# Membranous Nephropathy

## Challenges in diagnostics and treatment

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Cover illustration: Schematic drawing of the membranous glomerulus, by Johan Mölne

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*Carl, nu är mammas "saga" äntligen färdigskriven...*

# ABSTRACT

The variability in the pathogenesis, clinical presentation and outcome of membranous nephropathy (MN) poses major clinical challenges and raises different questions, both regarding diagnostics and treatment of patients with MN. The aims of this thesis were therefore to examine: 1) differences in the glomerular expression of different IgG subclasses and phospholipase A2 receptor ($PLA_2R$) between patients with idiopathic and malignancy-associated MN; 2) treatment pattern of patients with idiopathic MN; and 3) if the serum $PLA_2R$ antibody level at diagnosis can be used as a prognostic marker.

We found that absence of glomerular IgG4 and $PLA_2R$ indicates malignancy-associated MN. IgG2 was present in a large number of patients of both groups, and could not be used as an indicator of an underlying malignancy. Moreover, in our material we found no evidence for an IgG subclass switch during the disease process, as IgG1 and IgG3 were present in a low number of patients.

When investigating the treatment pattern of patients with idiopathic MN, we found that a majority of the patients (75%), had reached remission at the study end. 10% had developed end-stage renal disease, a fairly high number, given that 51% of the patients received immunosuppressive therapy at some point, and that 88% of the patients received supportive treatment with ACEIs and/or ARBs. The specific treatment varied, and there was a tendency to start treatment at an early point (21% of the patients) instead of awaiting a spontaneous remission. Not recommended therapy was used in a high proportion of these cases (47%).

In a retrospective cohort of patients with saved blood samples from the time of renal biopsy, we found a significant correlation between a high serum $PLA_2R$ antibody level at presentation, and a less favorable clinical outcome. Patients with higher autoantibody levels were more exposed to immunosuppressive therapy, but still there were less cases of complete remission among these patients.

We conclude that absence of glomerular IgG4 and $PLA_2R$ should raise the question of an underlying malignancy in a patient with MN. Moreover, the serum $PLA_2R$ antibody level at presentation seems to be the prognostic marker urged for, in the decision of whom and when to treat with immunosuppressive therapy.

# POPULÄRVETENSKAPLIG SAMMANFATTNING

Begreppet kronisk glomerulonefrit innefattar en rad olika njursjukdomar som alla kan ge upphov till avtagande njurfunktion. Vid kronisk glomerulonefrit är proteinuri ett vanligt symptom som obehandlat kan leda till skador i njurvävnaden. Proteinuri är det vanligaste kliniska fyndet vid membranös nefropati (MN). MN kan vara primär eller uppstå sekundärt till annan bakomliggande sjukdom eller läkemedel. Det är av stor vikt att sekundära fall identifieras, dels då behandlingen skiljer sig åt och dels för att inte fördröja handläggningen av exempelvis en bakomliggande tumörsjukdom.

Det kliniska förloppet vid primär MN skiljer sig åt kraftigt mellan olika patienter; en del tillfrisknar spontant medan andra utvecklar terminal njursvikt. Det saknas i nuläget en tillförlitlig prognostisk markör och för att inte riskera att utsätta patienter för onödig immundämpande behandling rekommenderas sex månaders observation med symtomatisk behandling, såvida patienten inte uppvisar tecken till en mer aggressiv sjukdom.

Målet med denna avhandling var att tydliggöra de kliniska utmaningar som omhändertagandet av patienter med MN innebär. Vi har studerat uttryck av olika IgG-subklasser samt fosfolipas $A_2$ receptor ($PLA_2R$) i njurvävnad hos patienter med primär respektive tumörorsakad MN. Vi fann att avsaknad av IgG4 samt $PLA_2R$ starkt talar för en bakomliggande tumörsjukdom. Vi har vidare studerat det kliniska utfallet hos patienter med MN i västra Sverige i början av 2000-talet. Vi fann att trots att en hög andel av patienterna erhöll immundämpande behandling, så var det kliniska utfallet sämre än vad man hade kunnat förvänta sig. Det förelåg skillnader i behandlingsstrategi, både inom och mellan klinikerna, vilket understryker svårigheterna i det kliniska omhändertagandet av denna patientgrupp, samt behovet av en prognostisk markör vid sjukdomsdebuten. Vi har därför studerat om halten $PLA_2R$-antikroppar i blodet hos patienter med primär MN spelar roll för det kliniska utfallet. Vi fann att en högre $PLA_2R$-antikroppsnivå starkt talar för en sämre prognos.

Kompletterande njurbiopsifärgning för IgG4 och $PLA_2R$ bör således kunna användas för att skilja mellan primär respektive sekundär MN. Sannolikt bör också halten $PLA_2R$-antikroppar i blodet, förutom att indikera primär sjukdom, också kunna användas som prognostisk markör vid MN, och därmed underlätta beslutet om att initiera immundämpande behandling.

# LIST OF PUBLICATIONS

This thesis is based on the following papers, referred to in the text by their Roman numerals.

I. **Glomerular IgG subclasses in idiopathic and malignancy-associated membranous nephropathy**
Jennie Lönnbro Widgren, Kerstin Ebefors, Johan Mölne, Jenny Nyström and Börje Haraldsson.
*Clinical Kidney Journal 2015 Aug;8(4):433-9;*
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II. **Treatment pattern in patients with idiopathic membranous nephropathy - practices in Sweden at the start of the millennium**
Jennie Lönnbro Widgren, Johan Mölne, Börje Haraldsson and Jenny Nyström.
*Clinical Kidney Journal 2016*;
doi: 10.1093/ckj/sfv152

III. **Initial anti-phospholipase A2 receptor antibody levels predict clinical outcome in patients with idiopathic membranous nephropathy**
Jennie Lönnbro Widgren, Kerstin Ebefors, Barbara Seitz-Polski, Christine Payré, Gérard Lambeau, Johan Mölne, Börje Haraldsson and Jenny Nyström.
*Submitted*

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# ABBREVIATIONS

| | |
|---|---|
| ACR | albumin to creatinine ratio |
| ACEI | angiotensin converting enzyme inhibitor |
| ACTH | adrenocorticotropic hormone |
| ARB | angiotensin receptor blocker |
| BMtx | bone marrow transplantation |
| C1q | complement 1q |
| C3c | complement 3c |
| C5b-9 | complement 5b-9 |
| CDK | chronic kidney disease |
| Ch | chemotherapy including alkylating agents |
| CR | complete remission |
| Cs | chemotherapy including steroids |
| CTLDs | C-type lectinlike domains |
| CyA | cyclosporine |
| CYP | cyclophosphamide-based treatment |
| CysR | cysteine-rich domain |
| DAB | 3-3-diaminobenzidine tetra hydrochloride |
| eGFR | estimated glomerular filtration rate |
| ESL | endothelial cell surface layer |
| ESRD | end-stage renal disease |
| FNII | fibronectin type II |
| GBM | glomerular basement membrane |
| H | hormonal therapy |
| HLA | human leukocyte antigen |
| HLA-DQA1 | HLA complex class II HLA-DQ alpha chain |
| HRP | horseradish peroxidase |
| IC | intracellular C-terminal |
| IgA | immunoglobulin A |
| IgG1-4 | immunoglobulin G, subclass1-4 |
| IgM | immunoglobulin M |
| KDIGO | Kidney Disease Improving Clinical Outcome |
| MAC | membrane attack complex |
| MDRD | modification of diet in renal disease |
| MMF | mycophenolate mofetil |
| MN | membranous nephropathy |
| NS | nephrotic syndrome |
| NSAID | nonsteroidal anti-inflammatory drug |
| PBS | phosphate buffered saline |
| $PLA_2R$ | phospholipase $A_2$ receptor |
| PR | partial remission |

| | |
|---|---|
| R | radiation |
| Ritux | rituximab |
| S | surgery |
| SEM | standard error of mean |
| SOD2 | superoxide dismutase 2 |
| $sPLA_2$ | secretory $PLA_2$ |
| THSD7A | thrombospondin type-1 domain-containing 7A |
| TM | transmembrane domain |
| uA1m | alfa-1-microglobulin |
| uB2m | beta-2-microglobulin |

# 1 INTRODUCTION

Most chronic kidney diseases can be progressive and lead to end stage renal disease, (ESRD) with the need for dialysis or renal transplantation. In December 2014, 9220 patients were under active uremic care in Sweden, 5361 patients had a functioning renal transplant, while 3859 patients were treated with dialysis [1]. The incidence rate of ESRD was 112 per million inhabitants, and the number is growing, a pattern seen in other parts of the world as well [2]. Glomerulonephritis has been, and still is, the dominant cause of uremia in the dialysis population, accounting for 25% of the cases. However, among new patients in dialysis, diabetic nephropathy is the most common cause of uremia [1]. All-cause mortality rate for dialysis patients is several times higher than for individuals in the age-matched population [3]. It is therefore of great importance to stop the progressive renal impairment before the patient develops ESRD, and requires active uremic care. Although glomerulonephritis is the most common cause of uremia, the pathophysiology and underlying mechanisms behind the different diseases are not clearly understood. The only available choices of treatment are therefore unspecific and can sometimes even be harmful.

## 1.1 The kidney

The kidneys play a crucial role in maintaining the body water and salt composition, excretion of metabolic end products and foreign substances, and production of enzymes and hormones. The nephron is the functional unit of the kidney, and there are approximately one million nephrons in one human kidney. Each nephron consists of a glomerular and a tubular part. The glomerulus is a capillary network enclosed by the Bowman´s capsule and the surrounding tubular system. Each day 150-180 liters of fluids are filtered through the glomerular capillaries. During the further tubular transport the primary urine is modified, and fluid is reabsorbed before becoming the final urine, approximately 1.5 liters per day.

### 1.1.1 The glomerular filtration barrier

The glomerular filter, through which the ultrafiltrate has to pass, consists of three distinct but interacting layers; the fenestrated endothelial cells, the glomerular basement membrane and the podocytes, with their foot processes and slit diaphragms (Figure 1). This complex filter is freely permeable to water and small molecules such as urea and glucose, but retains albumin and other large molecules (>70 kDa), as well as red blood cells [4]. The filtration barrier restricts passage of solutes depending not only on their size, but also on their charge and configuration, and the net filtration pressure in the glomerular tuft. Either genetic or acquired abnormalities in one of the three layers in the glomerular capillary wall, can lead to a defective glomerular filtration barrier and proteinuria. [5] The mesangial cells are not part of the glomerular filtration barrier, but are found in-between the glomerular capillaries, and provide structural and functional support. The glomerular cell components seem to interact more intensely than previously understood, and the intercellular signaling between endothelial, epithelial and mesangial cells may be crucial for the highly selective properties of the filtration barrier [6].

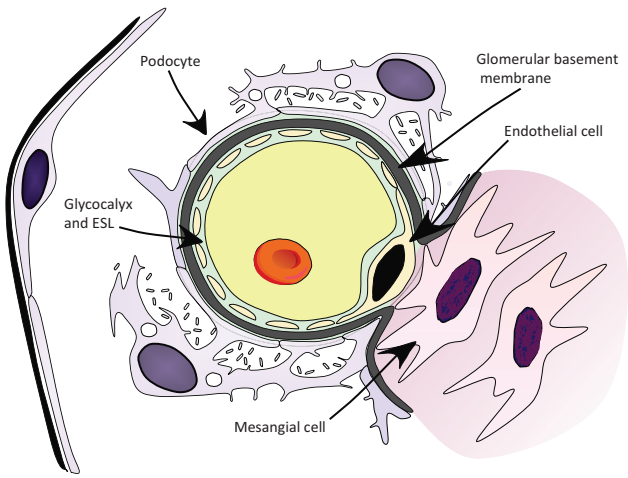


***Figure 1**. Structure of the glomerulus. One single capillary loop showing the endothelial cell surface layer (ESL) and the glycocalyx covering the endothelial cells. The outside of the glomerular capillaries is covered by the podocytes that are attached to the basement membrane by their foot processes. The basement membrane is attached to the mesangium that provides structural support. Illustration by Johan Mölne.*

## Glomerular endothelial cells

The heavily fenestrated endothelial cells allow high permeability to water and small solutes. The fenestrae are large, 60 nm in diameter, (compared to albumin 3.6 nm) and the endothelial cell layer has therefore previously not been considered essential to the selective glomerular filtration. However, the cells are covered with a thick negatively charged cell surface, which can be divided into the glycocalyx and the endothelial cell surface layer. The latter is more loosely attached to the luminal side of the glycocalyx and is suggested to consist of negatively charged glycoproteins and proteoglycans. Morphological alterations of the components in the cell surface layer lead to proteinuria, and the endothelium is therefore now recognized to have charge-selective properties that most likely contribute to the high permselectivity of the glomerular filtration barrier [7].

## Glomerular basement membrane

The glomerular basement membrane (GBM) is an acellular, extracellular matrix located between the endothelial cells and the podocytes. The membrane is a result of a fusion of basement membrane from the endothelial cells and the podocytes during the glomerulogenesis [8]. Both cell types seem to be of importance in maintaining the GBM´s structure and function, even after maturation [9]. The GBM is thicker compared to other basement membranes in the body, and the major components are laminin, collagen IV, nidogen and various proteoglycans. Numerous mutations in GBM proteins, such as collagen IV and laminin, are associated with proteinuria. The heparin sulfate proteoglycans agrin and perlecan are negatively charged, and for a long time this has been considered important for the charge-selective properties of the glomerular filtration barrier [10]. However, this is now questioned, due to the fact that selective removal of highly anionic substances neither influences the glomerular charge selectivity, nor increases proteinuria [11]. This may however be due to compensatory mechanisms in the experimental settings used and cannot be considered proven yet.

## The podocyte

The outer surface of the capillaries is covered with specialized epithelial cells called podocytes. These cells are highly differentiated cells that form an array of zipper-like foot processes over the outer layer of the glomerular capillaries, and face the Bowman´s capsule and the primary urine. The foot processes of adjacent podocytes are connected by a thin membranous

structure called the slit diaphragm, and this interposed slit diaphragm forms the final barrier to protein loss [4]. The structure of the foot processes is maintained by long actin filaments, which also connect adjacent processes. The foot processes are anchored to the underlying basement membrane by transmembrane receptors, such as α1β3-integrin and dystroglycans, which are in turn, linked to the actin cytoskeleton [12]. Nephrin is specifically expressed in the podocyte slit diaphragm, and plays a crucial role in maintenance of the function of the glomerular filtration barrier. This was originally shown by the discovery that a mutation in the nephrin gene causes congenital nephrotic syndrome of the Finnish type [13]. Since then, further research has shown that other cell adhesion molecules, located intracellularly and in the slit diaphragm, also are important for maintaining the properties of the slit diaphragm [14-17]. Besides being a size barrier to proteins, the negatively charged apical domain of the podocyte also limits the passage of albumin. The podocytes are further important for contributing to the synthesis and maintenance of the GBM, as well as for the fenestration of the endothelial cells, by production of vascular endothelial growth factor and angiopoetin [18].

Podocyte injury is involved in many forms of glomerular disease, and compared with single gene mutation-induced podocyte diseases, the pathogenesis for acquired podocytopathies is more complex. There are mainly four different types of alterations of podocyte morphology; foot process effacement, podocytopenia, arrested development, and dedifferentiation [19]. Foot process effacement; re-arrangement of the cytoskeletal actin filaments, is the adaptive response of stress to the podocytes. It is hypothesized that this adaptation is a protective response to detachment of podocytes from the GBM, rather than a result of injury. Podocytopenia; loss of podocytes, is encountered in podocytes with effaced processes, and it appears that foot process effacement proceeds detachment [20]. Podocytopenia is considered a significant contributor to the progression of glomerulosclerosis, with proteinuria developing correspondingly. It has been suggested that counting of podocytes in the urine, rather than measurement of proteinuria, would be a better marker of disease progress and its response to treatment [21, 22].

## The mesangial cells

The mesangial cells constitute the central stalk of the glomerulus, and provide structural support for the glomerular capillary loops. Furthermore, they have contractile properties, which enables them to alter and fine-regulate

the single nephron glomerular filtration rate (GFR). The mesangial cells are imbedded in their own matrix, which consists of type IV and V collagen, laminin, fibronectin, heparan sulphate, chondroitin sulphate, entactin, and nidogen. The composition thereby differs from the GBM [23]. Some of the components of the matrix provide structural support for the mesangium, and also influence mesangial cell growth and proliferation. Mesangial cell pathology plays an obvious part in a variety of glomerular diseases, for example IgA nephropathy and diabetic nephropathy. Cell proliferation and matrix expansion result in a reduction, and may eventually lead to occlusion, of the capillary lumen, and to glomerulosclerosis. The mesangial cells also seem to produce factors affecting the podocytes and the tubular system, and thereby contribute to the development of proteinuria and tubulointerstitial injury [24-26].

## 1.2 Proteinuria

Proteinuria is a cardinal sign of kidney damage and a clinical feature in all glomerular diseases. Historically, proteinuria has been considered a surrogate marker of the severity of the underlying glomerular damage. However, recent years it has become evident that proteinuria is a risk factor *per se*, and plays an important role in the pathogenesis of the progression of renal disease.

### 1.2.1 Nephrotic syndrome

Smaller proteins (< 30 kDa) are filtered in the glomerulus, but reabsorbed during the tubular transport, and the final urine normally consists of very small amounts of proteins [27]. Structural damage to the filtration barrier caused by primary kidney disease, systemic disease or medication, leads to proteinuria. Any type of glomerular disease can cause proteinuria, but not all proteinuria is of glomerular origin. Tubular damage can cause proteinuria, but it rarely exceeds 2 g/day.

Heavy protein traffic in the renal tubules, secondary to a damaged filtration barrier, is harmful in several ways. Experimental studies have shown that proteinuria leads to development of glomerulosclerosis, tubulointerstitial inflammation, and progressive tubulointerstitial fibrosis [28, 29]. The reabsorption of proteins in tubuli has been shown to activate fibrogenic and inflammatory factors, leading to scarring of the renal parenchyma. Proteinuria is therefore one of the most important factors for progression of renal disease, and loss of kidney function. When proteinuria is severe, it causes nephrotic syndrome (NS), characterized by massive proteinuria (>3.5 g/day), hypoalbuminemia, edema, and hyperlipidemia [17, 30]. Patients with

severe NS are at risk for thromboembolic complications due to loss of hemostasis control proteins such as antithrombin III. Plasma concentrations of the procoagulant proteins, including fibrinogen and factors V and VIII, are usually markedly elevated. These changes further amplify the prothrombotic state [31]. Other complications of the NS include infections secondary to loss of immunoglobulins, and toxic effect of medication with a high degree of protein binding [32]. Even in the long-term perspective, patients with heavy proteinuria have a less favorable outcome, both in terms of systemic complications and renal prognosis [32].

### 1.2.2 Treatment of the nephrotic syndrome

A reduction of proteinuria to non-nephrotic levels alleviates the negative effects of the nephrotic syndrome, and treatment of the underlying disease to reduce proteinuria is therefore of great importance. However, treatment options are few, often include immunosuppressants, and might not always be appropriate to use. Spontaneous remission of proteinuria occurs in some patients, but proteinuria may also continue to progress, and lead to renal impairment. Regardless of the underlying disease, reducing or eliminating proteinuria with supportive treatment is crucial. Angiotensin converting enzyme inhibitors (ACEIs) and angiotensin II receptor blockers (ARBs), used as single therapy or combined, are effective in reducing proteinuria. Therefore, they are recommended as the mainstay of treatment. Diuretics can be used if edema is profound and effects the patients breathing, skin status or mobility. Treatment of dyslipidemia should follow guidelines for patients at risk of cardiovascular events, and HMG-CoA-reductase inhibitors are recommended due to their effectiveness in correcting the lipid profile [33]. Infections should be treated aggressively with antibiotics, because of the elevated risk of severe infections in patients with nephrotic syndrome. The risk of thromboembolic events increases as the serum albumin concentration falls below 25 g/L. Other factors, such as immobilization, malignancy, or heart failure, can further aggravate the risk, and in many countries, including Sweden, prophylactic low-dose anticoagulant is indicated for patients at risk [33].

## 1.3 Membranous Nephropathy

Membranous nephropathy (MN) is one of the most common causes of nephrotic syndrome in the world [34]. Histologically, it is characterized by subepithelial immune deposits, with subsequent thickening of the basement membrane, without cellular proliferation and infiltration (Figure 2). The immune deposits appear as granular deposits of IgG with immunofluorescence or immunoperoxidase on light microscopy, or as electron-dense deposits on electron microscopy [35]. In approximately 80% of the patients, proteinuria, often nephrotic-range, is the typical clinical presentation. In a majority (75%) of the adult patients with membranous nephropathy the etiology is unknown, and the cases are considered idiopathic. In the remaining 25% patients, the cause of the disease is secondary to other diseases or drugs [36].

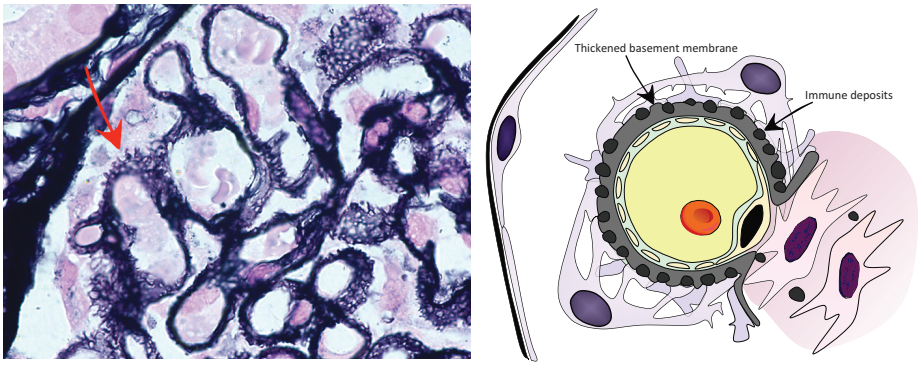


***Figure 2**. **A)** Light microscopy picture of the membranous glomerulus (silver staining). The thickened capillary wall shows numerous holes, indicating immune deposits (the deposits do no not take up the silver stain). The red arrow indicates spikes of the basement membrane silver-staining material that protrude from the basement membrane. **B)** Schematic drawing of the thickened basement membrane and the immune deposits. Illustration by Johan Mölne.*

## 1.3.1 Pathophysiology

### Autoantibodies

A considerable insight in the mechanisms of immune complex formation and nephritogenic potential was provided by the studies of Heymann nephritis in rats [37, 38]. The antigenic target in the rat disease is a podocyte membrane protein called megalin. Despite the finding that megalin is not present in human glomeruli, [39] the idea that an epithelial antigen is targeted in human disease, was further supported by a rare situation known as alloimmune antenatal MN [40]. In this pregnancy-induced immunization of the mother, with transplacental passage of nephritogenic antibodies, infants are born with the clinical and pathological features of MN. In both the rat model of Heymann nephritis, and alloimmune antenatal MN in human beings, antibodies against an antigen expressed on the podocytes, lead to formation of subepithelial deposits, podocyte injury and proteinuria.

It was recently discovered that approximately 70-80% of the patients with idiopathic MN, have circulating serum antibodies directed towards the M-type phospholipase $A_2$ receptor ($PLA_2R$) expressed on the podocyte [41]. The exact function of the $PLA_2R$ in the podocyte is unknown, but the receptor has been reported to promote replicative senescence in fibroblasts, and recent published data have suggested a role as a tumor suppressor in mammalian epithelium [42, 43]. The $PLA_2R$ is found to have significant expression not only in the human kidney, but also in the lung and placenta, and it is unclear why MN is limited to the kidney, given that $PLA_2R$ is expressed in other organs. $PLA_2R$ autoantibodies are not found in healthy individuals, in patients with other diseases causing nephrotic syndrome, or in cases of secondary MN.

$PLA_2R$ is a 180-kDa membrane receptor, which is composed of an N-terminal cysteine-rich domain (CysR), a fibronectin type II (FNII) domain, eight C-type lectinlike domains (CTLDs), a transmembrane domain (TM), and a short intracellular C-terminal (IC) tail [44], (Figure 3). Recent studies have obtained evidence for several epitopes in the $PLA_2R$ targeted by $PLA_2R$ antibodies [45, 46]. Epitopes in three different domains, recognized by distinct $PLA_2R$ antibodies, have been identified [47]; CysR, CTLD1 and CTLD7. The primary dominant epitope seems to be CysR, and a recent study provided evidence for epitope spreading to CTLD1 and CTLD7 [47]. Epitope spreading, development of immune responses to epitopes distinct from and non-cross-reactive with the original epitope, is described in many autoimmune diseases. The trigger of epitope spreading might be a second immune challenge (inflammation, allergy, infection), and is associated with a

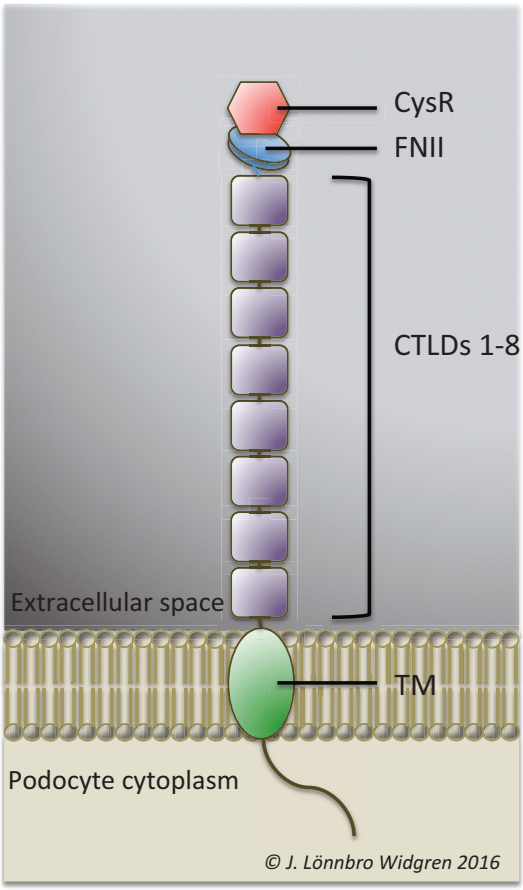


***Figure 3**. The domain structure of the phospholipase $A_2$ receptor, $PLA_2R$. The receptor is composed of an N-terminal cysteine-rich domain (CysR), a fibronectin type II (FNII) domain, eight CTLDs, a transmembrane domain, and a short intracellular tail.*

worsening of the disease [48-50]. Indeed, patients with anti-$PLA_2R$ activity against CysR, seem to have a more favorable outcome, compared with patients with anti-$PLA_2R$ activity against CTLD1 and CTLD7 [47]. Other reported antigen candidates in idiopathic MN are human leukocyte antigen (HLA) complex class II HLA-DQ alpha chain (HLA-DQA1), and the intracellular enzymes aldose reductase, superoxide dismutase 2 (SOD 2) and alpha-enolase [51-53]. These intracellular enzymes are not abundantly expressed in the normal glomeruli, but are induced with disease and thus are neoantigens. Recently, autoantibodies against the thrombospondin type-1 domain-containing 7A (THSD7A) were detected in 8 to 14% of $PLA_2R$-negative patients, suggesting a subgroup of patients with idiopathic MN [54]. The relative pathogenicity of each, as well as the possibility of synergistic effects, needs further investigation.

### Immunoglobulins and complement system

In idiopathic MN, antibodies against both $PLA_2R$ and THSD7A are predominantly of IgG4 subclass, and different theories on the pathophysiology have been proposed. Binding of the autoantibody to a specific epitope of the receptor on the podocyte, might form immune complexes leading to activation of the complement system. The membranolytic properties of the complement system may contribute to the podocyte damage by activation of C5b-9, also named membrane attack complex (MAC). C5b-9 stimulates the podocytes to produce inflammatory mediators and alter the cytoskeleton, which leads to detachment of podocytes, resulting in proteinuria [36, 55]. The glomerular epithelial cells can also be triggered by C5b-9 to activate signaling pathways, which results in further damage of the podocyte.

The different IgG subclasses differ in their ability to activate complement. IgG4 and IgG2 are less prone to activate complement compared to IgG1 and IgG3 [56]. Although complement activation occurs, infiltration of inflammatory cells is rarely seen, and crescent formation is not a characteristic feature in MN. In malignancy-associated MN, it has been reported a dominance of IgG1 and IgG2, rather than IgG4, as is typical in idiopathic MN. One study also reports an increased number of immune cells in the glomerulus in patients with MN and cancer, a feature that might be secondary to the differences in IgG subclasses [57]. The pathogenic mechanisms behind the formation of subepithelial immune deposits in secondary MN, are less well understood. Several mechanisms have been proposed, such as subepithelial incorporation of preformed circulating immune complexes, as seen in lupus nephritis [58]. In patients with solid tumors, antibodies may be generated against a tumor antigen identical to an endogenous podocyte antigen. Furthermore, extrinsic factors, such as infections with oncogenic virus or altered immune function, may be responsible for the development of both tumor and MN [58].

## 1.3.2 Idiopathic membranous nephropathy

The natural course of idiopathic membranous nephropathy varies and spontaneous remission, complete or partial, is reported in 30-60% of the patients [59-61]. Remission can occur at any time during the course of the disease, but is most likely to occur during the first two years after presentation [60]. Approximately one third of the patients experience a less favorable outcome and develop end stage renal disease. Factors indicating a

poor prognosis are high age at presentation, male gender, high amount of proteinuria, abnormal renal function at presentation, and presence of tubulointerstitial fibrosis and glomerulosclerosis [62, 63]. Additionally, it has been proposed that the serum $PLA_2R$ antibody can be used as a marker of immunological and clinical activity [64]. Changes in antibody levels seem to precede changes in proteinuria, and measurement of serum $PLA_2R$ antibodies may therefore be a useful method to follow, and predict response to treatment [65]. Moreover, patients who experience spontaneous remission, tend to have low $PLA_2R$ antibody levels at presentation, and reach remission faster compared to patients with high antibody levels [64].

### 1.3.3 Secondary membranous nephropathy

Approximately 25% of the cases of membranous nephropathy are associated with other conditions thought to secondarily cause membranous nephropathy. This estimate regards the Western countries in the world, and if one takes into account the cases of MN in tropical areas, where an indefinite number of MN are associated with endemic infections, the ratio idiopathic to secondary cases would probably decrease. A number of chronic infections such as hepatitis B and C, drugs and toxins such as D-penicillamin, nonsteroidal anti-inflammatory drugs (NSAIDs), captopril, and gold salts are associated with MN (Table 1). The most frequently associated diseases reported are diabetes mellitus, rheumatoid arthritis, systemic lupus erythematosus, and malignancies [35, 58].

***Table 1.*** *Examples of causes of secondary membranous nephropathy.*

| Autoimmune diseases | Malignancies | Infections | Drugs and toxic substances |
|---|---|---|---|
| Systemic lupus erythematosus | Lung | Hepatitis B | Captopril |
| Diabetes mellitus | Stomach | Hepatitis C | Clopidogrel |
| Rheumatoid arthritis | Colon | Strepotococcal infection | NSAIDs |
| Thyroiditis | Breast | Malaria | Penicillamin |
| Sjögren´s syndrome | Prostate | Tuberculosis | Gold |
| Psoriasis vulgaris | Kidney | Syphilis | Hydrocarbones |

In the clinical practice, it is of great importance to distinguish between idiopathic and secondary MN, since treatment is completely different. In idiopathic cases, specific treatment with immunosuppressive agents may be indicated, whereas in secondary cases, effort should be made to eliminate the

underlying cause of the renal disease. It is further important to reduce the risks associated with missing the diagnosis of an underlying malignancy, as well as the risks following unnecessary exposure to immunosuppressive therapy in secondary cases. Neither on the renal biopsy, nor in the clinical presentation, are there typical signs indicating a secondary disease. During the recent years, the possibility to test for serum $PLA_2R$ antibodies has provided a new tool to discriminate between idiopathic and secondary MN. It also seems like glomerular expression of $PLA_2R$ correlates with the presence of serum $PLA_2R$ antibodies, which further can help to discriminate between idiopathic and secondary MN. However, staining for glomerular $PLA_2R$ is not yet a routine investigation, and for $PLA_2R$ antibody-negative patients, a further investigation has to be performed. Most secondary causes can be excluded by a thorough medical history, combined with physical examination, laboratory studies and a review of the patient´s medical history.

### Malignancy-associated membranous nephropathy

The association between malignancy and MN is well known. The incidence of malignancy in MN is significantly higher than in the general population, and awareness of malignancy is especially important in the elderly [57, 66]. The causal relationship between malignancy and MN relies on several criteria [57, 67, 68]. There should be no other obvious alternative cause, and there should be a temporal relationship between the malignancy and MN. Thus, complete removal of cancer should lead to remission of MN and resolution of proteinuria. Similarly, recurrence of cancer should be accompanied by return of proteinuria. In the majority of the cases, malignancy is diagnosed within 12 months of the diagnosis of MN. Approximately 80% of the malignancies are discovered before or at the time of renal diagnosis, and the rest of the cases afterwards [58]. It is important to emphasize that the risk appears to persist for a longer period than the time surrounding the renal biopsy, and tumors may be discovered up to five years later [68]. Therefore, in cases with undetectable serum $PLA_2R$ antibodies, and no other obvious underlying cause, age- and sex appropriate screening for cancer should be performed. A close monitoring of the patient is necessary, even if no malignancy is found on the initial screening at the time of renal biopsy.

## 1.3.4 Immunosuppressive treatment

Supportive treatment of the nephrotic syndrome should be considered in all patients with membranous nephropathy [33]. However, the question regarding whom and when to treat with immunosuppressive therapy has been heavily debated in the past. There are few randomized controlled trials in

patients with idiopathic membranous nephropathy, precluding the provision of guidelines with the high-quality level of evidence. Due to the fact that a high proportion of spontaneous remission occurs in idiopathic cases, it is recommended to manage patients conservatively for six months after diagnosis. Specific treatment is recommended in patients with nephrotic syndrome and a combination of one or more of the following conditions: 1) persistent proteinuria that exceeds 4 g/day and stays over 50% of the baseline value, 2) presence of severe, disabling or life-threatening symptoms related to the nephrotic syndrome, or 3) if serum creatinine has risen by 30% or more within 6 to 12 months from the time of diagnosis, but the eGFR is not less than 25–30 ml/min/1.73m$^2$ [33, 69]. Patients with reduced kidney size on ultra-sound (< 8 cm in length) and serum creatinine > 309 µmol/L, or concomitant severe or life-threatening infections, should not be treated with immunosuppressive therapy due to a high risk of side-effects combined with a very small chance of a positive treatment response.

A reliable and accurate prognostic marker at the presentation of the disease would improve the management of patients with idiopathic MN. Such a prognostic marker would allow early treatment of high-risk patients, and minimize the exposure to unnecessary immunosuppressive therapy in patients with a favorable prognosis. Urinary markers, such as beta-2-microglobulin (uB2m) and alfa-1-microglobulin (uA1m), have been suggested to predict progression in idiopathic MN, especially when combined with the risk score which the KDIGO guidelines is based on [69-71]. However, the landmark discovery of $PLA_2R$ being the major target antigen in patients with idiopathic MN is a major advance in understanding of the disease, and recent studies have proposed that the autoantibody level at presentation might be the prognostic marker urged for [64, 72, 73].

# 2 AIM

The general aim of this thesis was to address the major challenges the clinical care of patients with a diagnosis of membranous nephropathy gives rise to. This includes identification of patients with a secondary disease, especially those with an underlying malignancy. Furthermore, the clinical outcome of patients with idiopathic MN is highly variable, which may lead to uncertainty regarding for what patient, and at which time in the course of the disease, immunosuppressive therapy should be started. Finally, a reliable prognostic marker at the presentation of the disease is lacking today. We therefore explored if serum $PLA_2R$ antibody level at presentation of the disease, can be used as a prognostic indicator, and we further explored the presence of epitope specific titers.

The specific aims of the papers included in this thesis were:

| | |
|---|---|
| Paper I | To test the hypothesis that patients with malignancy-associated MN could be identified based on the lack of glomerular staining for IgG4 and $PLA_2R$. |
| Paper II | To investigate the treatment pattern of patients with idiopathic MN in clinical practice. |
| Paper III | To test the hypothesis that a high serum $PLA_2R$ antibody level at presentation indicates a less favorable prognosis. |

# 3 PATIENTS AND METHODS

## 3.1 Patients

In 2003, the renal biopsy study at Sahlgrenska University Hospital was initiated, with the purpose to build a database on patients with renal diseases undergoing renal biopsy. Patients with the diagnosis of MN between 2003-2014 were identified through these files. To extend the number of patients in our studies, additional patients were identified through the renal biopsy files at the Department of Pathology at Sahlgrenska University Hospital. All patients with the histologic diagnosis of MN in the Western part of Sweden (covering a population of approximately 1.7 million inhabitants) between 2000-2013 were considered for inclusion, and medical data were retrospectively collected from 2000.

### 3.1.1 Patients paper I

In this study data from 85 patients with the histologic diagnosis of MN between the years 2000-2012 were analyzed. 19 of these patients were included through the renal biopsy study at Sahlgrenska University Hospital, and the remaining 66 patients were retrospectively included.

### 3.1.2 Patients paper II

This study included 73 patients with the diagnosis of idiopathic MN between the years 2000-2013, of which 19 patients were included through the renal biopsy study at Sahlgrenska University Hospital, and the remaining 54 patients were included retrospectively.

### 3.1.3 Patients paper III

In this study, we aimed to perform retrospective analyses on saved blood samples from the time of renal biopsy. The included 25 patients with a diagnosis of idiopathic MN were therefore recruited only from the renal biopsy study files at Sahlgrenska University Hospital between the years 2003-2014.

### 3.1.4 Ethical statement

The renal biopsy study at Sahlgrenska University Hospital and the additional retrospective study were approved by the regional ethical review board in Gothenburg (approval numbers S552-02, 432-13 and 423-09). Written

informed consent was obtained from all patients before collecting the clinical data and performing biopsy examinations.

## 3.2 Study design

### 3.2.1 Paper I

This study is a retrospective study comparing the glomerular expression of IgG1-4 and $PLA_2R$, between patients with idiopathic and malignancy-associated MN in six different nephrology clinics.

### 3.2.2 Paper II

During the process of going through the medical records of the included patients in the study in paper I, the idea of this study came up. The highly variable outcome of patients with idiopathic MN seemed to cause uncertainty concerning immunosuppressive therapy in terms of i) who should be given treatment, ii) the most accurate treatment to use, and iii) at which point in the disease process should therapy be initiated? In this study we retrospectively applied the treatment guidelines recommended by the Toronto group in 2000 [74] on the included patients to examine these questions.

### 3.2.3 Paper III

As a result of the study in paper II, this retrospective study was designed. The aim was to examine if the level of $PLA_2R$ antibody at presentation of the disease, can be used as a prognostic marker. Data from the medical records were collected retrospectively and frozen blood samples from the time of renal biopsy were used to detect presence and level of serum $PLA_2R$ autoantibody. We further measured epitope-specific titers, and investigated the presence of the recently discovered THSD7A antibody in the whole patient cohort.

## 3.3 Biochemical analyses

### 3.3.1 Routine analyses

With the exception of detection of serum antibodies against $PLA_2R$ and THSD7A, and $PLA_2R$ epitope-specific antibodies, all biochemical analyses were performed as accredited routine clinical laboratory tests by the Central Laboratory at Sahlgrenska University Hospital, or Central Laboratories at the different participating hospitals in the Western part of Sweden. Urinary albumin excretion was measured, and the results are presented as total

urinary albumin (g/day) in 24-hour urine collections or albumin to creatinine ratio (mg/mmol) in spot urine specimens. Complete remission (CR) was defined as albuminuria < 300 mg/day (or urine albumin to creatinine ratio (ACR) < 30 mg/mmol). Partial remission (PR) was defined as albuminuria falling by ≥ 50% from baseline albuminuria to a level between 300 mg/day and 3.5 g/day (urine ACR < 350 mg/mmol), accompanied by a normalization of serum albumin and a stable serum creatinine. End-stage renal disease (ESRD) was defined as progression of kidney failure to eGFR < 15 ml/min/1.73 $m^2$, measured GFR by Cr-EDTA or iohexol clearance, initiation of dialysis or kidney transplantation.

### 3.3.2 Measurement of serum $PLA_2R$ and THSD7A antibodies, and $PLA_2R$ epitope-specific antibodies

Patients participating in the renal biopsy study at Sahlgrenska University Hospital are evaluated using a standardized protocol. This includes collection of relevant clinical and laboratory data at the time of renal biopsy, as well as repeated collections during the follow-up period. Serum collections at the time of renal biopsy are centrifuged and the supernatant is stored in -80° freezer, making it possible to perform retrospective analyses.

In study III, we used the frozen blood samples for detection of serum $PLA_2R$ antibodies by an indirect immunoflourescence test. This test is performed at the Central Laboratory at Sahlgrenska University Hospital, and the titers are presented as <10, 10, 100 or 1000. To confirm these data a second ELISA-based assay was performed, and a detailed description of the protocol is found in [75]. $PLA_2R$ epitope-specific ELISAs were performed as described recently [47]. Serum THSD7A antibodies were detected by western blotting of recombinant proteins (THSD7A or $PLA_2R$) as described [41, 54]. Sera from $PLA_2R$-negative patients (n=6) were used as the primary antibody, diluted at a 1:100 ratio.

## 3.4 Histopathological evaluations

All biopsy specimens were examined using light microscopy, immunohistochemistry and electron microscopy. The light microscopy picture generally showed a membranous pattern, and excluded other glomerulonephritides including lupus.

### 3.4.1 Staining for glomerular immune deposits

Immune deposits such as IgG, IgA, IgM, light chains, C1q, C3c and C5b-9, were examined using a standardized immunoperoxidase method (Dako, Copenhagen, Denmark). The EnVision™ Flex high pH (Link) detection kit (DakoK8000) was used. This is an indirect immunohistochemical technique using unlabeled primary antibodies, and the procedure is described in detail in paper I. IgG subclass antibody expression was studied using the same immunohistochemical protocol, and the review of all renal biopsies was performed by one renal pathologist (J.M.), who was blinded to the clinical and laboratory data.

### 3.4.2 Staining for glomerular $PLA_2R$ and THSD7A

Staining for $PLA_2R$ and THSD7A was performed in a series of steps by JN and KE, blinded to the clinical and laboratory data. Consecutive series of paraffin sections were produced at a 4-µm constant thickness setting, floated on a 37°C water bath, and collected on serially numbered polylysin coated glass slides (Dako). Sections were de-paraffinized in xylene-ethanol at room temperature with an endogenous peroxidase blocking step. The sections were rehydrated in phosphate buffered saline (PBS) and heat induced epitope retrieval was performed, for $PLA_2R$ in citrate buffer pH 6.2, and for THSD7A in tris-EDTA buffer pH 9.2, followed by a blocking step and incubation with a primary antibody (polyclonal rabbit anti-$PLA_2R$, dilution 1:8000 or anti-THSD7A, dilution 1:400 (Atlas Antibodies, Sweden)) over night at 4°C. POLAP (Zytomed, Germany) was used as detection system. Labeled sections were analyzed by three independent scientists in a blinded fashion and for $PLA_2R$ a scoring method where 0= negative and 1= positive staining for $PLA_2R$ was used. For THSD7A, granular staining of THSD7A in the glomerulus was considered as a positive staining.

## 3.5 Statistical methods

Statistical analyses were executed with the SPSS software package, and all results are presented as mean ± SEM. In all papers, ANOVA was used for evaluation of differences between means. In paper I, the Chi square test (Fishers´exact test) was used to compare frequency of IgG4 and $PLA_2R$ between patients with idiopathic and malignancy-associated MN. In paper I and III, non-parametric Spearman´s rank coefficient of correlation, was used to analyze association between two variables. $P \leq 0.05$ was considered being statistically significant.

# 4 RESULTS AND DISCUSSION

## 4.1 Glomerular IgG subclasses in patients with idiopathic and malignancy-associated membranous nephropathy (Paper I)

### 4.1.1 Types of malignancies

The clinical picture of patients with malignancy-associated MN did not differ compared to patients with idiopathic MN, except for age (Table 2). The most frequent types of malignancies seen in this patient cohort were prostate cancer and lung cancer, together accounting for 57% of the cases. The characteristics of all patients with malignancy-associated MN are presented in table 3. In half of the cases, the malignancy was known at the time of renal biopsy, or discovered within a month from renal diagnosis. In the rest of the cases the malignancy was discovered within two years from renal biopsy, except for one patient with prostate cancer. In this patient, prostate cancer diagnosis was confirmed 31 months after renal biopsy, but serum prostate-specific antigen was elevated at least six months prior renal biopsy.

***Table 2.*** *Baseline characteristics of all patients. eGFR, estimated GFR calculated by Modification of Diet in Renal Disease formula (MDRD).*

| | **Idiopathic MN (n = 69)** | **Malignancy-associated MN (n = 16)** | **Significance, P-value** |
|---|---|---|---|
| **Sex (male/female)** | 45/24 | 10/6 | NS |
| **Smoking (yes/no), missing data 3 patients** | 40/26 | 9/7 | NS |
| **Age (years)** | 52 ± 16 | 68 ± 10 | <0.05 |
| **Serum albumin (g/L)** | 24 ± 8 | 21 ±7 | NS |
| **Urine albumin (g/day)** | 5.4 ± 3 | 5.5 ± 3 | NS |
| **eGFR (ml/min/m$^2$)** | 82 ± 32 | 76 ± 24 | NS |
| **Time from symptoms to biopsy (months)** | 15 ± 6 (5-360) | 3 ± 1 (0.5-9) | NS |
| **Length of follow-up, months (range)** | 82 ± 5 (12-164) | 37 ± 9 (2-164) | <0.05 |

***Table 3.*** *Characteristics of patients with malignancy. CR, complete remission; PR, partial remission; S, surgery; R, radiation; H, hormonal therapy; Cs, chemotherapy including steroids; Ch, chemotherapy including alkylating agents; BMtx, bone marrow transplantation; Ig, immunoglobulin; $PLA_2R$, phospholipase A2 receptor.*

| No | Age | Malignancy | Time from symptom to diagnosis (months) | Time from biopsy to discovery of malignancy (months) | Treatment of tumor | Remission | | Glomerular | | Follow-up time (months) | Outcome |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Tumor | Proteinuria | IgG4 | PLA2R | | |
| 1 | 61 | Prostate cancer | 6 | 31 | S | Yes | PR | - | - | 148 | Alive |
| 2 | 56 | Lung cancer | 8 | 11 | Cs+R | No | No | - | - | 23 | Dead |
| 3 | 70 | Uterus cancer | 2 | 9 | No | No | No | - | - | 10 | Dead |
| 4 | 76 | Lymphoma | 1 | 6 | Cs+Ch | No | PR | - | - | 30 | Dead |
| 5 | 83 | Prostate cancer | 1 | 0 | H | No | PR | - | - | 8 | Dead |
| 6 | 65 | Prostate cancer | 4 | 0 | S | Yes | CR | - | - | 72 | Alive |
| 7 | 68 | Buccal cancer | 1 | 12 | S+R | Yes | CR | - | - | 62 | Alive |
| 8 | 58 | Lymphoma | 1 | 0 | Cs+Ch+BMtx | Yes | CR | - | + | 60 | Alive |
| 9 | 78 | Prostate cancer | 9 | 1 | H | No | No | + | - | 2 | Dead |
| 10 | 49 | Leukaemia | 3 | 0 | Cs+Ch+BMtx | No | CR | - | - | 36 | Dead |
| 11 | 60 | Prostate cancer | 0,5 | 0 | H+R | No | No | + | + | 33 | Alive |
| 12 | 66 | Lung cancer | 1 | 18 | No | No | No | + | - | 27 | Dead |
| 13 | 65 | Breast cancer | 7 | 17 | S+H | Yes | CR | + | + | 33 | Alive |
| 14 | 79 | Prostate cancer | 2 | 1 | No | No | No | - | - | 8 | Dead |
| 15 | 73 | Lung cancer | 2 | 4 | S | Yes | CR | - | - | 18 | Alive |
| 16 | 80 | Colon cancer | 5 | 0 | S | Yes | CR | + | - | 16 | Alive |

### 4.1.2 IgG subclasses

Glomerular expression of IgG4 subclass was found in 45 of 69 patients with idiopathic MN, and in 5 of 16 patients with malignancy-associated MN (Table 4), a statistically significant difference ($p<0.05$). The positive predictive value for IgG4 as an indicator of idiopathic disease was 90% (95% CI 78-97). There was no difference in staining pattern for the other IgG subclasses (IgG1, IgG2 and IgG3) between the two groups. In the malignant group, 63% of the patients were positive for IgG2 and negative for IgG4, compared with 25% of the idiopathic patients. In patients with idiopathic MN, 55% of the cases were positive for IgG4 and IgG2, compared with 31% in the malignant group. Based on this marked difference in distribution pattern between patients with idiopathic and malignancy-associated MN, a recognition category score was created. However, we were not able to more accurately predict presence of malignancy using this score, compared to the use of glomerular IgG4 expression alone.

### 4.1.3 Glomerular $PLA_2R$

Staining for glomerular $PLA_2R$ was positive in 35 of 63 patients with idiopathic MN (lacking material for staining in six cases), and in 3 of 16 patients with malignancy-associated MN, a statistically significant difference ($p<0.05$). 86% of the patients with a positive $PLA_2R$ staining also expressed glomerular IgG4. However, IgG2 was positive in 83% of the cases, but no patient with a positive $PLA_2R$ staining expressed IgG1 (Table 4).

***Table 4.*** *Result of staining for glomerular IgG subclasses and $PLA_2R$. I-MN, idiopathic MN; M-MN, malignancy-associated MN; Pos, positive; Neg, negative.*

| | **I-MN (n=69)** | | | **M-MN (n=16)** | | | |
|---|---|---|---|---|---|---|---|
| | **Pos** | **Neg** | **% Pos** | **Pos** | **Neg** | **% Pos** | **Difference between groups, P-value** |
| **IgG4** | 45 | 24 | 65 | 5 | 11 | 31 | <0.05 |
| **IgG3** | 15 | 54 | 22 | 3 | 13 | 19 | NS |
| **IgG2** | 56 | 13 | 81 | 15 | 1 | 94 | NS |
| **IgG1** | 1 | 68 | 1 | 1 | 15 | 6 | NS |
| **$PLA_2R$** | 35 | 28 | 56 | 3 | 13 | 19 | <0.05 |

## Discussion

An association between malignancy and MN has been noted for decades, and the prevalence of cancer in patients with MN in whom other secondary causes have been excluded, is in case series reported to be 6-22% [67, 76]. However, some investigators have implied that the connection is overstated. It has been suggested a potential detection bias in reports published after the initial recognition of a link between MN and cancer [76]. Thus, patients with MN and no obvious underlying secondary cause might undergo more screening for cancer, compared to the age-matched population. Another major argument against a causal relationship between malignancy and MN is that both entities are diseases of the elderly, and therefore might represent two coincidental disease processes. However, when restricting malignancy cases to only those who are clinically evident prior to or at the time of renal diagnosis, the incidence of malignancy is higher than expected, compared with an age- and sex-matched population [57]. Moreover, the finding that tumor treatment produces resolution of the nephrotic syndrome, and that a tumor recurrence is followed by a relapse of the nephrotic syndrome, further suggests a causal relationship between malignancy and MN [77-79].

In our material, the prevalence of malignancy was close to 10%, which is consistent with previous findings [57]. Historically, the connection between MN and malignancy is most frequently reported in cases with solid tumors, for example lung- and gastrointestinal carcinomas [57, 67, 80]. In more recent reports, an association between MN and prostate cancer has been detected [57, 68], which might be explained by improved diagnostics and increased detection of this often slow-growing malignancy. A majority of the patients in our study had prostate cancer (38%) or lung cancer (19%), which is within the wide range of previous studies [57, 68]. Strikingly, there was only one case of breast cancer and no cases of skin cancer, despite the fact that these cancer forms are quite common in Sweden.

There are certain pathologic features that support the claim that malignancy-associated MN is an entity distinct from idiopathic disease. Detection of serum $PLA_2R$ antibodies, that predominantly are of IgG4 subclass, is highly specific for idiopathic MN, and glomerular staining for $PLA_2R$ has emerged as another method to define $PLA_2R$-associated disease [41, 81, 82]. Whereas $PLA_2R$ localizes to the cell body and the foot processes of the normal podocyte, the antigen is preferentially detected in subepithelial deposits in idiopathic MN, where it co-localizes with IgG. Furthermore, previous studies have reported a predominance of IgG1 and IgG2 in patients with malignancy-associated MN, rather than IgG4, that is most prevalent in idiopathic MN [41, 83, 84]. In our study, we found a significant correlation between absence of

glomerular IgG4 and $PLA_2R$, and malignancy-associated MN. IgG1 and IgG3 were present in a low number of cases, while IgG2 was found in a high number of cases in both groups. Therefore, IgG2 could not be used as an indicator of underlying malignancy (and neither could IgG1 or IgG3). Three patients in the malignant group had expression of glomerular $PLA_2R$, and it cannot be ruled out that the presence of MN and malignancy in these cases was coincidental.

The trigger of MN in patients with malignancy is unclear, as well as the underlying mechanisms behind the formation of subepithelial immune deposits. Different mechanisms have been postulated, such as formation of in situ and/or circulating immune complexes, tumor antigens or extrinsic factors such as viral infections. There might be different pathologic mechanisms depending on the patient´s immune system and the type of malignancy. Recent extensive studies on $PLA_2$ have revealed nine human genes coding for secretory $PLA_2$ ($sPLA_2$) [85]. Group IIA $sPLA_2$ seems to accumulate during inflammatory conditions, and has also been found to have a direct anti-bacterial activity against gram-negative bacteria. Group IIA and IIB $sPLA_2$ are also proposed to play a role in the development of cancer, although the exact mechanism on cell proliferation is unknown. Further, it seems like the inflammatory effect of $sPLA_2$ does not require lipolytic activity, but can be secondary to a direct binding to an antigen receptor on the target cell. One could therefore speculate that certain cancer cells produce $sPLA_2$, which affects the kidneys and leads to development of MN through a yet undefined immune response that less often includes antibodies of IgG4 subclass.

The dominance of IgG4 and IgG2 in our material fits well with the notion that these two subclasses are less prone to activate complement, compared to IgG1 and IgG3 [56]. Recently a subclass switch from IgG1 to IgG4 during the disease process in patients with idiopathic MN has been proposed [84], a phenomenon for which we found no evidence in our study. The poor ability of IgG4 to induce complement and cell activation depends on a low affinity for C1q (the q fragment of the first component of complement) and Fc receptors. Besides that, IgG4 possess an ability to exchange Fab-arms, which serves as an additional mechanism for generating anti-inflammatory activity. By Fab arm exchange the IgG4 molecules lose their ability to cross-link antigen and to form immune complexes [86]. As IgG4 is the dominant IgG subclass both within glomerular deposits and as circulating form of anti-$PLA_2R$, the podocyte damage may be caused by other mechanisms than direct complement activation through the classical pathway. Thus, mannan-binding lectin (MBL) is the initiator of the lectin pathway and has been shown to activate complement in patients with rheumatoid arthritis by

binding to a glycan on the Fc portion that is deficient in galactose. Preliminary results (presented in abstract form at the annual meeting of American Society of Nephrology (ASN) in 2011) suggest that similar mechanisms may be at work in the case of IgG4 anti-$PLA_2R$. Furthermore, the possibility of a direct interaction of IgG4 on $PLA_2R$, as has been shown for IgG4 autoantibodies to myosin-specific kinase in patients with myasthenia gravis, may be another possible mechanism [87]. A majority of patients with $PLA_2R$-associated disease have serum IgG4 anti-$PLA_2R$, but a recent study described MN caused by monoclonal IgG3κ anti-$PLA_2R$, with immune deposits of C1q [88]. To conclude, to further study the pathogenesis and the role of the different IgG subclasses, in both idiopathic and malignancy-associated MN, is necessary.

# 4.2 Treatment pattern in patients with idiopathic membranous nephropathy – Practices in Sweden at the start of the millennium (Paper II)

## 4.2.1 Overall outcome

The average age of patients at the time of renal biopsy was 52±2 years (range 15-83), and the patient cohort generally showed preserved renal function at that point (Table 5).

***Table 5.*** *Demographic data of the study population. Mean ± SEM. eGFR, estimated GFR calculated by Modification of Diet in Renal Disease formula (MDRD).*

| | |
|---|---|
| Patients, number | 73 |
| Males/females | 46/27 |
| Age, years | 52 ± 2 (15-83) |
| Serum creatinine, μmol/L | 102 ± 11 (45-789) n=73 |
| eGFR, ml/min/1.73m$^2$ | 80 ± 4 (7-188) n=73 |
| Serum albumin, g/L | 24 ± 1 (8-43) n=73 |
| Urine albumin, g/day | 5.3 ± 0.4 (1-14) n=72 |
| ≤ 4 g/day | 42% |
| 4.1-7.9 g/day | 38% |
| ≥ 8 g/day | 19% |
| Serum cholesterol, mmol/L | 8.7 ± 0.6 (3.5-16.7) n=24 |
| Systolic blood pressure, mmHg | 133 ± 2 (100-155) n=35 |
| Diastolic blood pressure, mmHg | 80 ± 2 (60-100) n=35 |
| Time from symptom to biopsy, months | Median 5 (0.5-360) |
| Length of follow-up, months | 82 ± 4 (12-164) |

At the study end, 43 of 73 of the included patients had reached complete remission (CR), 12 patients were in partial remission (PR), 3 patients had persistent proteinuria with only a slight increase in serum creatinine, 10 patients had developed end-stage renal disease (ESRD) and 5 patients had relapsed from previous CR (Figure 4). In total 14 patients experienced a relapse of the nephrotic syndrome at some point during the follow-up period. Three male patients died, mainly due to cardiovascular disease.

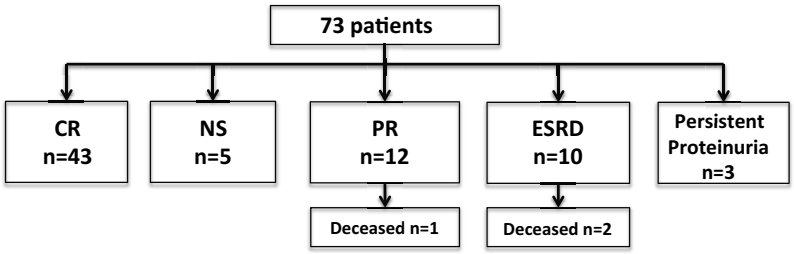


***Figure 4**. Total outcome of all patients at the study end. CR, complete remission; PR, partial remission; NS, nephrotic syndrome; ESRD, end-stage renal disease.*

### 4.2.2 Treatment

88% of the patients received supportive therapy with ACEIs and/or ARBs, and 66% of the patients received lipid-lowering therapy with HMG-CoA reductase inhibitors (statins). 49% of the patients received supportive therapy only and were not given immunosuppressive treatment, whereas 51% of the patients in this study received immunosuppressive treatment at some point during the follow-up.

All participating patients in this study were categorized into subgroups (group A-C) according to how well they fulfilled criteria for immunosuppressive treatment, based on the available clinical and laboratory data. The guidelines we used were those described by the Toronto group in 2000 [74].

**Group A** – Patients with persistent nephrotic range proteinuria, decline in renal function during follow-up, severe side effects of the nephrotic syndrome and/or ESRD at presentation.

**Group B** – Patients not fulfilling criteria for immunosuppressive treatment, still receiving treatment.

**Group C** - Patients not fulfilling criteria for immunosuppressive treatment, and not given treatment.

***Table 6.*** *Baseline albuminuria of patients of groups A-C. Data is missing from one patient (group A) who presented with end-stage renal disease and albuminuria was not measured. A large proportion of patients of each group received supportive therapy.*

| | Albuminuria (% of patients of each group) | | | Supportive therapy (% of patients receiving) | Mean follow-up time months (± SEM) |
|---|---|---|---|---|---|
| | ≤ 4 g/day | 4.1-7.9 g/day | ≥ 8 g/day | | |
| **A (n = 28)** | 26 | 55 | 19 | 75 | 69 ± 7 |
| **B (n = 15)** | 27 | 33 | 40 | 93 | 95 ± 9 |
| **C (n = 30)** | 63 | 27 | 10 | 90 | 87 ± 7 |

## Group A

28 patients were categorized into this group, of whom 22 received immunosuppressive therapy (Figure 5A). The choice of first-line treatment varied and the recommended cyclical Ponticelli regimen was not used in any case. Instead a modified version, based on the Dutch treatment scheme was used [89]. Ten patients received a second-line treatment due to resistance of the first given therapy. Six patients did not receive immunosuppressive therapy, and they all developed end-stage renal disease. The total outcome of group A patients at the study end is described in figure 5B.

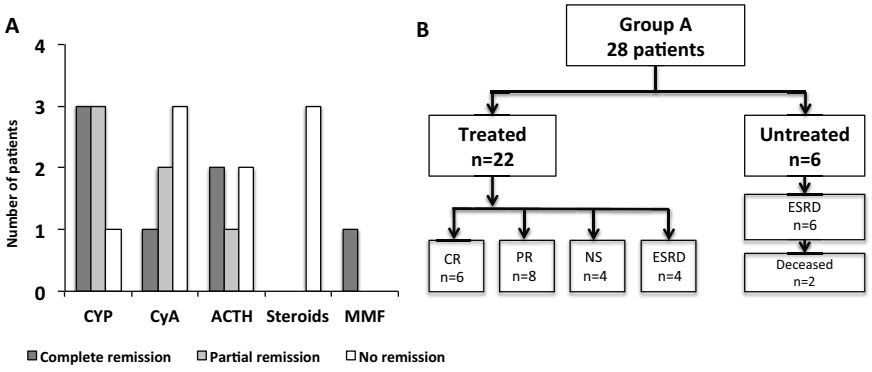


***Figure 5**. **A**) 22 of 28 patients of group A received immunosuppressive treatment, and choice of therapy varied. The bars represent the numbers of patients in complete, partial or no remission after first-line treatment. **B**) Total outcome of all patients of group A. 10 patients developed ESRD, and two of these patients died. Only 6 patients were in complete remission at the study end. CYP, Cyclophosphamide based therapy; CyA, cyclosporine; ACTH, adrenocorticotropic hormone; steroids, corticosteroids only; MMF, mycophenolate mofetil, CR, complete remission; PR, partial remission; NS, nephrotic syndrome and ESRD, end-stage renal disease.*

## Group B

We categorized 15 patients into this group, based on laboratory and clinical data collected from the medical records. The main reasons for not fulfilling criteria for immunosuppressive treatment were subnephrotic proteinuria (tU-albumin 2.0, 2.2, 2.4 and 2.5 g/day) at the time of initiation of therapy, and short time from renal biopsy to initiation of treatment. In 11 patients immunosuppressive therapy was initiated within 2 months from renal biopsy, in some cases a few days after the renal diagnosis. In none of the cases we found information regarding initiation of therapy at this early point. 7 of 15 patients received corticosteroids only, and the choice of first-line treatment is presented in figure 6A. At the study end, 14 patients had achieved complete remission and one patient partial remission (Figure 6B).

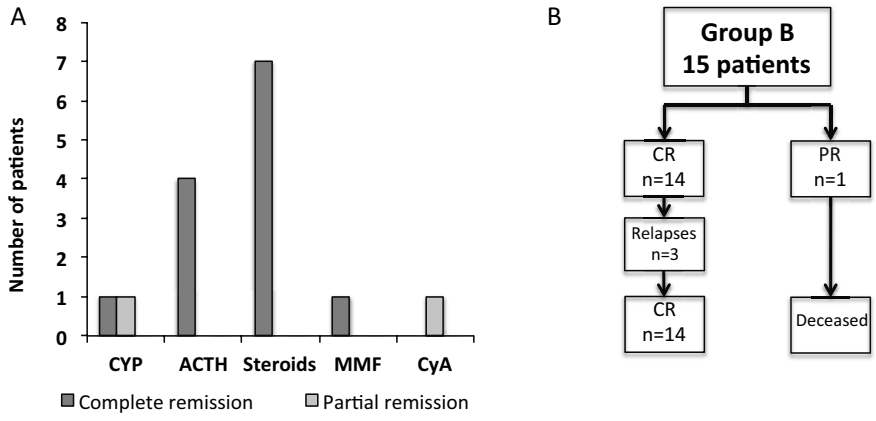


***Figure 6**. **A)** All 15 patients of group B received immunosuppressive therapy, and the bars represent numbers of patients in complete and partial remission after first-line treatment. **B)** Total outcome of group B patients at the study end. 14 patients had then achieved complete remission, and one patient with partial remission had died. CR, complete remission; PR, partial remission and ESRD, end-stage renal disease.*

## Group C

30 patients were categorized into this group, and at the study end a majority of the patients had attained complete (23 patients) or partial (3 patients) remission (Figure 7). One patient had persistent low-grade proteinuria during follow-up, but did not fulfill criteria for neither complete nor partial remission. Three patients, who initially reached complete remission, subsequently relapsed and were under follow-up with supportive treatment at the study end.

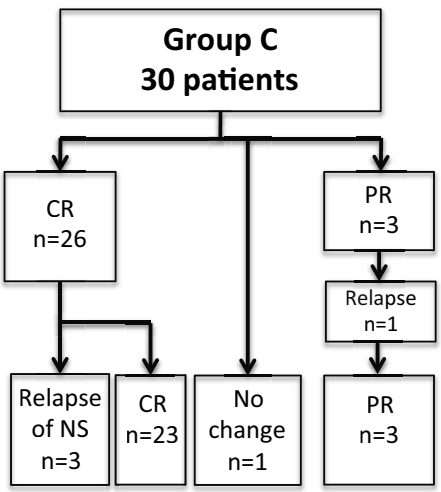


***Figure 7.** Outcome of group C patients at the study end. 26 patients had achieved remission and 3 patients were under follow-up with conservative treatment. CR, complete remission; PR, partial remission; NS, nephrotic syndrome.*

### 4.2.3 Relapses

14 of 73 patients experienced a relapse of the nephrotic syndrome at some point during the follow-up period (Table 7). Most relapses were seen among group A patients (7 cases), but relapses did also occur within group B and C (3 and 4 cases respectively).

***Table 7.*** *Relapses of the nephrotic syndrome during the follow-up period. Median time from previous remission to relapse was 22 months. Treatment of relapse was influenced by previous treatment; no patient received the same immunosuppressive therapy twice. Five patients relapsed shortly prior to the study end and received supportive therapy at that point. CR, complete remission; PR, partial remission; NS, nephrotic syndrome; ESRD, end-stage renal disease; ACTH, adrenocorticotropic hormone; MMF, mycophenolate mofetil; CyA, cyclosporine; Ritux, Rituximab; CYP, cyclophosphamide based therapy, ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker.*

| Previous treatment | Patients | Group | Previous remission | Relapse (months after remission) | Treatment of relapse | Outcome at study end |
|---|---|---|---|---|---|---|
| **No specific treatment** | 1 | C | CR | 30 | ACEI | NS |
| | 2 | C | CR | 20 | ACEI | NS |
| | 3 | A | CR | 22 | CyA/MMF | PR |
| | 4 | C | CR | 31 | ACEI | NS |
| **ACTH** | 5 | B | CR | 55 | ARB | CR |
| | 6 | A | CR | 41 | ACEI | NS |
| | 7 | A | CR | 32 | No | NS |
| | 8 | B | CR | 11 | No | CR |
| **Cyclophosphamide** | 9 | A | CR | 21 | CyA | ESRD |
| | 10 | A | CR | 39 | CyA | ESRD |
| **Corticosteroids only** | 11 | B | CR | 1 | ACTH | CR |
| **MMF** | 12 | A | CR | 2 | No | CR |
| **No specific treatment** | 13 | C | PR | 120 | No | PR |
| **CyA, Ritux** | 14 | A | PR | 8 | CYP | PR |

## Discussion

At the end of follow-up period 75% of the patients had achieved remission and 10% had developed ESRD. Previous studies report a better cumulative outcome and less cases of ESRD, despite a higher proportion of patients with nephrotic range proteinuria [90]. Even in patients with subnephrotic range proteinuria, not receiving immunosuppressive therapy, outcome is reported to be better [91]. In our material, 42% of the patients had proteinuria < 4 g/day and immunosuppressive treatment was given in 51% of the cases. 88% of the patients received supportive treatment, which is a high rate compared to other studies [92]. Outcome could therefore be expected to be better, which raises questions regarding implementation of treatment guidelines into clinical practice.

Not surprisingly, the best outcome was seen among patients with low-grade proteinuria at presentation, and/or declining proteinuria and a stable serum creatinine during follow-up period, group C patients. This result is consistent with previous reports [93, 94]. The outcome of group B patients was also excellent; 14 of 15 patients attained complete remission, despite the fact that a greater proportion of patients with heavy proteinuria were found in this group. All of these patients received immunosuppressive therapy, which was initiated despite low-grade proteinuria (< 3 g/day, measured as albuminuria) and/or short time from renal biopsy (0-2 months). The medical records provided no information regarding initiation of therapy in these patients, such as presence of severe, disabling or life-threatening symptoms of the nephrotic syndrome, persistent proteinuria or rapid decline in renal function. Since almost one third of patients with idiopathic MN attain remission without specific treatment [93], and patients with low-grade proteinuria have a more favorable outcome, these factors have probably influenced the outcome of group B patients. Moreover, according to our definitions and current guidelines, these patients were therefore over-treated with immunosuppressive therapy.

The least favorable outcome was seen in patients in whom conservative therapy was inefficient and/or combined with other features indicating a poor prognosis, group A. Of 28 patients, 22 patients received immunosuppressive therapy at some point. Despite that, only 14 patients had attained remission at the study end, the rest of the patients had persistent proteinuria (4 patients) or had developed ESRD (4 patients). Of group A, 10 patients developed ESRD, and six of these patients were not given any immunosuppressive therapy. In four of these cases, the patients presented with ESRD or low eGFR, or had co-morbidity contraindicating this kind of heavy medication. However, in two cases the patients might have benefited from immunosuppressive therapy

due to a progressive increase of serum creatinine during follow-up time, and an eventual loss of renal function. Despite a thorough investigation of the medial records, information regarding why treatment was withheld in these cases, could not be found.

In this study, the choice of immunosuppressive therapy varied, both within the clinics, but also between the different clinics, and some issues regarding the choice of therapy need to be highlighted. As previously mentioned, the cyclical Ponticelli regimen was not used in any case. Instead, a modified version based on the Dutch treatment scheme [89], was used. After the initial intravenous doses, cyclophosphamide and corticosteroids are administered orally every day, which probably facilitates coherence to the treatment, and makes it the preferential choice of therapy.

Another interesting finding is the frequent use of corticosteroids only, a phenomenon found in especially group B. The choice of this treatment might have been influenced by earlier studies, indicating a beneficial effect after two to three months of alternate-day prednisone. However, long-term beneficial effect has not been shown, and treatment with corticosteroids only was not recommended after year 2000 [74, 95-97].

ACTH was administered to 11 patients as subcutaneous injections (in nine patients as first-line therapy and in 2 patients as second-line therapy). ACTH has been shown to reduce proteinuria in patients with idiopathic MN [98, 99], and similar results have been obtained with natural ACTH gel in a small observational study. The mechanisms of action of ACTH may be related to the stimulation of the melanocortin receptors with inhibition of immune and inflammatory response, or a direct action of podocyte stability [100]. ACTH treatment is not recommended as first- or second-line therapy [33], but the patients in our study were also participating in a randomized controlled trial of ACTH treatment, which explains the high proportion of patients receiving this therapy. Some investigators have proposed ACTH to be a reasonable option of treatment for patients with contraindications to or who do not respond to cyclical alkylating steroid-therapy or calcineurin inhibitors [36]. However, in our material, after initial treatment attempts with these drugs, mycophenolate mofetil or rituximab seemed to be the preferred choices of therapies.

Rituximab, a monoclonal antibody to CD20 expressed on B-cells, has in observational studies been shown to significantly reduce proteinuria in patients with idiopathic MN, both in treatment naïve-patients and patients previously treated with other regimens [101-103]. Some investigators

propose rituximab to be the best first-line choice of treatment, due to a more favorable safety profile and better tolerance, compared with steroids and alkylating agents [104]. They also propose that in case of treatment failure, exposure to other toxic treatments should probably be avoided. However, some issues regarding rituximab treatment in patients with idiopathic MN are yet to be discovered. At this point, the optimal doses, spacing and duration of re-treatment, as well as the long-term benefit to harm ratio, and the value of monitoring of circulating B cell number, need further investigation.

By reviewing the medical records of the included patients, we could identify two major challenges in the clinical management of patients with idiopathic MN. Firstly, who should be given immunosuppressive therapy? As the clinical picture can follow a variety of courses, and a large proportion of patients with idiopathic MN achieve remission without treatment, a prognostic factor at the presentation of the disease would certainly facilitate the clinical practice. The levels of serum $PLA_2R$ antibodies have been shown to correlate with clinical disease activity, and their disappearance often heralds a subsequent decline in proteinuria [65, 72], which can be a useful tool to guide treatment. Furthermore, recent studies have suggested the autoantibody level at presentation to be a prognostic marker for the clinical outcome [64, 73], a promising finding and an eventual further tool in the management of patients with idiopathic MN.

Secondly, the treatment strategies varied, both between and within the different clinics. The same pattern has previously been shown in a study from US [92], which indicates the difficulties of implementing guidelines into clinical practice. The Swedish treatment recommendations during the actual time period were influenced by international guidelines, annually discussed on post-graduate courses and readily available in a Swedish text-book from 1997 [105]. In 2012, the KDIGO guidelines on glomerulonephritis treatment were published. Despite easy to find, on-line treatment guidance, the implementation of these guidelines still seems to be incomplete. A recent study reported a large variability in treatment, and a lack of standardized care tools and treatment protocols, and lack of physician access to educational glomerulonephritis rounds as possible explanations for the incomplete usage of the KDIGO guidelines [106]. Therefore, in order to implement treatment guidelines, minimize over-treatment, and improve best practice management, a collegial board for treatment discussions is a reasonable step to take. An alternative strategy to improve quality would be to centralize all treatment of Orphan diseases within nephrology to 1-3 specialized centers for a country with the size of Sweden (9 million inhabitants).

## 4.3 Initial anti-phospholipase A2 receptor antibody levels predict clinical outcome in patients with idiopathic membranous nephropathy (Paper III)

### 4.3.1 Anti-$PLA_2R$ antibodies and glomerular $PLA_2R$ deposits

The patient cohort generally showed a preserved renal function at the time of renal biopsy, and mean age at the presentation of the disease was 51±3 years (Table 8).

***Table 8.*** *Baseline characteristics of the patient cohort at the time of renal biopsy. The data are given as mean ± SEM. eGFR, estimated GFR calculated by Modification of Diet in Renal Disease formula (MDRD); $PLA_2R$, phospholipase A2 receptor.*

| | |
|---|---|
| Patients, numbers | 25 |
| Males/females | 16/9 |
| Age, years | 51 ± 3 |
| Creatinine, µmol/L | 82 ± 4 |
| eGFR, ml/min/$m^2$ | 85 ± 4 |
| Serum albumin, g/L | 22 ± 1 |
| Urine albumin, g/day | 4.7 ± 0.5 |
| Serum cholesterol, mmol/L | 8.4 ± 0.6 |
| Systolic blood pressure, mmHg | 130 ± 4 |
| Diastolic blood pressure, mmHg | 77 ± 2 |
| Time from symtoms to biopsy, months | 6 ± 1 |
| $PLA_2R$ antibody positive | 5.5 ± 1 |
| $PLA_2R$ antibody negative | 6.4 ± 2 |
| Length of follow-up, months | 63 ± 8 |

Serum autoantibodies against $PLA_2R$ at the time of renal biopsy were detected in 16 of the included 25 patients (Table 9) with the immunoflourescence test. There was a significant statistical correlation between antibody level and clinical outcome in terms of remission of proteinuria ($r=0.6$, $p<0.01$). Thus, patients with higher antibody levels at presentation were less likely to attain remission, compared to patients with lower antibody levels. As presented in table 9, patients with antibody levels of 100 or 1000, were also more frequently exposed to immunosuppressive therapy, compared to patients with low or undetectable autoantibodies at the time of renal biopsy. A second commercially available ELISA-based assay was performed, which confirmed the levels of $PLA_2R$ autoantibodies (Table 9). In one patient with $PLA_2R$ antibody level of <10, anti-$PLA_2R$ was

detected (patient no 17) with this ELISA-based assay. On the other hand, in one patient with $PLA_2R$ antibody level of 10, no antibodies were detected with the ELISA-based assay.

In 23 of the 25 cases, the renal biopsy material was sufficient to perform additional immunohistochemical staining. Glomerular deposits of $PLA_2R$ were detected in 15 of these patients, but only 13 of these patients had detectable serum $PLA_2R$ antibodies at that point. In addition, in two patients with undetectable serum autoantibodies, glomerular $PLA_2R$ deposits were found. Thus, 19 of 25 (76%) of the patients in this cohort were considered having a $PLA_2R$-associated MN.

### 4.3.2 THSD7A autoantibodies

One patient among the six $PLA_2R$-negative patients had a positive glomerular staining for thrombospondin type-1 domain-containing 7A (THSD7A), and detectable serum antibodies against THSD7A. However, serum THSD7A autoantibodies were not detected in any of the $PLA_2R$-positive patients (Table 9).

### 4.3.3 Antibodies targeting the CysR, CTLD1 and CTLD7 domains

All 17 patients (lacking data in one patient) with detectable $PLA_2R$ antibodies recognized CysR, except in one case (Table 9). Recognition of further epitopes was found in 12 of these patients; CysR, CTLD1 and CTLD7 (CRC1C7) in four patients, CysR and CTLD1 (CRC1) in two patients and CysR and CTLD7 (CRC7) in six patients. The eight patients with undetectable $PLA_2R$ antibodies were also negative for epitope-specific antibodies (lacking data in one patient).

Of the 12 patients with recognition of at least two epitopes, nine patients received immunosuppressive therapy at some point during the follow-up period (Table 9). At the study end, one patient had developed ESRD, 2 patients had on-going nephrotic syndrome, four patients had attained PR, and five patients had reached CR. In patients with reactivity against CysR only (three patients) and undetectable autoantibodies (eight patients) immunosuppressive therapy was given in two cases, and at the study end one patient had attained PR, and ten patients were in CR (Table 9).

***Table 9.*** *Baseline clinical and laboratory data of all patients at the time of renal biopsy and at the study end. The patients are divided into groups according to serum phospholipase A2 receptor ($PLA_2R$) antibody level at presentation. The mean follow-up period of the group of patients with antibody level <10, was 20 months shorter compared to patients with detectable antibodies. Still, there were less cases of complete remission in the groups of higher antibody levels (100-1000). eGFR, estimated GFR; tU-alb, total urine albumin; Glom, glomerular; THSD7A, thrombospondin type-1 domain-containing 7A; P+, $PLA_2R$ antibody-positive; T+, THSD7A-positive patient; DN, double negative ($PLA_2R$ and THSD7A); CysR; Cys-positive; CRC1, CysR- and CTLD1-positive; CRC7, CysR- and CTLD7-positive; CRC1C7, CysR-, CTLD1- and CTLD7-positive; CyA, cyclosporine; CYP, cyclophosphamide based therapy; ACTH, adrenocorticotropic hormone; RTX, rituximab; MMF, mycophenolate mofetil; CR, complete remission; PR, partial remission; NS, nephrotic syndrome; ESRD, end-stage renal disease. * indicates a relapse of proteinuria during follow-up.*

| | Presentation | | | | | | | | | | | Treatment and outcomes | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Patient | Age | Sex | eGFR (ml/min/$m^2$) | tU-alb (g/day) | Urine alb/crea (mg/mmol) | Glom $PLA_2R$ | Glom IgG4 | Serum $PLA_2R$ antibody level | Serum antibodies | Serum $PLA_2R$ antibody (ELISA OD450 nm) | Epitopes | Specific treatment | Outcome at study end | Serum $PLA_2R$ antibody level | Follow-up time (months) |
| 1 | 33 | M | 82 | 9.8 | | + | + | 1000 | P+ | 4.04 | CRC1C7 | CyA | PR | <10 | 51 |
| 2 | 63 | M | 93 | 7.9 | | missing | + | 1000 | P+ | 6 | CRC1C7 | CYP, CyA | PR | missing | 38 |
| 3 | 70 | M | 55 | 6.2 | | + | + | 1000 | P+ | 4.41 | CRC1 | CYP | PR | <10 | 37 |
| 4 | 36 | M | 87 | 3.2 | | + | + | 100 | P+ | 5.02 | CRC7 | CyA, CYP, RTX MMF | NS | 10 | 37 |
| 5 | 30 | F | 116 | 4.6 | | + | + | 100 | P+ | 2.59 | CysR | No | PR* | 100 | 104 |
| 6 | 59 | F | 66 | 4.0 | | - | + | 100 | P+ | 2.31 | CRC1C7 | ACTH | ESRD | <10 | 111 |
| 7 | 53 | M | 66 | 6.6 | | + | + | 100 | P+ | 1.71 | CRC1C7 | CyA | CR | <10 | 15 |
| 8 | 40 | F | 100 | | 277 | + | + | 100 | P+ | 0.48 | CRC7 | CyA | PR | <10 | 24 |
| 9 | 46 | M | 124 | 5.7 | | + | + | 100 | P+ | 0.84 | CRC7 | ACTH | NS* | 100 | 93 |
| 10 | 45 | M | 84 | 1.8 | | - | - | 100 | P+ | 0.34 | CysR | No | CR | 10 | 28 |
| 11 | 67 | M | 74 | 4.0 | | missing | missing | 100 | P+ | 0.64 | CysR | No | CR | missing | 107 |
| 12 | 36 | F | 81 | 2.5 | | + | + | 100 | P+ | missing | missing | ACTH | CR* | <10 | 141 |
| 13 | 63 | M | 54 | 6.3 | | + | + | 10 | P+ | 0.75 | CRC7 | No | CR | <10 | 35 |
| 14 | 38 | F | 117 | 2.0 | | + | + | 10 | P+ | 0.31 | CRC7 | No | CR | <10 | 66 |
| 15 | 61 | F | 74 | 4.5 | | + | + | 10 | P+ | 0.2 | CRC1 | CYP, CyA | CR | <10 | 23 |
| 16 | 72 | M | 49 | 4.8 | | + | - | 10 | P+ | - | - | CYP | CR | <10 | 49 |
| 17 | 44 | M | 90 | 4.4 | | + | + | <10 | P+ | 0.45 | CRC7 | No | CR | missing | 96 |
| 18 | 36 | F | 124 | | 1000 | + | - | <10 | DN | - | - | No | CR | <10 | 31 |
| 19 | 32 | F | 74 | 7.1 | | + | + | <10 | DN | - | - | No | CR | missing | 28 |
| 20 | 42 | F | 94 | 1.8 | | - | + | <10 | - | missing | missing | No | CR | <10 | 146 |
| 21 | 54 | M | 83 | 9.1 | | - | + | <10 | T+ | - | T+ | ACTH, CyA, RTX | CR | <10 | 124 |
| 22 | 69 | M | 84 | 3.1 | | - | + | <10 | DN | - | - | No | CR* | <10 | 92 |
| 23 | 61 | M | 92 | 3.2 | | - | - | <10 | DN | - | - | No | CR | <10 | 59 |
| 24 | 57 | M | 73 | 2.3 | | - | + | <10 | DN | - | - | No | CR | <10 | 22 |
| 25 | 64 | M | 88 | | 410 | - | - | <10 | DN | - | - | No | CR | <10 | 18 |

## Discussion

In our material, we found that high serum $PLA_2R$ autoantibody levels at presentation indicate a less favorable clinical outcome, a result consistent with previous studies [64, 73, 107]. Interestingly, the amount of proteinuria at presentation did not correlate with the $PLA_2R$ levels. We could therefore not define a potential pathogenic role of $PLA_2R$ levels, despite the fact that the levels seem to influence the clinical response.

In patients (n=12) with antibody levels of 100 or 1000 at presentation, only four patients had attained complete remission at the end of follow-up period. This should be compared to patients with antibody levels of 10 or < 10 (n=13). All these patients were in complete remission at the study end, and at that point, autoantibodies were undetectable in serum (lacking data in two cases). Time from renal biopsy to remission of proteinuria was in median 16 months, and did not differ between patients with low antibody levels (< 10 and 10) and patients with higher antibody levels (100 and 1000). However, it should be noted that fewer patients with higher antibody levels reached complete remission, despite an extended exposure to immunosuppressive therapy in this group. Only 3 of 13 patients with antibody levels of 10 or <10, received immunosuppressive therapy, compared to 9 of 12 patients with higher antibody levels.

In most patients with antibody levels of 100 and 1000, a decline in proteinuria followed a decline in antibody levels (lacking data in two cases). However, in three patients with residual proteinuria at the study end, antibodies were undetectable at that point. This might reflect a time lag between reduction of autoantibody levels and proteinuria. Thus, recovery of the altered podocyte structure and damaged basement membrane needs to occur, before proteinuria can decline. On the other hand, a prolonged disease period might lead to tubulointerstitial damage and secondary glomerular changes, leading to an eventual loss of renal function. One patient, who developed end-stage renal disease, had undetectable autoantibody level at the study end, and experienced a subsequent decline in proteinuria during the follow-period (111 months). This might reflect a progressive loss of renal function, with a subsequent decline in proteinuria, due to reduced glomerular filtration rate.

In our cohort of patients, 17 of 25 patients had detectable $PLA_2R$ autoantibodies at the time of renal diagnosis. By adding the glomerular $PLA_2R$ staining results, 19 of 25 patients (76%) of the patients were considered having a $PLA_2R$-associated MN. This result is consistent with previous studies from Europe and US [41, 65, 72, 82]. Some investigators

have previously proposed that patients negative for serum $PLA_2R$ antibodies and glomerular $PLA_2R$, would be classified as having a secondary MN [81]. Despite a thorough investigation of our patient material it cannot be ruled out that some of our patients wrongly have been classified as idiopathic. However, it is more likely that antigens other than $PLA_2R$ are relevant in these cases.

The recent finding of the THSD7A as an antigen in 2.5-5% of patients with idiopathic MN, indicates a subgroup of patients with idiopathic disease [54]. The clinical picture of patients with $PLA_2R$-related disease is similar to that of THSD7A-associated MN, as antibody levels disappear before full resolution of the clinical disease activity. Moreover, the majority of THSD7A antibodies are of IgG4 subclass, and THSD7A and $PLA_2R$ have quite the same biochemical and structural properties. In our material, none of the patients with $PLA_2R$-associated MN had serum antibodies targeting THSD7A. However, one of the six $PLA_2R$-negative patients had THSD7A antibodies and a positive glomerular staining for THSD7A (Patient no 21, table 9). This particular patient received three courses of different immunosuppressive therapy before resolution of disease activity, but outcome was excellent; at the study end complete remission was achieved and sustained.

In our patient material, all but one sera from patients with detectable $PLA_2R$ antibodies, recognized the CysR domain of the $PLA_2R$. This result is consistent with a previous study [47] and indicates that CysR is the dominant epitope of the $PLA_2R$. Furthermore, patients with recognition of two or three epitopes were more exposed to immunosuppressive therapy compared with patients with low or undetectable antibody levels. In this patient cohort, except for one patient, the indications for initiation of immunosuppressive therapy were persistent heavy proteinuria, decline in renal function during the follow-up period, and/or severe side effects of the nephrotic syndrome. The clinical outcome was also less favorable compared to patients with reactivity against only one epitope, or patients with undetectable autoantibodies. A previous recent study identified CTLD1 and CTLD7 antibodies as risk factors for a poor renal prognosis, while reactivity against CysR only was associated with a stable and mild disease. Our patient material is too small for further statistical analysis, but there seems to be a clinical relationship between higher levels of $PLA_2R$ antibodies, recognition of more than one epitope and a less favorable clinical outcome. In the future, maybe epitope profiling, in addition to measurement of $PLA_2R$ antibodies, could be used as a prognostic factor in the clinical practice.

# 5 CONCLUDING REMARKS

The clinical approach to patients with membranous nephropathy requires responding to a series of questions. In this thesis, three major challenges in the management of these patients have been highlighted and discussed.

## 5.1 Paper I

The aim of paper I was to test the hypothesis that patients with malignancy-associated MN could be identified based on the lack of glomerular staining for IgG4 and $PLA_2R$.

We found a statistically significant association between absence of glomerular IgG4 and $PLA_2R$ and malignancy-associated MN. Glomerular IgG2 was present in a majority of all included patients, and could therefore not be used as an indicator of an underlying malignancy. Furthermore, as IgG1 and IgG3 were present in a low number of cases, we found no evidence for an IgG subclass switch during the disease process.

A majority of the malignancies were solid organ cancers, especially prostate and lung cancer, and except for age, we found no differences in baseline characteristics between patients with idiopathic and malignancy-associated MN. Even if the recent landmark discovery of $PLA_2R$ being the major target antigen in idiopathic MN, we still lack a reliable tool for identification of patients with an underlying malignancy. We therefore conclude, that in patients negative for glomerular IgG4 and $PLA_2R$, suspicion of an underlying cancer should be raised, especially in the older patient.

## 5.2 Paper II

The aim of paper II was to investigate the treatment pattern of patients with idiopathic MN in clinical practice.

We found that a high proportion of the patients received supportive therapy, and at the study end, 75% of the patients had attained remission and 14% had developed ESRD. Immunosuppressive therapy was given to 51% of the patients at some point during the follow-up period, and compared to other studies, the number of patients that developed ESRD, was higher than expected. In some cases, immunosuppressive therapy was withheld due to contraindications, but in some cases the patients might have benefited from

specific therapy. Furthermore, we found a tendency to start treatment at an early point, instead of awaiting a spontaneous remission, and non-recommended therapy was used in a high proportion of these patients.

We conclude that a prognostic marker at the presentation of the disease would certainly facilitate the management of patients with idiopathic MN. Moreover, to implement current guidelines, we suggest a collegial board for treatment discussions and further education and support of the treating physician.

## 5.3 Paper III

The aim of paper III was to test the hypothesis that a high serum $PLA_2R$ antibody level at presentation indicates a less favorable prognosis.

We found a significant correlation between a high $PLA_2R$ antibody level and a less favorable clinical outcome. Compared to patients with lower antibody levels (<10 or 10), patients with higher antibody levels (100 or 1000) were more exposed to immunosuppressive treatment. Despite that, complete remission at the study end was attained in only a few patients with higher antibody levels, and one patient had at that point developed ESRD. We further measured the epitope-specific titers of CysR, CTLD1 and CLTD7, and found CysR to be the most common epitope in our material. The phenomenon of epitope spreading was more prominent in patients with higher $PLA_2R$ antibody levels.

We conclude that there seems to be a clinical relationship between a high $PLA_2R$ antibody level, recognition of more than one epitope, and a less favorable clinical outcome.

# 6 FUTURE PERSPECTIVES

During the recent decade, substantial advances in understanding of the pathogenesis of human MN have been made, such as the landmark discovery of $PLA_2R$ being the major target antigen in patients with idiopathic MN. A further understanding of the disease pathogenesis in MN, and identification of other antigens of importance, will hopefully continue to improve diagnosis and monitoring of this fascinating, yet challenging disease. Many questions are still to be elucidated:

- The initiating events leading to production of $PLA_2R$ antibodies

A recent genome-wide study presented a highly significant association between idiopathic MN and the risk alleles of HLA-DQA1 and $PLA_2R$ [51]. HLA-DQA1 is a heterodimer (consisting of an alpha chain and a beta chain), which is anchored in the membrane and plays an important role in the immune system by presenting epitope peptides derived from extracellular antigenic proteins. Both the alpha chain and the beta chain contain polymorphisms that determine peptide-binding specificities, and sequence variance in HLA-DQA1 could therefore contribute to altering the specificity of immunogen presentation [108, 109]. The antibody response in MN might therefore be a result of presentation of a mutated $PLA_2R$ epitope by an "idiopathic MN" HLA-DQA1 allele. Maybe a third factor, such as an infection, is additionally required to development of disease [110].

- The phenomena of epitope-spreading in MN; why does it occur, and can it be prevented?

Anti-CysR antibodies are more frequently seen in younger patients, who are probably at the beginning of the disease, and anti-CysR restricted activity is associated with a stable and mild disease, despite detectable anti-$PLA_2R$ antibody activity [47]. On the contrary, anti-CTLD1 and CTLD7 antibodies seem to disappear during disease remission and reappear during disease relapse, and are associated with a poor clinical outcome. Several common genetic variants associated with idiopathic MN have been localized in the domains of CysR, CTLD1 and CTLD7. CysR appears to be the primary dominant epitope and intramolecular spreading in the $PLA_2R$ toward the C-terminal end maybe initiated by a second immune challenge such as an infection, resulting in a more active disease [47]. Apparently, this is not the case for all patients with idiopathic MN, as 1/3 of the patients achieve complete remission and have a very favorable prognosis [61]. One can

therefore speculate that a genetic susceptibility is necessary in the cases where an active disease is linked with epitope spreading.

• How the knowledge of the $PLA_2R$ antibody level being a prognostic marker for clinical outcome can contribute to an update of current clinical guidelines, in order to optimize timing of immunosuppressive treatment

The KDIGO GN-treatment guidelines [33] were presented in 2012 and based on current knowledge regarding renal prognostic factors at that point. Since the finding of $PLA_2R$ being the major target antigen in 2009, seven years have passed, and the number of studies on idiopathic MN and if/how the $PLA_2R$ antibody level can be used in the clinical practice is still increasing. Our data on the initial $PLA_2R$ antibody level being a predictor for clinical outcome support recent studies, and can hopefully contribute to the up-coming update of international treatment guidelines.

• When will we see new, more specific and effective treatment with less side effects than the therapeutic options we have today?

The ultimate goal of treatment is termination of immune response to $PLA_2R$ or other antigens, but slow decline of antibody titers after treatment can place the podocyte at risk for prolonged injury. Complement inhibition with newer generations of complement inhibitors might be one possible way to interrupt the effector mechanisms of anti-$PLA_2R$ until immunological remission occurs [111]. However, the optimal treatment would probably be targeted deletion of B-cells and plasma cells producing pathogenic antibodies, but such targeted therapy is not yet available in clinical practice. Recently, a new mechanism to eliminate antigen-specific B cells was presented, in which nanoparticles containing the antigen and a carbohydrate ligand for SigLec, confers an inhibitory signal to the B cell receptor when complexed with antigen [112]. In the future, other specific interventions, such as development of antibody traps may become feasible, now when the primary epitope seems to be defined. However, epitope spreading to other parts of the $PLA_2R$ may make this treatment strategy quite challenging.

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