	6	0.027
+	8	4	
IgG1	2	7	0.037
+	7	3	
IgG3	3	6	0.096
+	6	4	
C3 staining			
Low (0-1)	2	7	0.037
High (2-3)	7	3	

Table 10 IgG subclasses, proteinuria and serum creatinine with C3C status in glomeruli

	C3C+ (n=14)	C3C- (n=5)	P
IgG1 +	9	5	0.089
−	1	4	
IgG3 +	8	6	0.510
−	2	3	
IgG4 +	10	4	0.637
−	3	2	
Proteinuria (mean ± SD) g/24h	6.6 ±4.2	5.5 ± 6.2	0.472
Creatinine (mean ± SD) mg/dl	148.3 ± 109.4	109.2 ± 49.0	0.117

Table 11 IgG4 subclasses and C3C by anti-PLA2R antibody titer status in circulation

	aPLA2R + (n=5)	aPLA2R − (n=14)	p
IgG1 +	3	6	0.510
−	2	8	
IgG3 +	1	7	0.243
−	4	7	
IgG4 +	5	7	0.047
−	0	7	
C3C staining			
Low (0-1)	2	7	0.701
High (2-3)	3	7	

aPLA2R-positive patients than -negative patients (P=0.047). There was no significant difference for IgG1, IgG3 and C3C between the two groups (P>0.05).

3.4.5 ALBIA measured anti- PLA2R antibody or anti-α-enolase antibody titers with proteinuria and serum creatinine

Pearson correlation analysis was performed to investigate the possible correlations within anti-α-enolase antibody titers and anti- PLA2R antibody titers as measured by ALBIA, proteinuria and serum creatinine. No significant correlations were identified (P>0.05) (Table 12)

3.4.6 IMN and SMN

The difference of baseline characteristics, IgG subclasses, PLA2R antibody, antigen, α-enolase and C3C staining between IMN and SMN were also analysed (Table 13). No significant difference was identified. However, due to the small sample size of SMN (n=3), clear-cut conclusions could not be drawn from the analysis.

3.4.7 PLA2R, α-enolase with clinical outcomes

A few studies have addressed the relevance of autoantigen and antiantibodies levels and clinical outcomes. The current study first analysed the correlations of circulating anti- PLA2R antibody titers or anti-α-enolase antibody titers identified by ALBIA with proteinuria after 1 year follow up of 18 IMN and 3 SMN patients (one IMN data was missing). No significant correlations were identified (Table 14). However, when comparing the follow up proteinuria levels between aPLA2R antibody -positive versus –negative patients identified by IIF-CBA, there was a statistical significance: the circulating anti-PLA2R positive group had higher

Table 12 Correlation analysis of anti-PLA2R antibody (apla2r), anti-α-enolase antibody (aENO) titers measured by ALBIA with proteinuria and serum creatinine in IMN patients

Correlations

		apla2r	aENO	proteinuria	creatinine
apla2r	Pearson Correlation	1	.194	.025	-.193
	Sig. (2-tailed)		.427	.919	.429
	N	19	19	19	19
aENO	Pearson Correlation	.194	1	.117	.118
	Sig. (2-tailed)	.427		.632	.632
	N	19	19	19	19
proteinuria	Pearson Correlation	.025	.117	1	.202
	Sig. (2-tailed)	.919	.632		.407
	N	19	19	19	19
creatinine	Pearson Correlation	-.193	.118	.202	1
	Sig. (2-tailed)	.429	.632	.407	
	N	19	19	19	19

Table 13 Comparing of baseline characteristics, IgG subclasses, PLA2R antibody, antigen, and C3C staining between IMN and SMN

	IMN (n=19)	SMN (n=3)	P
Age (mean ±SD)	55.5±15.4	56.0±18.7	0.821
Gender (m:f)	14:5	1:2	0.163
Pathological stage			0.490
Low (I,I- II)	2	1	
Med (II, II-III)	12	1	
High (III, III-IV)	5	1	
Proteinuria (mean) (mean ± SD) g/24h	6.4±4.6	6.2±3.4	0.675
Serum creatinine (mean ± SD) mg/dl	138.1±97.4	121.7±68.6	0.582
IgG1 −	4	2	0.099
+	15	1	
IgG3 −	9	3	0.143
+	10	0	
IgG4 −	6	0	0.254
+	13	3	
C3 staining			0.650
Low (0-1)	9	1	
High (2-3)	10	2	
PLA2R −	10	1	0.534
+	9	2	
Anti-α-enolase	323.9±360.0	571.0±633.8	0.128

Table 14 Correlations of circulating anti- PLA2R antibody titers (apla2r) or anti-α-enolase antibody titers (αENO) identified by ALBIA with proteinuria after 1 year follow-up (ProteinuriaFU)

Correlations

		aENO	apla2r	ProteinuriaFU
aENO	Pearson Correlation	1	.070	.174
	Sig. (2-tailed)		.757	.452
	N	22	22	21
apla2r	Pearson Correlation	.070	1	.327
	Sig. (2-tailed)	.757		.148
	N	22	22	21
ProteinuriaFU	Pearson Correlation	.174	.327	1
	Sig. (2-tailed)	.452	.148	
	N	21	21	21

proteinuria after one year follow up ($P<0.01$) (Table 15). This did not reach statistical significance when analysis the correlation with glomeruli PLA2R status ($P=0.08$) (Table 16).

Table 15 Comparison of proteinuria after 1 year follow-up with anti-PLA2R antibody status in circulation

	aPLA2R − IIF-CBA (n=15)	aPLA2R + IIF-CBA (n=6)	P
Proteinuria FU g/24h (mean ± SD)	1.1 ±1.3	3.9±2.9	0.005

Table 16 Comparison of proteinuria after 1 year follow-up (Proteinuria FU) with PLA2R status in glomeruli

	PLA2R − (n=10)	PLA2R + (n=11)	P
Proteinuria FU g/24h (mean ± SD)	1.5 ±1.7	2.3±2.6	0.08

Chapter Four: **Discussion**

4.1 Diagnostic value of PLA2R and α-enolase in MN

Since the discovery of anti-PLA2R autoantibodies in 2009 (Beck, Bonegio et al. 2009), a number of studies confirmed that autoantibodies directed against PLA2R were present in approximately 57%-89% of IMN patients (Qin, Beck et al. 2011, Debiec, Ronco 2011, Hoxha, Harendza et al. 2011, Behnert, Fritzler et al. 2013). To date, four techniques have been reported to detect PLA2R antibodies in serum: western blot, IIF-CBA, ALBIA and ELISA. IIF-CBA and ELISA are commercially available (Hoxha, Harendza et al. 2011, Dahnrich, Komorowski et al. 2013), while western blot and ALBIA are employed in specialized laboratories (Behnert, Fritzler et al. 2013). In this study, anti-PLA2R antibody titers were initially detected in MN patients' sera by a commercially available IIF-CBA using both IgG and IgG4 as secondary antibodies. The results showed a high correlation between total IgG and IgG4, although subtle differences existed. This result suggested that IgG4 is the major IgG isotype for anti-PLA2R antibody in sera. Other isotypes may also be present occasionally.

It has been reported that serum anti-PLA2R antibody levels are correlated with the clinical status (Hofstra, Beck et al. 2011, Hoxha, Thiele et al. 2014). For instance, meta-analysis of diagnostic test studies have demonstrated that serum anti- PLA2R level is of diagnostic value for IMN in the active stage only (Hu, Wang et al. 2014). Because of the limitations of access to biomaterials, I could not obtain the serum samples at the time of diagnosis to detect the baseline anti-PLA2R titers for all MN cases. Serum samples were collected from different clinical phases.

The relatively lower anti-PLA2R positivity in this study might be due to the inactive status of some cases after treatment or spontaneous remission.

Recently, Behnert *et al.* compared three different immunoassays: IIF-CBA, ELISA and ALBIA for the diagnosis of IMN (Behnert, Schiffer et al. 2014). The authors concluded that ALBIA represents a promising assay for the detection of anti-PLA2R antibodies by showing similar performance to the IIF-CBA. In this study, anti-PLA2R antibody titers were quantitated using the new developed ALBIA assay. This assay yielded consistent results with the IIF-CBA assay, suggested that ALBIA is reliable in detecting circulating anti-PLA2R antibodies with the advantage of ease of use and suitability for high throughput diagnostic testing. Quantification of anti-PLA2R antibodies using ALBIA assay will probably become an invaluable tool for monitoring of disease immunological activity and guiding of immunosuppressive treatments in the near future.

Although most studies have evaluated the role of PLA2R antibodies in the serum of patients with MN, some recent studies have pointed to the value of PLA2R staining in kidney biopsies. For example, Svobodova *et al.* indicated that in cases of delayed serum sampling, assessment of PLA2R antigen in renal biopsy specimens is more sensitive than the serological test for the diagnosis of PLA2R-related MN (Svobodova, Honsova et al. 2013). This could be due to the depletion of serum autoantibodies owing to therapeutic interventions, their deposition in the kidney or by periodic decreased expression of the B cell autoimmune responses, as observed in other autoimmune diseases.

In the current study, PLA2R was assessed in renal biopsies utilizing a commercially available antibody and standard indirect immunofluorescence assay. PLA2R was detected in approximately 50% IMN kidney biopsies. 5/14 anti-PLA2R serum-negative patients also showed positive PLA2R in glomeruli. These observations are consistent with previous findings that the presence of autoantibodies in sera is not always consistent with antigens as detected in glomeruli (Debiec, Ronco 2011, Svobodova, Honsova et al. 2013). The use of glomerular tissue biopsies and serum might serve as platforms to stratify different stages of the disease. The consistently detected PLA2R staining in repeat biopsies suggested that immunostaining of archived kidney biopsy specimens can be utilized for the retrospective diagnosis of PLA2R-related MN. The availability of these specimens will also enable the establishment of more meaningful disease classification and perhaps more effective therapeutic interventions in the future.

PLA2R antigen or autoantibodies were not detected in SMN in an earlier study (Beck, Bonegio et al. 2009), however, subsequent studies demonstrated weakly positive results in lupus MN (Kanigicherla, Gummadova et al. 2013, Qin, Beck et al. 2011) and in approximately 20% of MN patients with hepatitis B virus, sarcoidosis or malignancy (Oh, Yang et al. 2013, Kanigicherla, Gummadova et al. 2013, Svobodova, Honsova et al. 2013, Larsen, Walker 2013). The systemic lupus case in our study did not have circulating anti-PLA2R and there was no detectable glomerular PLA2R, a finding that is consistent with previous reports (Gunnarsson, Schlumberger et al. 2012, Svobodova, Honsova et al. 2013). Conversely, the renal cell carcinoma- associated MN had both circulating anti-PLA2R autoantibodies and PLA2R detected in glomeruli. In addition, the breast cancer-associated MN did not have elevated levels of circulating anti-PLA2R but positive glomeruli PLA2R. Although it is possible that IMN and

SMN can be concurrent conditions, a secondary cause cannot be fully excluded in all IMN PLA2R positive patients at the moment.

In the current study, an additional autoantigen involved in IMN, α-enolase, was also investigated in renal sections and its cognate autoantibodies in circulation. The serum anti-α-enolase levels measured by ALBIA were significantly higher than those in normal controls ($P<0.01$). In contrast, the detectable α-enolase antigen in glomeruli was rare. Murtas C *et al*. measured anti-α-enolase and anti-PLA2R antibodies levels using western blot and found that the two antibodies were correlated (Murtas, Bruschi et al. 2012). However, an association between these two in circulation measured by ALBIA was not detected in this study, although α-enolase antigen identified by indirect immunofluorescence coexisted with PLA2R in 2 MN glomeruli. This data indicated that circulating anti-α-enolase antibodies might not be specific for MN. In fact, several autoimmune diseases have been reported to be associated with circulating anti-α-enolase antibodies (Gitlits, Toh et al. 2001). Thus, there is no obvious diagnostic value of circulating anti-α-enolase antibodies as a diagnostic biomarker for MN. However, the coexistence of antigens in IMN suggests a complex pathogenic pathway that might involve different podocyte targets.

4.2 Clinical correlations

Recent studies have indicated that levels of circulating anti-PLA2R are related with proteinuria and clinical outcomes (Hofstra, Beck et al. 2011, Hofstra, Debiec et al. 2012, Kanigicherla, Gummadova et al. 2013). Kanigicherla *et al.* measured the anti-PLA2R by ELISA and found that high levels of PLA2R antibodies are linked with active diseases and a higher risk

of declining renal function during follow-up (Kanigicherla, Gummadova et al. 2013). Further, as reported by Hoxha *et al.* (Hoxha, Thiele et al. 2014), the PLA2R antibody level was associated with the time to remission. Hofstra *et al.* also showed that patients with high titers of antibodies were less likely to acheive spontaneous remission (Hofstra, Debiec et al. 2012), and PLA2R antibodies measured at the end of therapy predicted long-term outcome. In the present study, the relationships of both PLA2R antigen in glomeruli and anti-PLA2R antibody titers with proteinuria and serum creatinine at the time of biopsy were analysed. The glomerular PLA2R autoantibody positive group tended to have higher proteinuria levels, but this did not reach statistical significance (P=0.068). There was no association between either anti-PLA2R antibody titers or anti-α-enolase titers with proteinuria and/or serum creatinine at the time of diagnosis. However, proteinuria after one year follow-up was significantly different in the context of anti-PLA2R positive and negative groups. The anti-PLA2R positive cases exhibited a higher proteinuria after one year follow up. The glomerular PLA2R positive group also showed a higher tendency to proteinuria after one year follow up, although this did not reach statistical significance (P=0.08). These observations indicate that circulating anti-PLA2R autoantibodies and PLA2R in renal biopsy specimens are related to important clinical outcomes. A positive anti-PLA2R or positive glomeruli PLA2R showed a lower remission rates (higher proteinuria) than those with negative tests. These data supported previous findings that PLA2R and anti-PLA2R antibody may be utilized as a monitoring tool to guide treatment decisions (Hofstra, Beck et al. 2011). However, the quantitative measurement of PLA2R antibody levels by ALBIA did not predict outcome of the MN patients. This might be due to: 1) the relatively small number of patients enrolled in this study; 2) the failure to obtain baseline levels of circulating PLA2R titers for all cases, which in turn limit the analysis for clinical correlations.

4.3 IgG subclasses in the differential diagnosis of IMN and SMN

The exact pathophysiology of IgG subclasses in MN has remained an enigma. Several studies have shown that IgG4 predominated in the glomerular immune complex in IMN. However, other subclasses, especially the IgG1 and IgG3, had also been reported (Segawa, Hisano et al. 2010, Bannister, Howarth et al. 1983, Noel, Aucouturier et al. 1988). In the present study, the distribution of IgG1, 3 and 4 was assessed in both IMN and SMN cases. There was no significant difference between the distribution of the IgG subclasses (IgG1, IgG3 and IgG4), although IgG4 and IgG1 staining tend to be stronger than IgG3. When subclasses were analyzed by the PLA2R staining status in glomeruli, both IgG1 and IgG4 but not IgG3, displayed a highly positive frequency in PLA2R positive group than PLA2R negative group ($P<0.05$). Moreover, IgG4 was the only one that was associated with anti-PLA2R antibodies status in sera ($P<0.05$). As indicated by my results, IgG4 was often codominant with either IgG1 or IgG3 or both in PLA2R positive glomeruli. It is generally believed that IgG4 does not effectively activate complement through the classical pathway. By contrast, IgG1 and IgG3 have strong affinity for C1q and can induce complement activation. The IgG1 and IgG3 status of kidney biopsies may thus worthy of more attention in the future to help elucidate the pathogenic mechanisms of MN. Huang C et al showed that in early histological stage MN, antibody response is different from later sages, with IgG1 dominant deposits (Huang, Lehman et al. 2013). The data in this study couldn't confirm the association, as most of the cases were in stage II or III.

In the setting of SMN, especially malignancy associated MN, IgG subclasses other than IgG4 were usually found to be predominant. Qu Z *et al.* detected IgG subclasses in 8

malignancy-associated MN glomeruli and observed absence of IgG4 in 7 cases (Qu, Liu et al. 2012). They concluded that the absence of glomerular IgG4 deposits in patients with MN may indicate the presence malignancy. Ohtani H *et al.* reported that the glomerular IF intensities of IgG1 and IgG2 were significantly stronger in the malignancy group than those in the IMN groups (Ohtani, Wakui et al. 2004). In this study, 6 out of 19 IMN cases showed negative IgG4 staining and 5 of them were also glomerular PLA2R-negative. All cases of SMN with positive PLA2R showed IgG4-predominant staining. IgG4 predominance in SMN raises the possibility that these cases were more pathogenically related to IMN than secondary. Consistent with this notion, the results of our study suggested that MN is most likely a heterogeneous disease and that IgG4 and PLA2R negative cases may have different pathogenic mechanisms. It is still not clear whether IgG subclasses contribute to the differential diagnosis for IMN with malignant associated MN. A large retrospective cohort for further investigation of glomerular IgG subclass deposition and further investigation of MN pathogenesis would help to clarify this issue.

4.4 Antigen, IgG subclasses and Complement roles in MN pathogenesis

The key to substantial improvement of disease monitoring and treatment is the identification of relevant pathogenic mechanisms. The evidence drawn from experiments using Heymann models of MN indicated that subepithelial immune deposits provoke local complement activation and podocyte injury. In fact, in human MN, complement C3 and the membrane attack complex were present in subepithelial immune deposits in 80% and 50% of patients with idiopathic MN, respectively (Lai, Lo et al. 1989). Elucidation of the role of complement in MN would let us delve deeper in the mechanisms of this autoimmune disease. The data of the present

study showed that the PLA2R positive groups had a more prevalent and stronger C3 staining as compared with PLA2R negative group, which suggested that PLA2R and its specific antibody interaction might activate the complement cascade. It is possible that PLA2R is the inciting antigen in the cascade and other antigens such as AR, SOD2 or α-enolase are recruited through the mechanism of intermolecular epitope spreading. In addition, it is also possible that the inciting podocyte autoantigen still remains to be identified and all the identified antigens to date result from intermolecular epitope spreading.

The complement cascade can be activated by classical, lectin and alternative pathways which all lead to the activation of C3 components (Sarma, Ward 2011). Previous reports showed that IgG4 is the most predominant IgG subclass in glomeruli. Unlike other subclasses, IgG4 does not activate complement by the classical pathway and behaves mostly as a monovalent, low –affinity immunoglobulin (Papadea, Check 1989). According to the current results, IgG4 autoantibodies directed to PLA2R were codominant with either IgG1 or IgG3 in most of the PLA2R positive cases. Additionally, a positive correlation between IgG1 with complement C3C expression in glomeruli was observed. The concomitant presence of IgG1 and IgG3 with IgG4 could possibly be responsible for complement activation through the classical pathway. However, the alternative or lectin pathways might also be involved in complement activation and formation of the C5b-9 complex, as indicated by some studies in which most patients with IMN had very low levels of C1q in deposits (Jennette, Hipp 1985).

Recently, Huang *et al.* reported that in early stage IMN, IgG1 was the dominant IgG subclass and IgG4 became dominant in all later stages (Huang, Lehman et al. 2013). The exact pathogenic meaning is not clear, as progression from one stage to another does not necessarily indicate worsening disease but can also be seen with resolution (Forland, Spargo 1969). IgG4 has

been considered anti-inflammatory in other autoimmune diseases, by decreasing crosslinking of antigen (van der Neut Kolfschoten, Schuurman et al. 2007). The data of our study showed that IgG4 detected in glomeruli was not associated with C3C. It might be speculated that IgG4 in IMN represents a protective response against cross-linking of antigens induced by the IgG1 antibodies that were developed earlier and activated complement. In fact, it appears that there is an inverse relationship between the intensity of glomerular IgG4 and C1q staining as indicated by Huang *et al.* (Huang, Lehman et al. 2013). Further research is required to investigate the differences in the glomerular deposition of IgG subclasses, especially IgG4 and complement proteins in different clinical phases.

4.5 Significance, limitations, and future directions

The lack of understanding of the mechanisms involved in the pathogenesis of MGN limits early, effective and appropriate therapeutic management. To date, nonspecific severity criteria can be used as the hallmarks of adopted treatment approaches. The finding of the podocyte transmembrane antigen PLA2R provides evidence that idiopathic MN is an autoimmune disease. It is conceivable that the binding of anti-PLA2R antibodies to PLA2R (or other antigen antibody binding) could activate complement leading to recruitment of additional inflammatory components, cell and tissue damage and the release of more target autoantigen(s). The study of the PLA2R, IgG subclasses and complement components in the circulation and glomeruli would elucidate the passible mechanisms underlying the pathogenesis of IMN and guide therapeutic strategies. Despite the fact that there was relatively a small sample size especially for SMN cases and the MN cases were selected based mainly on the biomaterial availability could cause selection bias, the data together with other related studies suggested that

secondary causes cannot be rule out based on IgG4 status alone. In addition, this study is the first to attempt correlating PLA2R and IgG subclasses with complement components in glomeruli. The current studies also add relevant data to the PLA2R and IgG subclasses in the differential diagnosis between IMN and SMN. Further prospective studies are however needed in order to elucidate the complement's role in the PLA2R-related MN. A meaningful outcome of such studies is that perhaps the measurement of serum autoantibodies directed to PLA2R and the detection of IgG subclasses in renal biopsy specimens might, in parallel, provide more meaningful diagnostic information. Such directions are being contemplated in the future diagnostic setting in Calgary.

It is now appreciated that MN is a rather heterogeneous disease. In fact, more than 20 intracellular proteins were identified as possible antigens in the model of passive Heymann nephritis mice (Ronco, Debiec 2012). Similarly, it is expected that more potential antigens in human IMN will be identified in the future. The coexistence of autoantigen, antibodies suggests a complex pathogenic pathway that involves different podocyte targets. Studies of a prospective MN cohort would help elucidate the temporal appearance and the role of each podocyte antigen and antibody in MN development and progression. Work on the Heymann nephritis model as well as cultured rat glomerular epithelial cells, would help to elucidate how the B cell response to target autoantigens is triggered and progressed. Additionally, using immune EM to assess the subcellular localization of PLA2R antigen and antibody in glomeruli will add more information to the understanding of MN molecular pathogenesis. It is important to note that anti-PLA2R autoantibodies or PLA2R antigen are not found in all cases of IMN. Actually, only approximately half of the IMN are PLA2R negative cases in our study. The PLA2R negative

cases might use different pathogenic mechanisms than the PLA2R positive cases. It would be of interest to investigate this in the future.

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