IMMUNE DYSFUNCTION IN NEPHROTIC SYNDROME

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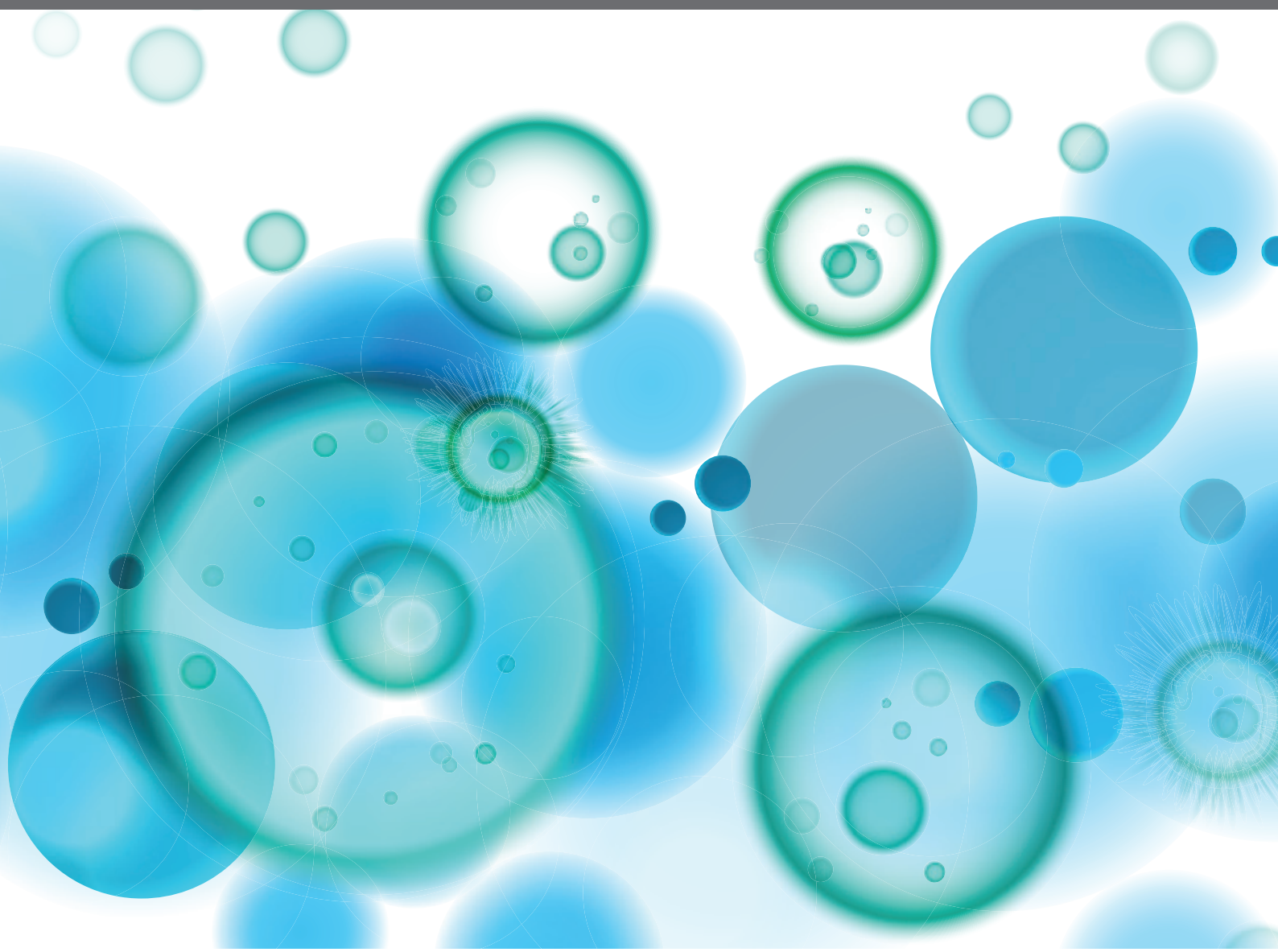

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IMMUNE DYSFUNCTION IN NEPHROTIC SYNDROME

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Editorial: Immune dysfunction in nephrotic syndrome - recent advances and new roads ahead

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Editorial on the Research Topic
Immune dysfunction in nephrotic syndrome

Introduction

Nephrotic syndrome (NS) is a clinical condition characterized by proteinuria, a reduction in serum levels of albumin and other proteins, edema, hypercholesterolemia, and often predisposition to thrombosis. Prerenal acute kidney injury or acute tubular necrosis may occur in most serious cases. NS occurs in children and adults as a consequence of specific pathological conditions that have age-specificity and that are characterized by certain common histological findings, such as the effacement of podocyte foot processes and loss of the slit diaphragm architecture, leading to a disruption of the glomerular filtration barrier and loss of plasma proteins into the urine (Figure 1). The resulting clinical signs and symptoms are consequences of the urinary loss of transport proteins, coagulation factors, metabolites and others.

Underlying pathological renal lesions associated with NS have been categorized over the past decades. Membranous nephropathy (MN), minimal change disease (MCD) and focal segmental glomerulosclerosis (FSGS) represent the most common primary glomerular disorders underlying a nephrotic syndrome and now build the basis for a morphologic classification that has the merit to create a common reference

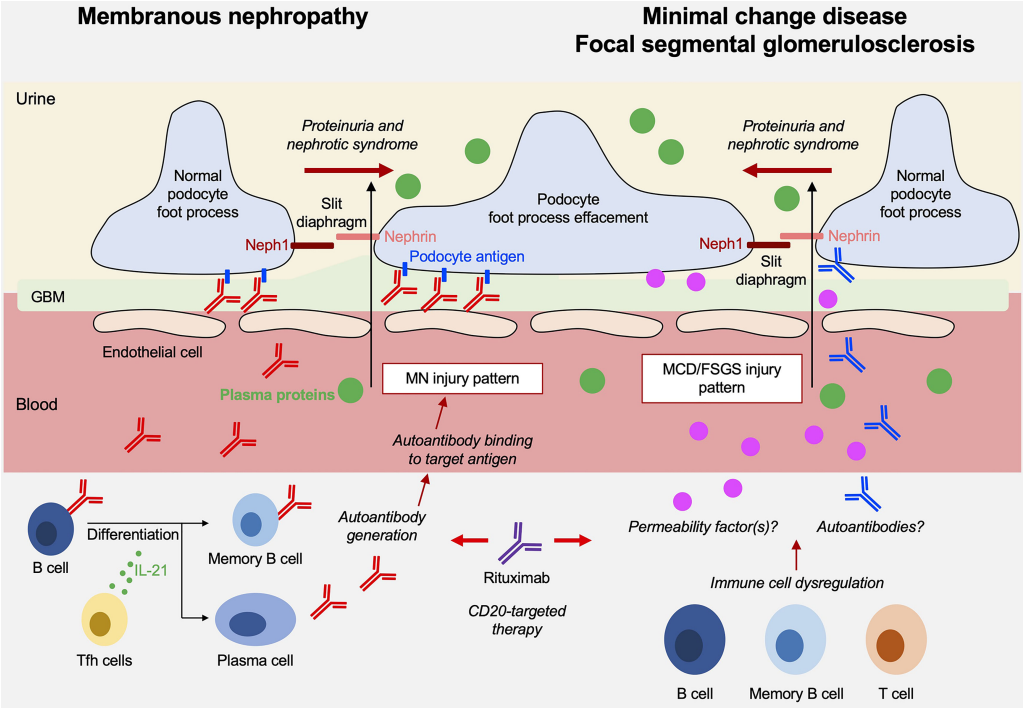

FIGURE 1
Overview on the pathogenesis of membranous nephropathy (MN) and minimal change disease (MCD)/focal segmental glomeruloscleroses (FSGS).

for clinicians and a starting point for studies on therapies. The underlying molecular mechanisms are various and include genetic modifications, degenerative and infectious causes, inflammatory conditions, and autoimmunity, but unknown causes represent an empty chapter that still needs be filled despite almost 50 years of research in the field. It is remarkable that diseases characterized by different pathology backgrounds (e.g. MN, MCD, FSGS patterns) are still often treated with the same drugs. This highlights the need to define NS and its major causative entities on a deeper molecular level and justifies a special issue in *Frontiers in Immunology* dedicated to 'Immune dysfunction in nephrotic syndrome'.

The indications of the special issue were wide but have been intended by Authors to be limited to those conditions with a clear immunologic origin and MN had the major interest. This is justified by recent discoveries of disease-specific circulating autoantibodies (1–10). MCD/FSGS and more in general childhood idiopathic NS were second in the number of papers. The pathogenesis of MCD and FSGS have not yet been resolved, and the major focus was on immunomodulatory therapies targeting CD20, which have been developed over the last few years which and are linked with the production of antibodies. They suggest a key role of B lymphocytes in MCD/FSGS pathogenesis that deeply has modified our knowledge about these two glomerular diseases. Points of interest that bridge MN with MCD/FSGS are the recent discovery of anti-nephrin antibodies in the latter condition (11) that would become, if the finding will be confirmed, an antibody-mediated autoimmune disease, further justifying the use of anti-CD20 monoclonal antibodies.

Membranous nephropathy

Primary MN is one of the most frequent causes of nephrotic syndrome in adults without diabetes. It is histologically characterized by granular deposition of IgG and complement factors at the glomerular filtration barrier, a finding that has long indicated a critical role of autoantibodies in the pathogenesis of this disease. In 2009, the identification of antibodies targeting PLA2R1 – which represents the main antigenic target in MN – confirmed this pathophysiological concept (2). Since then, a number other renal autoantigens were identified using different methodological approaches. These new autoantigens are frequently associated with unusual clinical presentations as well as secondary etiologies (lupus, autoimmune diseases, neoplasia…) that require special attention and therapeutic measures. These discoveries were reviewed by Caza et al. in this Research Topic. In addition to PLA2R1 (60-70% of all MN patients) (2), the list includes other podocyte antigens such as

THSD7A (2-4% of patients) (3), HTRA1 (6) and SEMA3B (12), as well as NELL1 and PCDH7 (5), which are not expressed by normal podocyes. Finally, a few new antigens are associated with other clinical conditions, such as EXT1/2 (13), NCAM1 (10) and TGFBR3 (9) with lupus nephritis, or CTNTI (7) with demyelinating polyneuropathy and myositis. The authors also discussed in detail medications, infectious triggers and malignancies and finally suggested the implementation of an antigen-based classification in place of the simplified distinction between primary and secondary MN. For practical use, an interesting algorithm has been proposed that distinguishes between anti-PLA2R positive and negative forms and, in the latter case, considers the age of presentation (SEMA3B is frequent in pediatric cases), the association with other autoimmune conditions (EXT1/2, NCAM1, TGFBR3 with lupus and CTNTI with neuropathy/myositis) and, finally, the IgG pattern (segmental IgG suggests NELL1-, global IgG indicates THSD7A-, PCDH7- or HTRA1-associated MN).

Furthermore, De Menezes Neves et al. and He et al. described two particular forms of MN associated with Grave's disease and IgA nephropathy, respectively, which are rare occurrences but seem to represent an overlap syndrome between two distinct immune disorders. MN associated to Graves' disease was histologically characterized by thyroglobulin deposition along the capillary loop. Interestingly, proteinuria normalized only after radioiodine therapy (De Menezes Neves et al.), indicating potential necessity of a thyroid assessment in patients with MN. The huge population with IgA/MN (137 patients) had a lower median level of galactose-deficient IgA1 and were less proteinuric than MN (He et al.).

Despite these advances and the comprehensive clinical characterization of the role of antibodies for diagnosing and monitoring patients with MN, insights on pathogenic molecular mechanisms are still limited. Current research focuses on the characterization of the B cell response with the identification of new antigenic targets as well as the role of the complement system. In addition, there are many arguments in favor of an involvement of T cellular immunity in the pathogenesis of MN. Zhao et al. reviewed the role of follicular helper T cells in the pathogenesis of MN and summarized many studies showing the orientation towards the Th17 pathway in MN, paving the way for new targeted therapies.

Minimal change disease and FSGS

The molecular mechanisms underlying MCD and primary FSGS are only partially defined. Both entities are considered diseases of podocytes, or podocytopathies, that are characterized by podocyte foot process effacement, loss of podocyte architecture and slit diaphragm integrity in the absence of any inflammatory hallmarks. Both conditions are considered of immunologic origin and linked to T and B cells, and more recently also to autoimmune processes. Secondary forms also exist as part of more complex settings, such as viral infections (e.g. cytomegalovirus, Epstein Barr virus, SARS-CoV2) and cancer, notably Hodgkin's lymphoma, may be associated with MCD (14).

The theory on T cell involvement dates back to 1974 (Shalloub hypothesis) and was based on the favorable response to steroids and on the association of MCD with T cell malignancies (15). However, T cell involvement has not been confirmed across studies (16–18). Experimental induction of proteinuria by injection of supernatant from a T cell hybridoma into rats was a part of the Shalloub hypothesis (19) and served as the basis to the hypothesis on the existence of a still uncharacterized "extra renal" circulating glomerular permeability factor (20). An implication of Treg is indirectly suggested by the association of MCD/primary FSGS (but also of MN) with IPEX, a FoxP3 X-linked congenital immune pathology characterized by altered Tregs, polyendocrinopathy and enteropathy (21, 22). Treg expansion with drugs, e.g. IL2 and anti-CD20, is not associated with a clear anti-proteinuric effect in patients with MCD/FSGS (23–25) limiting the idea on a direct Treg involvement. More recently, a growing body of evidence linked MCD/primary FSGS pathogenesis to B cells and also autoimmunity. An involvement of B cells is supported by the increasing and succesful use of anti-CD20 monoclonal antibodies in both conditions (26). Autoimmunity is linked to the observation of several antibodies in the serum of patients with MCD/primary FSGS, such as anti-nephrin, anti-annexin A2 and anti-UCHL1 antibodies (11, 27, 28).

Two papers in this issue addressed the crucial aspect of T/B cells subsets in MCD. Fribourg et al. utilized time of flight mass cytometry (CyTOF) for studying patients with steroid-dependent nephrotic syndrome treated with either chimeric or human anti-CD20 monoclonal antibodies. CyTOF utilizes metal isotopes as cell surface markers that display specific mass spectrometry signatures for simultaneous quantification of over 40 circulating cells. The authors confirmed previous findings on a significantly higher number of switched memory B cells in relapsing patients (29) and identified among 5 subsets of switched memory B cells, IgD- CD27+ CD38+ CD95+ antibody-secreting cells as the subset most strongly associated with disease relapse. Positivity for CD38 of this cell subset is of particular interest since it is a marker of plasma cells and immature plasmablasts (30) and is absent in memory B cells. A second interesting finding of the study above was that patients undergoing disease relapse had a faster recovery of B_{REG} that are cells capable of inhibiting the T cell compartment through IL10 and IL35. Major modifications of T cells were not found.

The exact molecular mechanism underlying the B cell-mediated detrimental effect in children and adult patients with

MCD/primary FSGS remain to be determined and the accurate mechanisms associated with therapeutic efficacy of B cell-depleting agents are not well elucidated. In a second paper, Colucci et al. discussed current knowledge regarding the role of B cell dysregulation in the pathogenesis of MCD/FSGS in both pediatric and adult patients. This review summarized the most relevant clinical and experimental findings suggesting a key role of B lymphocytes in the pathogenesis of MCD/primary FSGS. Strikingly, at onset disease, during the first episode of nephrotic syndrome, alteration in B cell homeostasis and distribution of B cell subpopulation seem to be widely different in children compared to adult populations. The production of pathogenic autoantibodies (anti-nephrin and anti-UCHL1) targeting podocytes and/or slit diaphragm structure but also the secretion of B cell-derived cytokines may play a crucial role in the increase of glomerular capillary permeability leading to podocyte cytoskeleton disorganization and proteinuria. In addition, the B cell production of hyposialylated IgM directed against T cells may effect on corticosteroid response.

Therapies: Anti-CD20 monoclonal antibodies and more

Scolari et al. reviewed current treatment strategies for MN. They presented data on the comparison between drugs utilized in early 2000 that is historically known as the Ponticelli regimen (cyclophosphamide plus steroids) with more recent approaches that utilize anti-CD20 monoclonal antibodies (e.g. rituximab). The conclusion was that the two treatments had comparable effects opening *de facto* the discussion on which therapies should be utilized first.

In addition, original work by Teisseyre et al. demonstrated the importance of rituximab plasma levels 3 months after rituximab infusion for remission induction in MN. They also highlighted other mechanisms that may limit rituximab efficacy (e.g. anti-rituximab antibodies). Teisseyre et al. additionally reviewed in this issue the recent therapeutic advances in MN, the mechanisms for rituximab resistance, and proposed personalized management based on immunomonitoring of rituximab, anti-rituximab antibodies and autoantibodies.

The knowledge on autoantibodies targeting specific renal proteins in patients with MN principally enables the use of antigen-specific treatments. Such treatments would ideally target the immunological disease mechanisms while sparing protective immunity, thus bearing an enormous potential to enhance specificity, efficacy and compatibility. The gap between the increasing knowledge on the pathogenic role of autoantibodies and autoantigens in MN on the one side and the currently available treatments with limited specificity on the other side was discussed in the perspective by Köllner et al.. The authors highlight two potential strategies targeting both the pathogenic antigens and the antibody-producing B cells.

The use of chimeric anti-CD20 in MCD/FSGS has been established over the last decade with good results. Basu et al. extended to the use of the human anti-CD20 antibody ofatumumab that was developed in substitution of chimeric products with the hope to improve therapeutic efficiency and reduce risks. A first report indicated that ofatumumab utilized in very high doses (about 10 times higher than rituximab) successfully normalized proteinuria in 6 patients with FSGS resistant to other drugs (31). This finding has never been confirmed in other studies, probably for the sake of side effects linked with the very high dosage. When ofatumumab was utilized in doses that doubled rituximab, no effects in patients with multi-drug resistant nephrotic syndrome were achieved (32). A recent randomized clinical study comparing ofatumumab and rituximab in patients with steroid and/or multidrug dependence showed no superiority of the former in comparison with rituximab and a better outcome of children under 9 years treated with the chimeric molecule (25).

Conclusions

In conclusion, this special issue on 'Immune Dysfunction in Nephrotic Syndrome' contains important and timely manuscripts that covered years of research in the broad area of nephrotic syndrome. New antibodies involved in MN, therapy evolutions with anti-CD20 monoclonal antibodies and new proposals for biomarkers and targeted treatments have been major topics of interest. Proposals for new drugs have also been presented and mechanisms of resistance to drugs that are currently utilized have been discussed. Overall, this issue witnesses the evolution made in recent years and opens to new discoveries that will complete the knowledge on molecular mechanisms in different conditions associated with nephrotic syndrome and, we hope, consolidate new therapeutic approaches.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflict of Interest

VA received consulting fees from Addmedica, Sanofi Genzyme, Travere, Alnylam, and Astrazeneca outside of the submitted work.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Helper T Cells in Idiopathic Membranous Nephropathy

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Idiopathic membranous nephropathy (IMN) is an autoimmune disease in which the immune system produces an antibody response to its own antigens due to impaired immune tolerance. Although antibodies are derived from plasma cells differentiated by B cells, the T-B cells also contribute a lot to the immune system. In particular, the subsets of helper T (Th) cells, including the dominant subsets such as Th2, Th17, and follicular helper T (Tfh) cells and the inferior subsets such as regulatory T (Treg) cells, shape the immune imbalance of IMN and promote the incidence and development of autoimmune responses. After reviewing the physiological knowledge of various subpopulations of Th cells and combining the existing studies on Th cells in IMN, the role model of Th cells in IMN was explained in this review. Finally, the existing clinical treatment regimens for IMN were reviewed, and the importance of the therapy for Th cells was highlighted.

Keywords: idiopathic membranous nephropathy (IMN), helper T cells (Th cells), autoimmune, antibodies, germinal center (GC)

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INTRODUCTION

In 2009, Beck et al. (1) discovered the podocyte autoantigen, i.e., M-type receptor of secretory phospholipase A2 1 (PLA2R1), in the immune deposits of IMN, providing a key evidence of IMN as an autoimmune disease. Later, in addition to PLA2R, more IMN antigens were identified, including thrombospondin type-1 domain-containing 7A (THSD7A), neural epidermal growth factor-like 1 protein (Nell-1), and semaphorin 3B (sema3B), which were all self-components of podocytes (2). In recent years, the incidence of IMN has been increasing year by year, making it the most common primary glomerular disease (3). At present, it is widely accepted that the autoimmune reaction of antibodies and the circulation and combination of target antigens on the cell, formed *in situ* immune complex deposition in cells and basement membrane space, lead to cell destruction, basement membrane thickening, and glomerular filtration barrier damage, as well as proteinuria and low plasma protein concentration (4).

As a key component of the human adaptive immune system, Helper T (Th) cells play an auxiliary or regulatory role in the immune response by expressing CD4 (5). Before being stimulated by antigens and cytokines, CD4+T cells are in their initial state, namely, naive CD4+ T cells.

Upon being stimulated, the naive T cells begin to differentiate into different lineages. The differentiation direction is influenced by T cell receptor (TCR) signaling and specific cytokines in the microenvironment, and the cell fate is determined by major activated transcription factors. At present, 5 subsets of Th cells are relatively well-defined: Th1, Th2, Th17, regulatory T (Treg) cells and follicular helper T (Tfh) cells (6).

Due to their importance to autoimmune response, possible roles of various subsets of Th cells in the induction, immune disorders, and antibody generation of IMN will be discussed, and new clinical therapeutic strategies will be presented.

UNDERSTANDING HELPER T CELLS

The subsets of helper T cells are balanced and coordinated with each other, as shown in **Figure 1**. Th1 and Th2 subsets were the first ones discovered and explained by Mosmann et al. in 1986 (7). When the organism was infected with intracellular pathogens, such as viruses and bacteria, the naive T cells could be induced to differentiate into Th1 cells (8). Such differentiation is mainly promoted by IFN-γ and IL-12, which activate the major transcription factor T-bet through signaling transducer and activator of transcription (STAT)1 and STAT4 signaling, respectively, thus producing more IFN-γ in turn. IFN-γ is the major effector of Th1 cells functions to activate macrophage-mediated cellular immunity (6). IFN-γ also urges T-bet to produce a cascading amplification effect of Th1 cells through autocrine and positive feedback mechanisms (9). In contrast, Th2 cells mainly mediate humoral immune response and assist B cells to produce antibodies. IL-4 activates STAT6 signaling to promote the transformation of naive T cells to Th2 cells, which is regulated by GATA3, the major transcription factor of Th2 cells (10). IL-2 is also important for the formation of Th2 cells by activating STAT5 (11). IL-4 in Th2 cells also plays a similar role to the positive feedback mechanism of IFN-γ in Th1 cells, promoting Th2 cells differentiation (10). Th2 cells can also produce IL-5 and IL-13, etc., and participate in allergic reactions (6). There is an antagonistic relationship between Th1 and Th2 cells. First, when naive T cells receive antigen-presenting signals through TCR, a stronger TCR signal promotes Th1 differentiation, while a weaker TCR signal promotes Th2 differentiation (12). In addition, their major transcription factors T-bet and GATA3 are also inhibiting each other at both gene expression level and protein level (13, 14).

Approximately 10 years later after the discovery of Th1/Th2 cells, Sakaguchi et al. found a subpopulation (Treg cells) of CD4+T cells expressing the IL-2 receptor α (CD25) in mice that exacted immunosuppressive effects and maintained immune tolerance (15). Treg cells were derived from initial T cells induced by TGF-β alone and mainly regulated by the transcription factor forkhead box P3 (FoxP3) (16). Due to the grouping expression of CD25, Treg cells had a higher affinity with IL-2 than other Th subsets as it helped to achieve optimal

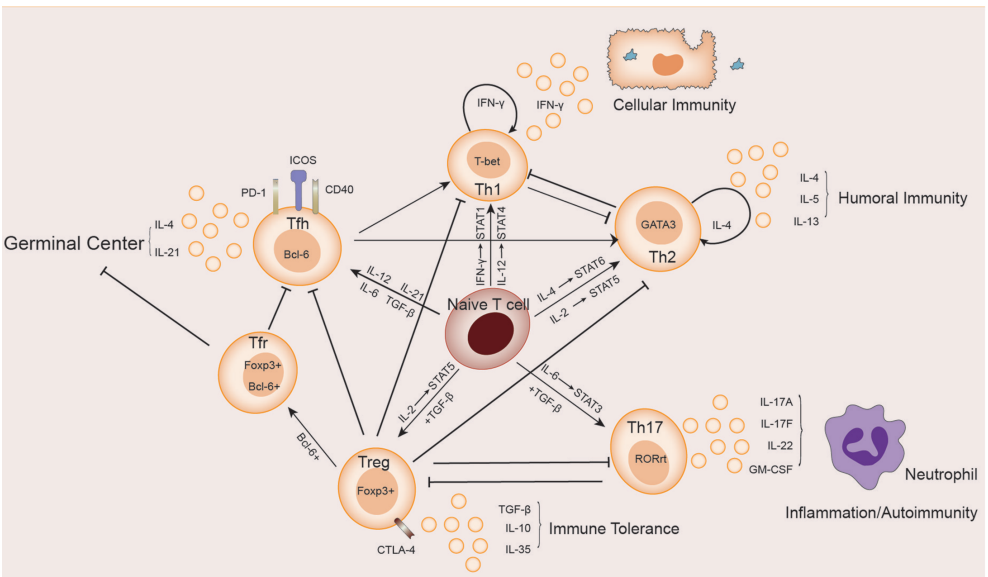

FIGURE 1 | Relationship between Th cells subpopulations. Naive T cells differentiate in different directions under different conditions: IL-12 and IFN-γ activate STAT4 and STAT1 signaling, respectively, inducing the expression of the major transcription factor T-bet, and naive T cells differentiate in the direction of Th1 cells, which secrete cytokines such as IFN-γ and participate in cellular immunity. Th2 cells secrete cytokines such as IL-4, IL-5, and IL-13, which are involved in humoral immunity. In the presence of IL-6 and TGF-β, naive T cells differentiate towards Th17 cells, whose main transcription factor is RORγt. Th17 cells secrete cytokines such as IL-17A, IL-17F, IL-22 and GM-CSF, which are involved in autoimmune diseases or inflammatory responses. Treg cells secrete IL-10, IL-35 and TGF-β to maintain immune tolerance. The naive T cells differentiate towards Tfh cells in response to cytokines such as IL12, IL-21, IL-6 and TGF-β. Treg cells differentiate into Tfr cells in the germinal center. Tfh cells and Tfr cells together participate in the germinal center response.

inhibition of Treg cells through activation of STAT5 signaling (17). In general, Treg cells were found with high expression of CD4, simultaneous expression of CD25, Foxp3 (cytoplasm), and low expression of CD127 (IL-7 receptor α chain), constituting phenotype for such cells (18). Decrease in the number and/or function of Treg cells has been observed in patients with a variety of autoimmune diseases and mouse models (19). Treg cells have been identified with functional plasticity and different transcriptional characteristics in response to different types of immune responses and environments, thus playing a greater role of immunosuppression. There is also a subset of follicular regulatory T cells (Tfr) located in the germinal center (GC) that, in addition to expressing Foxp3, also express the chemokine CXCR5 and transcription factor Bcl-6, which are also markers of Tfh cells (20). The function of Tfr cells is to inhibit GC reaction and plasma cell differentiation, which is in balance with Tfh cells.

Until 2005, a new subset of Th cells known as Th17 cells which can secrete IL-17 to regulate tissue inflammation was discovered (21, 22). The development of Th17 cells rely on both the induction of TGF-β and the action of the inflammatory factor IL-6. They activate the major transcription factor RORγ-T through the STAT3 signaling pathway, which determines the differentiation of naive T cells to Th17 cells. This induction of IL-6 can also be enhanced in the presence of other cytokines, including IL-1β, TNF-α, IL-23, and IL-21 (23–25). Th17 cells produce IL-17A, IL-17F, IL-22 and granulocyte-macrophage colony-stimulating factor (GM-CSF), recruit inflammatory cells such as neutrophils, and promote inflammation at the infected site (26). An increase in Th17 cells has been observed in a variety of forms of autoimmune diseases, including inflammatory bowel disease (IBD), psoriasis, rheumatoid arthritis (RA), etc. (26), which is contrary to the observed reduction or suppression of Treg cells. There is a balance between Th17 and Treg cells: first, they compete for TGF-β at the site of differentiation; second, both STAT5 and Foxp3 in Treg can inhibit Th17 differentiation, while STAT3 signaling in Th17 can down-regulate Foxp3. All these lead to the differentiation of naive T cells in two different directions under different conditions. It was much believed that the imbalance of Th17/Treg cells was the key to the pathogenesis and therapeutic target of autoimmune diseases (27, 28). Yet, the cause for such imbalance still remains unknown.

Several groups of studies have identified a type of CXCR5+Th cells that have a specific and preferred helper function to B cells in follicles (29–31), known as follicular helper T(Tfh) cells. The main transcription factor of Tfh cells is Bcl-6, which is essential to Tfh formation, assistance to B cells and GC formation (32–34). The expression of Bcl-6 inhibits differentiation of CD4+T cells in directions other than Tfh cells (33), and also hinders the expression of Th1, Th2, Th17 and Treg-related functional receptors (35, 36). In humans, IL-12, IL-21, IL-6, IL-23, and TGF-β synergistically promote Tfh cells, but TGF-β inhibits Tfh cells development in mice (37–40). IL-2 inhibits STAT3 and Bcl-6 by phosphorylating STAT5, and upregulates Blimp-1, thereby inhibiting Tfh cells (41, 42). Tfh cells also secrete IL-21 and express surface molecules programmed cell death protein 1 (PD-1) and recombinant Inducible T cell co-stimulator (ICOS) (43–45), which are critical for regulating the development, migration and function of Tfh cells. Differentiation and development of Tfh cells is mainly accomplished in secondary lymphoid organizations (SLOs). Through the interaction with B cells, Tfh cells gradually migrate from the T cell zone, through the T-B border, to the B cell follicles and germinal center, and finally form GC Tfh cells (46–49), as shown in **Figure 2**. GC Tfh cells are necessary to maintain GC response and cause three outcomes of B cells: A, differentiation into long-term memory B cells, waiting to be exposed to antigen again; B, differentiated into long-lived plasma cells to continue to produce antibodies; C, re-entry into the dark zone for more proliferation and somatic hypermutation (50–52). Owing to its heterogeneity and plasticity, GC Tfh cells are also able to adapt to different types of immune responses. In addition to secreting IL-21, Tfh cells can also produce IL-4 in response to Th2-mediated antibody response (53).

Since it is difficult to obtain SLOs from patients, attention has been paid to circulating cells with a Tfh phenotype. Some CD4+T cells in the blood with a Tfh-like phenotype (CXCR5+) subpopulation, but without Bcl-6 expression, are referred to as circulating Tfh (cTfh) cells (52). Although the relationship between cTfh cells and true Tfh cells in SLOs is unclear, the frequency of cTfh and its subsets are associated with influenza vaccines, chronic infections, and autoimmune diseases (54–58). Therefore, circulating CXCR5+CD4+T cells are currently considered to be the circulating responders of Tfh cells. According to the different expressions of CXCR3 and CCR6, cTfh can be divided into three subsets expressing different cytokines: A, CXCR3+CCR6-cTfh1, which can secrete IFN-γ; B, CXCR3-CCR6-cTfh2, which can secrete IL-4, IL-5 and IL-13; C, CXCR3-CCR6+cTfh17, which can secrete IL-17A, IL-17F and IL-22 (59). In addition, the activation status of cTfh cells can be distinguished according to the expression of ICOS and PD-1 cTfh2 and cTfh17 can secrete IL-21, which can effectively induce proliferation and differentiation of juvenile B cells and antibody class conversion (59–62).

HELPER T CELLS IN IMN

Th Cells and Induction of IMN

There are many inducing factors of autoimmune diseases, such as the change in autoantigen, the abnormality of immune system, genetic factors, gender and age, etc., as well as their combined forces (63). IMN is usually caused by a single antigen, of which PLA2R accounts for 75%, and 10%-20% of IMN patients have not yet been identified with their antigens (64). Exposure to autoantigen is the major incidence reason, and no direct evidence has been found to reveal this process in IMN. Considering PLA2R as an example, anti-PLA2R antibodies in serum of IMN patients can bind to PLA2R antigen *in vitro* in a non-reduced state (65), which suggests that the antibody-bound epitopes require PLA2R spatial epitopes and are maintained by disulfide bonds (66). In China, the incidence of IMN is positively correlated with air pollution reflected by PM2.5 (67). We and

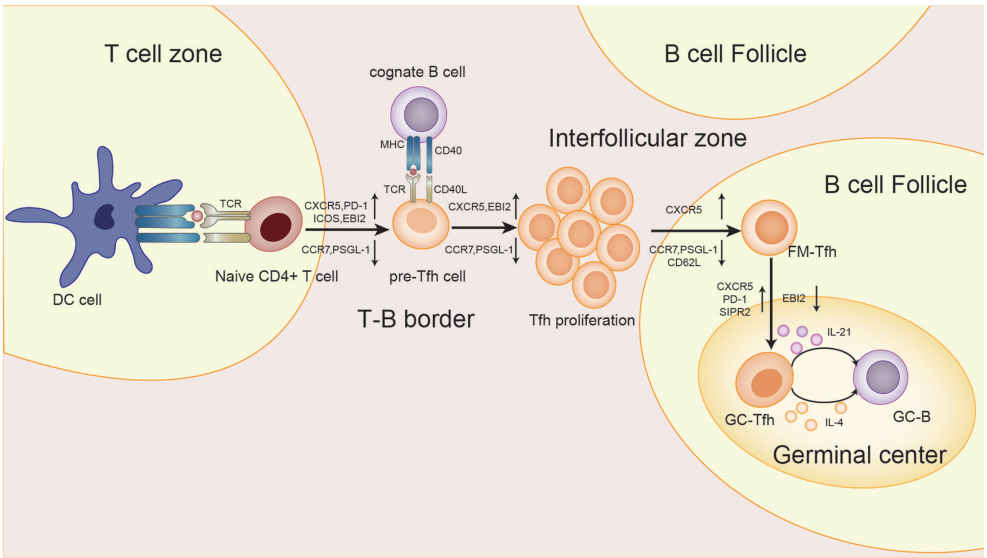

FIGURE 2 | Differentiation and development of Tfh cells. First, in the T cell zone, the naive T cells receive the antigen presentation signal from the DC cells, and Tfh cells begins to differentiate. T cells expressed CXCR5, PD-1, ICOS, and epstein-barr virus-induced gene 2(EBI2), while CCR7 and P-selectionglycoproteinligand-1 (PSGL-1) were down-regulated to obtain the pre-Tfh cell phenotype. At the T-B border, cognate B cells interact with T cells to maintain the Tfh cell phenotype. After that, the T-B cell complexes move from the border to the interfollicular zone, where more proliferation takes place. Next, Tfh cells are about to enter the follicle, and the signal from the bystander B cells further upregulates CXCR5 and suppresses CCR7, PSGL-1, and CD62L. Finally, Tfh cells in the follicular fimbria up-regulated CXCR5, PD-1, and sphingosine-1-phosphate receptor 2(S1RP2) surface molecules, down-regulated EBI2, and became GC-Tfh cells. The expression of IL-21 and IL-4 by GC Tfh is essential for the survival, proliferation and differentiation of germinal center B cells.

Paul Brenchley et al. have proposed the hypothesis that lung tissue is stimulated by PM2.5 to cause an inflammatory environment, leading to exposure of PLA2R1 pathogenic epitopes in a strong oxidative microenvironment and then inducing the pathogenesis of IMN (68, 69). Recently, several studies have indeed found enhanced expression of Th17 cells and up-regulation of IL-17 and other cytokines in IMN, suggesting that there is indeed an inflammatory environment in IMN (70–72). Why has PLA2R become the main autoantigen of IMN? This may be related to genetic predisposition. At present, HLA-DQA1 and PLA2R allele risk loci have been found in IMN, which can promote the delivery of antigen epitopes to T cells through major histocompatibility complex (MHC) class II (73, 74), and CD4+T cells receive antigen signals through TCR.

In addition to the exposure of epitopes, the pathogenesis of IMN also involves the breakdown of autoimmune tolerance, including central and peripheral immune tolerance. The production of autoreactive T cells and B cells matters a lot, and the question is how they can escape the numerous tolerance checkpoints. In the process of thymus development of T cells, the V region gene of TCR is rearranged (75). This process may produce TCR against autoantigen, which can be eliminated by negative selection. However, this process may be abnormal in autoimmune diseases, causing abnormalities in the TCR library of T cells arriving at the periphery. Not long ago, Yu Zhang et al. (76) used T-cell receptor repertoire high-throughput sequencing (TCR-HTS) to analyze the TCR β chain repertoire of the circulating T lymphocytes of IMN patients. The result showed that IMN had lower diversity of VJ cassette combination in peripheral blood and a decrease in TCR lineage diversity. A decrease in TCR diversity of peripheral T cells has also been observed in patients with compulsive spondylitis and systemic lupus erythematosus (77, 78). This may explain why autoreactive T cells have escaped central immune tolerance, or why TCR has a shared sequence in patients, increasing the risk of autoimmune diseases (79). Peripheral immune tolerance may also play a key role in autoimmune diseases (80). Treg cells are the key to maintaining peripheral immune tolerance. A large amount of evidence shows that IMN has a reduced proportion of Treg cells in serum and decreased expression of Foxp3 (72), as well as impaired activation and inhibition of Treg cells (81). However, the expression of Treg cells in patients improved by rituximab treatment was significantly up-regulated, and the proportion of Treg cells had a prognostic effect on the treatment of rituximab (82, 83).

Immune response of IMN is dominated by humoral immunity (84), during which differentiation and development of autoreactive B cells are crucial, and Tfh cells play the role of peripheral immune tolerance checkpoint (85, 86). The B cell pool of healthy adults contains a large number of autoreactive B cells, but they have a low affinity and therefore do not cause disease (87, 88). Autoimmune diseases, including IMN, require high affinity with disease-causing antibodies (89, 90), suggesting that these plasma cells that produce these antibodies have undergone affinity maturation and somatic hypermutation (SHM) in GC. In fact, most GC-B cells experience apoptosis, and only a small

portion survives and differentiates into memory B cells or plasma cells to leave GC (91–93). GC-B cells can survive and develop only with the assistance of T cells. A competitive model was first proposed, which was positively correlated with B cell receptor (BCR) affinity and antigen presenting ability (52). Professor Carola G. Vinuesa later described this competitive mechanism as positive selection and negative selection of Tfh cells (85, 94). Positive selection meant that Tfh cells provide survival signals to GC-B cells through CD40L, IL-4, IL-21 and other cytokines (94). Negative selection referred to the process Tfh cells transmit death signal to GC-B cells *via* CD95L. Mice with CD95L deficiency would develop autoimmune diseases (94). B cells without CD40L signal went to apoptosis (95), and the homologous interaction of T-B cells could make B cells enter the dark area again for further division and SHM (85). Studies have shown that the reduction of SHM is associated with the impairment of B cell tolerance, and the increase of cTfh cells and IL-21 in such patients (86). Restrictions on the number and quality (secreting cytokines) of Tfh cells create an environment in which GC B cells must compete for help, making it difficult for some low-affinity B cells, such as autoreactive B cells, to proliferate and differentiate. However, when the amount of Tfh cells increase abnormally, this checkpoint will be damaged, and the loosening of the floodgate will allow some autoreactive B cells to proliferate and differentiate, producing antibodies, and leading to autoimmune disease, which has been confirmed in Sanroque mice (45, 96, 97). In addition, Tfh cells were associated with the occurrence of autoimmune responses in chronic inflammation (98) as well as the process of antigen simulation (99), which has not been further investigated yet.

In fact, no matter the central or peripheral immune tolerance is abnormal or not, the resulting diseases are often multi-antigen pathogenic, such as systemic lupus erythematosus (100). In IMN, although more than one antigen or antibody has been reported (101, 102), the majority of patients are single-antigen pathogenic. Therefore, the abnormalities of Th cells may not be the main cause of the induction of IMN, and the greater significance of such abnormalities lies in the maintenance of the disease state.

Th Cells Involved in the Immune Dysregulation of IMN

The differentiation diversity of Th cells is affected by at least two aspects: on the one hand, the differentiation of naive T cells is affected by cytokine signals in the microenvironment; on the other hand, such differentiation is regulated by TCR downstream signals in the cell. Recently, Mikel Ruterbusch et al. proposed a new differentiation model of CD4+T cells *in vivo* (103). The studies on Th cell subsets and related cytokines in IMN were reviewed and recorded in **Table 1**. IMN is identified with obvious Th cells subgroups imbalance, which is mainly reflected in the following aspects:

First, the CD4+/CD8+T cell ratio increased, and then the Th2/Th1 cell ratio increased, indicating that humoral immunity was dominant in IMN (84). In CD4+T cells, the expression of IL-4 was up-regulated, which was positively correlated with antibody production and disease severity (111). The representative cytokine IFN-γ secreted by Th1 was decreased in IMN (111). Cellular immune-mediated diseases are usually infiltrated by local monocytes and cytotoxic T cells. Although IMN presents as an organ-specific autoimmune disease, there is a local lack of cell infiltration that mediates cellular immunity in the glomerulus, and the generation of proteinuria may be caused by antibody activation of complement that damages the podocytes or antibody affecting podocyte function (115–117). The predictive value of anti-PLA2R antibody titers for clinical prognosis has also been vigorously described (69).

Many studies (112, 115, 118), represented by the rituximab clinical trial conducted by Ronco et al., have shown that IMN reduces Treg cells and destroys immune tolerance, and whether Treg cells can be increased after treatment can predict the therapeutic effect of rituximab. A recent study showed impaired inhibition of Treg cells in IMN (81), which might be attributed to the continuous exposure of antigen and the weakened ability of human immune regulation. TGF-β, IL-35 and IL-10 are the main cytokines secreted by Treg cells that play immunomodulatory roles. Although reductions or no significant changes in TGF-β and IL-35 were observed in IMN, there was an increase in IL-10, and this contradiction could be explained by upregulated regulatory B (Breg) cells in IMN (81). They can also secrete IL-10, but it does not suffice to block the development of the immune response. It was further speculated that the elevated Breg subsets were Br1 cells (119). As mentioned above, there is an antagonistic relationship between Treg and Th17 cells in terms of differentiation, function and other aspects, and imbalance of Th17/Treg has been observed in many autoimmune diseases. Th17 cells in IMN have been a heated topic recently, and studies from different research groups suggested the up-regulation of Th17 and the increase of IL-6 and IL-17A. This indicates that IMN is conducive to Th17 cells differentiation, and also strengthens our confidence that IMN is originated from extrarenal inflammation (115). Increased Th17 cells are also associated with a higher recurrence rate and a higher risk of venous thrombosis (71), which is a concern for clinical treatment.

Autoantibodies are essential to the development and maintenance of IMN, and the production of antibodies requires GC reactions. Tfh cells are professional GC helper B cells, and also serve as the novae of Th cells. In fact, the discovery of Tfh cells has challenged the previous classification of Th cells because their differentiation had been made earlier (103). In addition, it was previously held that the humoral immunity of IMN was driven by IL-4 secreted by Th2 cells, but the present study shows that IL-4 promoting antibody production may come from Tfh cells. Reduction of memory B cells and increase of initial B cells are present in IMN (83), which is consistent with the reports of some other autoimmune diseases (120, 121). An increase in initial B cells, such as Tfh cells, may be associated with the breakdown of tolerance checkpoints (83). The decrease of memory B cells may be caused by the induction of B cells into local tissues by chemokines, or the differentiation into plasma cells to produce antibodies under the action of Tfh cells,

TABLE 1 | Studies of helper T cells in IMN.

Author	Year	Patients	Th cells changes	Related cytokine changes	Reference
Chatenoud L/ Cagnoli L/ Bannister KM/ Rothschild E et al.	1981/ 1982/ 1983/ 1984	12/27/ 14/8	**Increase:** Ratio of the Th/cytotoxic (OKT4+/OKT8+) or suppressor T cells Decrease: Ratio of the OKT8+T cells	No testing	(104–107)
Zucchelli P et al.	1988	39	**Increase:** Ratio of helper/suppressor T cells(LEU3+/LEU2+)	No testing	(108)
Ozaki T et al.	1992	30	**Increase:** Level of suppressor inducer T (Leu3a+8+) **Decrease:** Level of suppressor T cells (Leu2a+15+)	No testing	(109)
Hirayama K et al.	2002	8	**Increase:** Ratio of Th2 (IL-10+CD4+T cells) **Decrease:** Ratio of Th1 (IL-2+CD4+T cells)	As shown in the left	(110)
Masutani K et al.	2004	24	**Increase:** Ratio of IL-4+Th cells **Decrease:** Ratio of Th1/Th2 (IFN-γ+/IL-4+), positively correlated with urinary protein.	As shown in the left	(111)
Kuroki A et al.	2005	14	**Increase:** Ratio of CD4+T cells, CD4+/CD8+T cells **Decrease:** Ratio of CD8+T cells	**Increase:**IL-10mRNA, IL-13mRNA in PBMC	(84)
Wang B et al.	2011	66	**Increase:** Ratio of CD4+/CD8+ T cells **Decrease:** Number of Treg cells(CD4+CD25+Foxp3+)	No testing	(112)
Shi X et al.	2016	39	**Increase:** Ratio of Tfh cells (CD4+CXCR5+, CD4+CXCR5+PD-1+,CD4+CXCR5+ICOS+, CD4+CXCR5+IL-21+)and ratio of ICOS+/PD-1+Tfh cells	**Increased:** IL-21 in serum	(113)
Michelle Rosenzwajg et al.	2017	25	**Increase:** Frequency of effector memory CD4+T cell **Decrease:** Frequency of Treg (CD25hiCD127lo/-Foxp3+)in CD4+T cells	**Increased:** TNF-α, IL-5, IL-2RA **Decrease:** IL-17, IL-1α, IL-7, and granulocyte-macrophage colony-stimulating factor (GM-CSF) **No change:**IL-35	(83)
Zhang Z et al.	2017	45	**Increase:** Ratio of Tfh cells (CD4+CXCR5+,CD4+CXCR5+ICOS+,CD4+CXCR5+CD154+,CD4+CXCR5+IL-21+,CD4+CXCR5+CD28+), negatively correlated with eGFR and positively correlated with urinary protein.	**Increase:** IL-21, IL-4, IL-10, IL-2, IL-17A, IFN-γin serum Serum IL-21 concentration was negatively correlated with eGFR and positively correlated with urinary protein.	(114)
Cantarelli C et al.	2020	30	**Decrease:** Frequency of Treg (CCR4+CD45RA-CD25+CD127low) **No statistical difference:** Tfh cells, etc	**Increase:** TNF-α in serum **No significant difference:** IFN-γ, IL-4, and IL-17 in CD4+ and CD8+ T cells	(81)
Li, H. et al.	2020	29	**Increase:** Frequency of Th17 (IL-17A+CD4+T),Th2 (IL-4+CD4+T) **Decrease:** Frequency of Th1 (IFN-γ+)and Treg	**Increase:** IL-17A(positive correlation with antibody titer and proteinuria), IL-6, IL-10, IL-13 in serum **No significant difference:** IL-4, IFN-γ, IL-2 in serum	(70)
Cremoni, Marion et al.	2020	56	No testing	**Increase:** IL-17A, IL-4, IL-6 in serum **Decrease:** IFN-γ, IL-10 in serum **No significant difference:** TNF-α, IL-5, IL-13 and GM-CSF	(71)
Roza Motavalli et al.	2021	30	**Increase:** Ratio of Th17/Treg cells **Decrease:** Ratio of Treg (CD4+CD25+CD127−) **No significant difference:** Th17 (CD4+IL-17+)	**Increase:** IL-21, IL-4, IL-10 mRNA in PBMC **Decrease:** expression of FOXP3 in PBMC **No significant difference:** expression of IL-17, IL-23, STAT3, RORγT in PBMC	(72)

or both (85). Two studies from the same group have shown an abnormal increase in Tfh cells in IMN patients, which was correlated with disease severity (113, 114). Earlier studies have also shown that the proportion of CD4+CXCR5+T cells was also up-regulated in the classic model of Heymann nephritis rats (122), a classic animal model of IMN. Nevertheless, there are still many shortcomings, such as discrepancies in the results of studies from the same group. In addition, many questions remain to be explored, such as what causes the abnormal increase in Tfh cells? What is the function of the increased Tfh cells? Are Tfh cells involved in the recurrence of IMN? Research on various lymphocytes in IMN is still insufficient (4), and the role of Tfh cells in the overall immune system of IMN remains to be explored. In addition to the balance between Tfh cells and Treg cells, Tfr cells also form a balance in germinal center. An elevated proportion of circulating Tfh/Tfr cells is found in some autoimmune diseases (123–125), but unfortunately Tfr cells have not been studied in IMN.

We have to point out that although there are many studies on Th cells in IMN, their results are not in good agreement, which is

a big obstacle for us to reveal the immunological mechanism of IMN. The underlying reasons may include: A. The included patients are heterogeneous and can be classified by etiological type; B. There are differences in detection methods, especially for cytokines. Measuring cytokine levels after *in vitro* stimulation may differ a lot compared to direct serological tests, because cytokines can be lost in proteinuria; C. The changes of Th cells and cytokines in IMN are small, suggesting that we should include patients with more active immune responses for observation, such as those with higher autoantibody titers. D. All the above studies are based on non-antigen-specific immunity, so the changes of autoreactive T cells may be attributed to a minority of the total T cells that are neglected. Although PLA2R-specific IgG-producing plasma cells have been identified in IMN (126), the changes of self-reactive T cells, such as PLA2R-specific T cells, are unknown in IMN. IMN is an autoimmune disease with antibody response as its core. In this process, abnormal Th cells provide an immune-promoting environment for autoreactive B cells by secreting cytokine. Therefore, the changes in the population and subpopulation of circulating Th cells can still reflect the immunological and pathological state of IMN, but such changes cannot be completely equivalent to those of antigen-specific Th cells. Further studies shall be conducted to clarify this problem, such as the use of flow cytometric analysis or major histocompatibility complex (MHC) tetramer (IST) staining to detect antigen-specific T cells in IMN. Such a study would be beneficial because Treg and Tfh cells do depend on antigen specificity to a certain extent when acting through cellular contact (127), and the study can also provide a basis for specific immunotolerance therapy in IMN. In rheumatoid arthritis, the degree of CD4+ T cell autoreactivity can determine the mode of immune response and influence the treatment prognosis, which is also enlightening for the study of IMN (128, 129).

The local immunological appearance in the kidney is also of concern. The pathological process of IMN is the binding of circulating antibodies to podocyte antigens and the formation of immune complexes deposited on the basement membrane. This process has been widely recognized. In fact, prior to the discovery of autoantigens on podocytes, it was assumed that the antigens of idiopathic membranous nephropathy were located in the tubules under the influence of Heymann nephritis rats, and CD20+B cell infiltration was observed in the tubulointerstitial area of approximately 50% of patients with membranous nephropathy, of which about 50% were focal infiltration (130). This structure is similar to the ectopic lymphoid structure (ELS) but has not been further described. Data from experimental animal models and patients suggest that Tfh cells or cells with Tfh phenotypic characteristics contribute to the maintenance of the structure and function of ELS (131–133). ELS was associated with interstitial inflammation and poor prognosis in IgA nephropathy (134). In autoimmune diseases, Eels or locally infiltrating clusters of B cells often caused harmful effects (135). For example, local autoantibodies were produced in patients with rheumatoid arthritis (136). A recent study by Kyriaki Kolovou et al. has shown that there is localized B cell infiltration in the kidney in renal diseases characterized by podocyte injury, including membranous nephropathy (137). Huimin Li et al. found infiltrating IL-17+ cells in the renal tubule of IMN patients (138). These findings suggest that T-B cell interaction can play a role in the renal tissue of IMN, especially in the renal tubules, and affect the prognosis of the disease, thus providing a new focus for renal pathological diagnosis.

Th Cells Participating in the Production of IMN Antibodies

In IMN, antibodies against autoantigens are predominantly IgG4, both in renal pathology and in serum, although a small number of other subtypes are also present (139, 140). Why IgG4 is the main pathogenic antibody in membranous nephropathy has always been a problem to be solved. IgG4 is the lowest IgG subtype in the blood of healthy adults, accounting for only 5% (141). Although it has about 90% homology with amino acid sequences of other IgG subtypes, due to changes in individual amino acids, IgG4 is identified with different characteristics, such as Fab arm exchange, weak complement binding force, etc. (142–144). Depending on the environment, IgG4 can play a protective or pathogenic role. In the autoimmune diseases mediated by IgG4, such as IMN and pemphigus, the pathogenic effect of IgG4 is often reflected in blocking the binding of antigen to other proteins and thus affecting its function (90, 144). In IMN, IgG4 combined with THSD7A affects cell adhesion, and thus proteinuria (145), while IgG4 combined with PLA2R may affect IV type collagen fiber adhesion (144, 146), but there are still controversies.

According to V (D) J gene rearrangement, some scholars speculated that IgG4 antibody should be the one with the highest affinity among all IgG subclasses and appear the latest (147). In GC, Tfh cells provide promoting or inhibiting signals to B cells through the competitive mechanism according to their affinity, which is crucial to the production of high-affinity antibodies (148). Among IgG4-mediated autoimmune diseases, including IMN, some other diseases have also demonstrated abnormalities in cTfh cells (83, 84, 110, 113, 114, 149–153), as shown in **Table 2**. These diseases may share some similarities in pathophysiology. Factors that promote the production of IgG4 mainly include two aspects: long-term exposure of allergens or antigens, and the influence of microenvironment created by cytokines, such as IL-4, IL-13, IL-10, IL-21, etc. (90, 154–156). IMN is an autoimmune disease with long-term exposure to autoantigens, and most of the cytokines involved in IgG4 production are abnormal in IMN (see **Table 1**). IL-4 or IL-13 combined with IL-10 can promote antibody conversion to the IgG4 category, while IL-4 combined with IL-21 can stimulate plasma cells to produce IgG4 antibodies (154–156). IL-4 is considered to be the hallmark cytokine of Th2 cells, while IL-21 is believed to be the hallmark cytokine of Tfh cells, even though neither of them serves as the sole source (157, 158). Studies have shown that Tfh cells can also express IL-4 and regulate germinal center response, independent of Th2 cells (159). In fact, Tfh cells may express both IL-21 and IL-4 simultaneously, or in sequence (160). These two cytokines all

TABLE 2 | Tfh cells in IgG4-mediated autoimmune diseases.

Disease	Antigen	Target organ	Symptoms	Circulating Tfh	Tfh whether associated with antibodies production	Tfh whether influence the development of disease	Circulating B cells	References
Membranous nephropathy	PLA2R/ THSD7A /others	Kidney (podocytes)	Proteinuria	Both the number and the frequency in Th cells increase	Unclear	Yes, positive	Increase: naive B cells(IgD +CD27-) Decrease: memory B cells(IgD-CD27+,IgD +CD27-)	(83, 84, 113, 114)
Pemphigus	Dsg1/ Dsg2	Skin	Flaccid blisters and erosions of the skin and mucous membranes	Frequency in Th cells increases	Yes, positive	Unclear	Unclear	(149, 150)
Myasthenia gravis	MuSK	Muscle/ neuro-muscular junction	Muscle weakness	Frequency in Th cells increases, Tfh17/ Tfh1 increases	Yes, positive	Yes, positive	Decrease: B10, CD24+CD38+B cells	(151–153)

play a key role in the survival and proliferation of B cells, maturation of antibody affinity and class conversion, and the combined effect of IL-21 and IL-4 can promote the production of IgG4 antibody with the support of CD40 co-stimulatory signal (161), which may be related to the regulation of germinal center response. In addition, IL-21 can promote the production of autoantibodies (158).

The Role Model of Th Cells in IMN

Based on the above discussions, genetic, immune, and environmental factors may co-participate in the incidence and development of IMN. In the presence of genetic susceptibility and in extrarenal inflammatory environment, autoantigens represented by PLA2R are presented to T cells. The initial cytokine environment pushes the immune response in a Th2-dominated direction. An abnormal increase in Tfh cells enables the proliferation and differentiation of autoreactive B cells, and assists B cells in completing somatic hypermutation in the germinal center, thus promoting the differentiation of B cells into plasma cells to produce IgG4 antibodies. Inflammation up-regulates Th17 cells and affects autoimmune response and inflammation by secreting cytokines such as IL-17. In addition, Th17 cells, Tfh cells and B cells may be partially liable for the damage of the renal tubulointerstitial region in IMN. The number and function of impaired Treg cells could not be maintained under autoimmune tolerance. The autoimmune response of IMN eventually produces antibodies, which bind to the target antigen on the podocytes, resulting in the classical pathological appearance of IMN, as described in **Figure 3**.

TREATMENTS FOR IMN

IMN, as an autoimmune disease, is mainly treated with immunosuppression, which is initiated after a full assessment of the condition, and the patient's disease status is monitored during the course of treatment. Corticosteroids alone do not work much for the treatment of IMN but are effective when combined with alkylating agents represented by cyclophosphamide (162–164). Cyclophosphamide was originally designed as an antitumor agent and is metabolized by cells to produce phosphoramide mustard (165), which forms cross-links with DNA to achieve cytotoxic effects (166). Cells with high proliferative potential, such as hepatocytes and hematopoietic stem cells, are relatively resistant to cyclophosphamide due to the expression of high levels of aldehyde dehydrogenase (ALDH) (167). Conversely, cyclophosphamide is cytotoxic to mature hematopoietic progenitor cells and almost all lymphocyte subsets (167–169), inducing systemic leukocyte and lymphocyte ablation resulting in rapid suppression of the immune response. However, alkylating agents are associated with a high incidence of adverse events, mainly leukopenia, infection, thrombosis, gonadotoxicity, and increased risk of cancer (170, 171). Calcineurin inhibitors (CNIs) are also widely used in the treatment of IMN, such as tacrolimus and cyclosporine. CNIs can target and block the NFAT signaling pathway, primarily producing an inhibitory effect on T cells, impairing the helper effect of T cells on B cells and thus reducing antibody production. Moreover, some studies have shown that CNIs also have a regulatory effect on the podocyte cytoskeleton (172). The limitations on the clinical use of CNIs lied in their high rate of relapse after drug discontinuation (173) and the association of multiple relapses with progressive renal function (174). Recent studies have shown that the relapse rate after discontinuation of CNIs for IMN can be reduced by the addition of rituximab (175). Rituximab targets the B-cell surface antigen CD20 and cuts the number of B cells other than plasma cells, which can directly reduce antibody titers and induce disease remission (176). In addition to the above drugs, the use of other drugs such as mycophenolate mofetil and belimumab in the treatment of IMN is still being testified.

In addition to remission rates, immunosuppressive therapies for IMN shall also take into account the issues of relapse rates and safety. In terms of the immunological mechanisms of IMN, treatment targeting T or B cells alone may not be comprehensive, and immunosuppressive therapies with multiple targets are yet to be proposed. It has been shown that renal transplant recipient

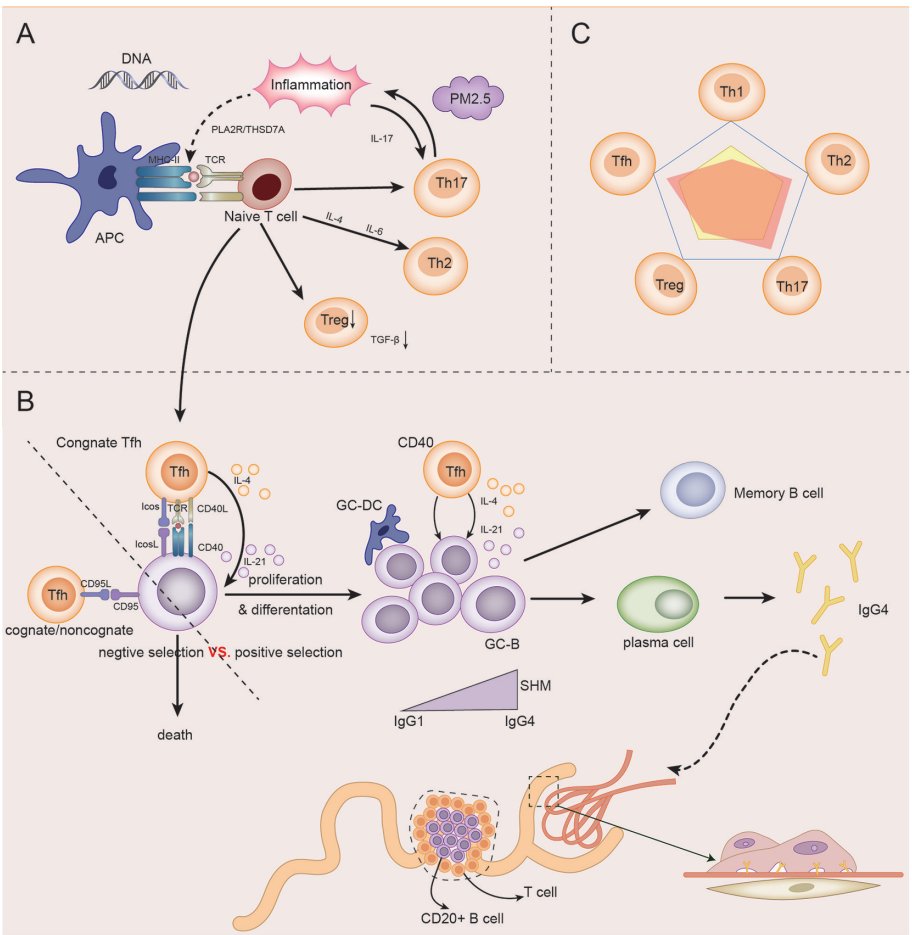

FIGURE 3 | The role model of Th cells in IMN. **(A)** Under the influence of genetic, inflammatory, and environmental factors (PM2.5), antigen-presenting cells (APCs) present their own antigens to juvenile T cells, and then in the initial microenvironment, the immune response develops towards Th2-dominated direction. Infant T cells differentiate into Th17, which in turn participates in and maintains inflammation and promotes immune response. The differentiation of naive T cells to Treg cells decreased, and the immunosuppressive ability decreased. Naive T cells differentiate into Tfh cells and participate in GC reaction. **(B)** In germinal centers, homologous Tfh cells transmit survival signals to B cells *via* CD40L and cytokines (positive selection). Homologous or non-homologous Tfh cells transmit death signals to B cells *via* CD95L (negative selection). The abnormal increase in Tfh cells, which transmit survival signals, gives autoreactive B cells a chance to proliferate and differentiate. Under the action of IL-4 and IL-21 secreted by GC Tfh cells, GC B cells underwent somatic hypermutation (SHM) and antibody affinity maturation. After GC reaction, some B cells become memory B cells and some plasma cells, and begin to secrete IgG4 antibodies. IgG4 circulates to the glomerulus and binds to podocyte antigens (such as PLA2R) to form immune complexes that lead to the pathological appearance of IMN. In addition, under the influence of some factors, T-B cell infiltration may occur in renal tubules, and even form ectopic lymphatic structure, affecting the prognosis of the disease. **(C)** The relationship between the five major Th cell subpopulations in IMN was dominated by Th2, Th17, and Tfh cells, while Treg and Th1 cells were impaired.

patients treated with a combination of rituximab, tacrolimus, and mycophenolate mofetil are found with Tfh or cTfh cells in the circulation and lymph nodes even when B-cell counts are reduced and GC responses are suppressed (177). Once the B-cell subpopulation recovers after treatment cessation, the residual Tfh may rapidly facilitate B-cell production of auto-reactive antibodies, so the combined or sequential use of rituximab and treatment against Tfh cells may have the potential to reduce relapse rates. Indeed, tacrolimus has a specific inhibitory effect on Tfh cells, which may be due to the greater dependence of Tfh cells on the NFAT signaling pathway (178). In addition, rituximab does not affect increased Th17 cells in IMN, which is associated with relapse and thromboembolism (71). Although many patients can achieve clinical remission with rituximab, maintenance treatment for post-remission immunosuppression, such as targeting Th17 and other Th cells, is also worthy of concern, especially in those patients at high risk of recurrence. It is worth pointing out that the potential therapeutic role of IL-2 in the treatment of autoimmune diseases is gaining increasing attention (179), and recently, a double-blind placebo-controlled trial has demonstrated the efficacy and safety of low-dose IL-2 in the treatment of SLE (180). Different T subpopulations of cells have different affinities with IL-2, with the CD4+FOXP3+ Treg cells subpopulation having a high

affinity with IL-2 (181), and Treg cells can be induced to proliferate even at low IL-2, while such dose of IL-2 makes it impossible for other Th cells to proliferate. In addition, IL-2 can inhibit TFH cells responses without relying on Treg cells, which in turn inhibits GC responses and antibodies production (41, 182). By promoting the proliferation of Treg cells and inhibiting the responses of Tfh cells, which are indispensable for the treatment of IMN, IL-2 may have a greater potential in the clinical treatment of IMN.

CONCLUSION

IMN is a special autoimmune disease mainly caused by autoantibodies. Although antibodies are secreted by plasma cells, T-B cells also contribute a lot in the immune system, and the imbalance of Th1/Th2, Th17/Treg, Tfh/Tfr cells, and other Th cells subsets in IMN jointly shapes the immunological pathological state of IMN. More studies are needed to fully understand the pathological mechanism of IMN. The application of rituximab shifts the scholars' attention to the study of B cells in IMN, but Th cells are located in the upstream of B cells, and convincing explanation of the changes in B cell subsets hinges on a good understanding of Th cell subsets, which should also be focused on in clinical treatment.

AUTHOR CONTRIBUTIONS

QZ, HD, XL, and BL contributed to the conception and design of the review study. QZ, HJ, ZWL and ZF wrote the first draft of the manuscript. NZ, YG, ZD, XZ, JD and NQZ wrote sections of the manuscript. HR, LY and BL discussed and revised the content of the review article. All authors contributed to the article and approved the submitted version.

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CyTOF-Enabled Analysis Identifies Class-Switched B Cells as the Main Lymphocyte Subset Associated With Disease Relapse in Children With Idiopathic Nephrotic Syndrome

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B cell depleting therapies permit immunosuppressive drug withdrawal and maintain remission in patients with frequently relapsing nephrotic syndrome (FRNS) or steroid–dependent nephrotic syndrome (SDNS), but lack of biomarkers for treatment failure. Post-depletion immune cell reconstitution may identify relapsing patients, but previous characterizations suffered from methodological limitations of flow cytometry. Time-of-flight mass cytometry (CyTOF) is a comprehensive analytic modality that simultaneously quantifies over 40 cellular markers. Herein, we report CyTOF-enabled immune cell comparisons over a 12-month period from 30 children with SDNS receiving B cell depleting therapy who either relapsed (n = 17) or remained stable (n = 13). Anti-CD20 treatment depleted all B cells subsets and CD20 depleting agent choice (rituximab *vs* ofatumumab) did not affect B cell subset recovery. Despite equal total numbers of B cells, 5 subsets of B cells were significantly higher in relapsing individuals; all identified subsets of B cells were class-switched. T cell subsets (including T follicular helper cells and regulatory T cells) and other major immune compartments were largely unaffected by B cell depletion, and similar between relapsing and stable children. In conclusion, CyTOF analysis of immune cells from anti-CD20 antibody treated patients identifies class-switched B cells as the main subset whose expansion associates with disease relapse. Our findings set the basis for future studies exploring how identified subsets can be used to monitor treatment response and improve our understanding of the pathogenesis of the disease.

Keywords: nephrotic syndrome, B cell, T cell, predictor, relapse, immune phenotype

INTRODUCTION

Pathogenesis of idiopathic nephrotic syndrome (INS), the most frequent pediatric glomerular disease (1), remains poorly understood. Familial forms of INS are characterized by genetic abnormalities (2, 3), whereas non-genetic INS forms are hypothetically immune-mediated, possibly *via* unknown circulating permeability factors (4). The prevailing hypothesis, enshrined by a classic 1974 Shalhoub article in *The Lancet*, posits INS as a T cell disorder (5). Accordingly, first-line treatment for INS are corticosteroids with a complete remission rate of about 80%. However, after initial responses, 40%–50% of patients can experience frequent relapsing-remitting episodes and become steroid-dependent or, eventually, steroid-resistant (6–8).

Pathogenic contribution from B cells was suggested in 2006, when Pescovitz et al. described the case of a 7-year-old boy with post-transplant focal segmental glomerulosclerosis recurrence [FSGS, thought to represent an evolution of minimal change disease, MCD (9)] who underwent remission after he received B-cell depletion to treat post–transplant lymphoproliferative disease (PTLD) (10). Subsequently, numerous case reports and clinical trials documented efficacy of B cell depleting therapies in inducing/maintaining long-term remission (11–14). Some reports document temporal correlation between B cell reconstitution and relapse (15, 16), while other patients remain in remission despite B cell recovery (17, 18). This formed the basis for our studies testing the hypothesis that differential immune cell frequencies during B cell recovery underlie differences in relapsing *versus* non-relapsing patients after B cell depletion.

Past analyses of B cell depleted patients relied on flow cytometry, which limited the scope of immune compartment analysis. We hypothesized that comprehensive and longitudinal peripheral immune cell analysis of reconstituted B and T cell subpopulations would confirm or identify new associations to predict/identify disease relapse post B cell depletion therapy. To address challenges of previous studies, we used time-of-flight mass cytometry (CyTOF) which utilizes heavy metal labeled antibodies, instead of fluorophores, to create immunophenotyping panels that are quantified by mass spectrometry. We analyzed and compared PBMC samples from steroid–dependent nephrotic syndrome (SDNS) patients who relapsed to those that did not after initially responding to B cell depletion (**Figure 1A**). CyTOF allows deconvolution analysis of over 40 distinct antibody markers to comprehensively characterize immune phenotypes. Using unbiased clustering, we assessed changes and tested for relationships with disease activities in (i) B cells subsets, ii) $CD4^+$ and $CD8^+$ T cell subsets, and iii) the major immune compartments.

METHODS

Subjects and Sample Collection

We performed CyTOF analyses on frozen PBMCs serially collected from patients with SDNS maintained in remission with oral steroids and calcineurin inhibitors (CNI). SDNS was defined by two consecutive relapses during corticosteroid therapy tapering or within 14 days of steroid withdrawal that responded to the association of prednisone with cyclosporine or tacrolimus.

All the patients were in complete remission at enrollment and received a single dose of either ofatumumab (OFA, 1500 mg/1.73 m^2) or rituximab (RTX, 375 mg/m^2) infusion, as part of a randomized trial (NCT02394119 and Eudract.ema.europe.eu: 2015-000624-28) (19). After infusion, steroids were progressively tapered off until complete withdrawal, which happened within 3 weeks. At one week after complete steroid withdrawal, CNI were withdrawn within 3 weeks. For the present study, we included 17 patients who underwent a relapse of NS after full immunosuppression withdrawal and 13 controls who did not undergo relapse over the same follow-up period. PBMC were collected before OFA or RTX infusion (T0), during remission after immunosuppression withdrawal (T1), and at the time of relapse or at a similar time-point after OFA or RTX therapy in non-relapsing patients (T2). At T2, relapsing patients had already received steroid therapy to promote remission.

Complete remission was defined by urinary protein over creatinine ratio (uPCR) <200 mg/g (<20 mg/mmol) or 1+ protein on urine stick for 3 consecutive days (20). Partial remission was defined by proteinuria reduction of 50% or greater from the presenting value and absolute uPCR between 200 and 2000 mg/g (20-200 mg/mmol). NS relapse was defined as uPCR ≥2000 mg/g (≥200 mg/mmol) or ≥3+ protein on urine dipstick for 3 consecutive days.

The study was approved by the Institutional Review Board at the participating centers.

Study coordinator illustrated the project, delivered the information material and collected written informed consent from parents and child assent for treatment and collection of samples. Participants or their families could withdraw the consent at any time during the study.

CyTOF Sample Preparation

To limit batch effect, we barcoded samples collected from the same subjects at 3 different times with anti-CD45 antibodies conjugated to unique metal isotopes before pooling the samples together. CyTOF sample preparation was conducted as previously reported by others (21). Antibodies were either purchased preconjugated from Fluidigm (formerly DVS Sciences, San Francisco, CA) or purchased purified and conjugated in-house using MaxPar X8 Polymer Kits (Fluidigm, San Francisco, CA) according to the manufacturer's instructions. Ninety samples (30 subjects, 3 timepoints) were processed in 3 separate batches barcoded using 3 barcoding antibodies and pooled together. All PBMCs were stained with a panel of 38 antibodies (37 for clustering, 1 for viability) (**Supplementary Table 1**).

CyTOF Data Acquisition

CyTOF data were acquired at Icahn School of Medicine at Mount Sinai as previously reported by others (21). Samples were acquired on a CyTOF2 (Fluidigm) equipped with a SuperSampler fluidics system (Victorian Airships) at a concentration of 1 million cells/ml in deionized water containing a 1/20 dilution of EQ 4 Element

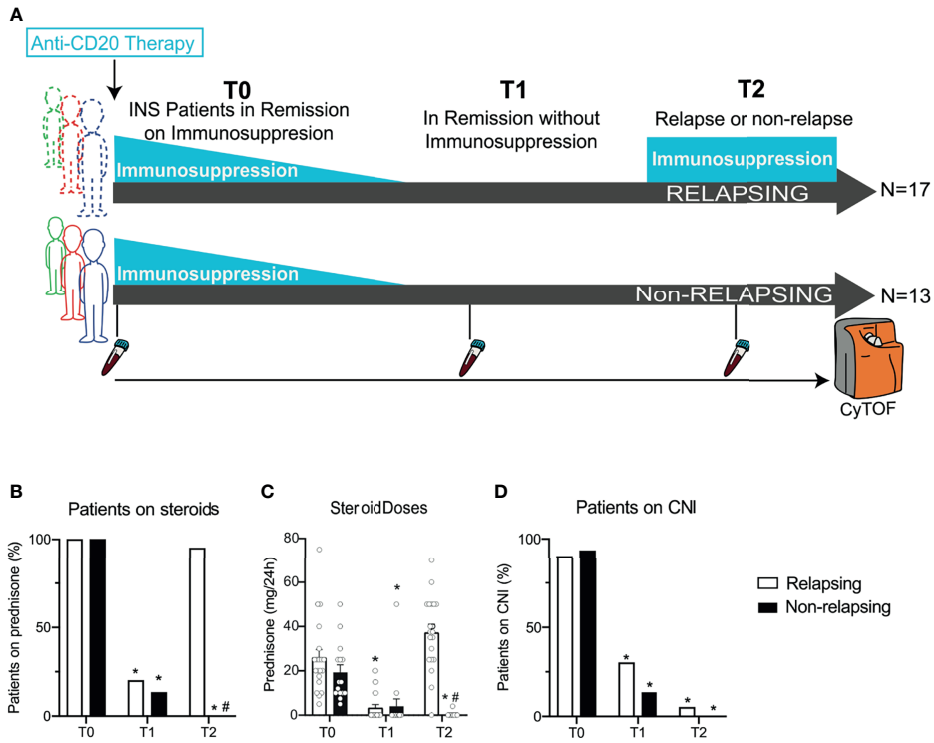

FIGURE 1 | Serial high-dimensional profiling of INS patients and concomitant immunosuppressive treatments. **(A)** Study design of thirty children with steroid-dependent nephrotic syndrome in remission with steroids ± calcineurin inhibitors who received either rituximab (RTX) or ofatumumab (OFA) and then underwent withdrawal of immunosuppression in 3 months. After withdrawal, 17 patients developed a relapse, while 13 stayed in remission. Blood was collected before RTX or OFA therapy (T0), after immunosuppressive withdrawal (while still in remission; T1), and at the time of relapse (or at the same time after B cell depleting therapy in patients in remission; T2). Cells were barcoded for patient and time, pooled, and stained with 38 antibodies conjugated to unique metal isotopes. Single-cell data acquired from time-of-flight mass cytometry (CyTOF) was clustered using Phenograph to identify cell clusters and how they evolved over time in each patient (see Methods). **(B)** Percentage of patients treated with prednisone and **(C)** average daily prednisone doses in relapsing and non-relapsing patients before anti-CD20 therapy (T0), in remission after immunosuppression withdrawal (T1), and at relapse (or at the same time point after anti-CD-20 therapy in non-relapsing patients; T2). **(D)** Percentage of patients treated with calcineurin inhibitors (CNI) at the same visits. n=30. *$p < 0.05$ *vs*. T0. $^{\#}p < 0.05$ *vs*. relapsing at the same visit. Bar plots depict mean ± SEM.

Beads (Fluidigm) and at an event rate of < 500 events/second. After acquisition, the data were normalized using bead-based normalization in the CyTOF software. Barcodes were demultiplexed using the Fluidigm debarcoding software. The data were gated to exclude residual normalization beads, debris, dead cells, and doublets, leaving live $CD45^+$ events for subsequent clustering and high-dimensional analyses.

CyTOF Data Analyses

We first clustered cells using the PhenoGraph algorithm (21) and we then curated the 20 metaclusters obtained. The frequencies for each common population were obtained by summation of the frequencies in each metacluster and subsequently debarcoded to obtain frequencies for each timepoint and patient. To minimize variability in measurement, our analysis strategy was structured as follows:

i. Application of FlowSOM to computationally pooled samples: we pooled *in silico* all labeled cells from all timepoints together for each patient to analyze the major immune compartments (10,000 cells per timepoint for a total of 30,000 cells per patient), we applied FlowSOM, and then demultiplexed them. This allowed mapping of the same subsets in all the samples.
ii. Equal contribution of the samples: to avoid bias in FlowSOM for the subsets present in the samples with a greater number of $CD4^+$ or $CD8^+$ we maintained an equal contribution in the number of cells from every sample determined by the sample with the lowest number of $CD4^+$ or $CD8^+$ T cells.
iii. Reiteration: to increase power of the analyses for the cell subsets with low number of events, we reiterated the entirety of the sampling process and FlowSOM clustering up to 3 times to achieve robustness in the results.

Despite the level of stringency imposed by the repeated sampling and clustering process, we observed little dispersion in the results indicating that our findings were robust.

We did not observe significant differences in the average and SD of the signal for each marker nor in the frequencies of the major immune compartments across batches.

The number of $CD45^+$ cells/mm^3 was derived from measurements of the number of lymphocytes (T, B and NK cells) in each patient based on the frequencies of those major immune compartments.

Cell Number Counts

Absolute cell number counts were obtained from total lymphocyte counts available from CBCs performed on these samples multiplied by the frequencies derived from the CyTOF analysis.

Statistics

Statistical significance was determined by GraphPad Prism or R. Statistical tests used are reported in the figure legends. To establish all comparisons within relapsing and non-relapsing at different timepoints we performed a two-way ANOVA test, without assuming sphericity (Geisser-Green house correction) correcting for multiple comparisons by controlling the False Discovery Rate using the two-state step-up method of Benjamini and Yekutieli. Differences are considered significant at $p \leq 0.05$.

RESULTS

Baseline Patients' Characteristics

We analyzed serial peripheral blood samples from 30 children with SDNS: baseline characteristics of included patients are shown in **Table 1**. At time of anti-CD20 therapy (before RTX or OFA; given at T0), all but one patient were receiving steroids and calcineurin inhibitors (**Figures 1B–D**). After receiving anti-CD20 therapy, patients underwent immunosuppressive withdrawal, and at T1 [after an overall median of 4.1 months (IQR 3.0 - 7.1); 3.0 (3.0-7.3) and 3.0 (3.0-7.0) months for relapsing and non-relapsing patients, respectively], all patients were still in complete remission with reduced or no immunosuppression (**Figures 1B–D**). During a median follow-up period of 7.2 months (IQR 6.0 - 11.8) (T2), NS relapse occurred in 17 patients [T2: 6.5 months (5.0-7.8)] who restarted steroids, while remission persisted in 13 [T2: 8.0 months (6.0-9.0)]. At the same visit (T2), non-relapsing patients were in remission with no immunosuppression (**Figures 1B–D**). There was no difference in relapse rates between RTX and OFA treated patients.

Changes in the Major Circulating Immune Compartments After B-Cell Depletion

We quantified and compared major immune compartment kinetics using unbiased clustering (Phenograph Clustering Algorithm (22), see Methods) with 12 markers and viSNE to visualize high-dimensional data in two dimensions while preserving single-cell resolution (23) (**Figure 2A**). B cell frequencies were significantly reduced after anti-CD20 treatment (OFA and RTX) and returned toward baseline values at T2 (**Figures 2B, C**). Frequencies of other major immune compartments, including macrophages, dendritic cells (DCs), plasmacytoid dendritic cells (pDCs), monocytes, NK cells, $CD4^+$ T cells, $CD8^+$ T cells and $CD4^-CD8^-$ T cells were not significantly affected by B cell depletion (visits T0 and T1; **Figures 2B, C**). At T2, relapsing patients had significantly decreased NK and pDCs and increased monocytes and $CD8^+$ T cell frequencies compared to non-relapsing patients (**Figures 2B, C**).

TABLE 1 | Baseline characteristics of study participants.

	Total (n = 30)	Relapsing (n = 17)	Non-relapsing (n = 13)	P-value
Demographics				
Age (yrs)	11.6 ± 6.9	11.1 ± 6.7	12.3 ± 7.4	0.65
Sex n (%)				0.24
Female	6 (20)	2 (12)	4 (31)	
Male	24 (80)	15 (88)	9 (69)	
Treatment				0.29
Rituximab n (%)	15 (50)	10 (59)	5 (38)	
Ofatumumab n (%)	15 (50)	7 (41)	8 (62)	
Prior rituximab use, n (%)	12 (40)	6 (35)	6 (46)	0.36
Number of prior relapses, n	1.5 (1-10)	2 (1-10)	1 (1-6)	0.52
Immunosuppressive drugs at enrollment (T0), n (%)				
Steroids	30 (100)	17 (100)	13 (100)	1.0
Tac	16 (53)	9 (53)	7 (58)	0.96
CsA	13 (43)	8 (47)	5 (38)	0.63
Labs				
Serum Creatinine (mg/dL)	0.54 ± 0.24	0.53 ± 0.27	0.55 ± 0.20	0.85
Serum Albumin (g/dL)	3.73 ± 0.64	3.74 ± 0.55	3.71 ± 0.77	0.88
Cholesterol (mg/dL)	210 ± 61	192 ± 57	232 ± 61	0.09
eGFR (ml/min)	137 ± 38	142 ± 39	129 ± 37	0.35
IgA (mg/dL)	658 ± 231	638 ± 243	685 ± 221	0.59
IgM (g/dL)	166 ± 109	162 ± 127	171 ± 84	0.82
IgG (mg/dL)	144 ± 83	150 ± 99	134 ± 60	0.60
Proteinuria (mg/24 h)	99 ± 83	116 ± 104	78 ± 36	0.18

Data are mean ± SD number (%), or median (range). p-value compares the two treatment groups in a two-tailed t-test of heteroscedastic variance or chi squared. Tac, tacrolimus; CsA, cyclosporine.

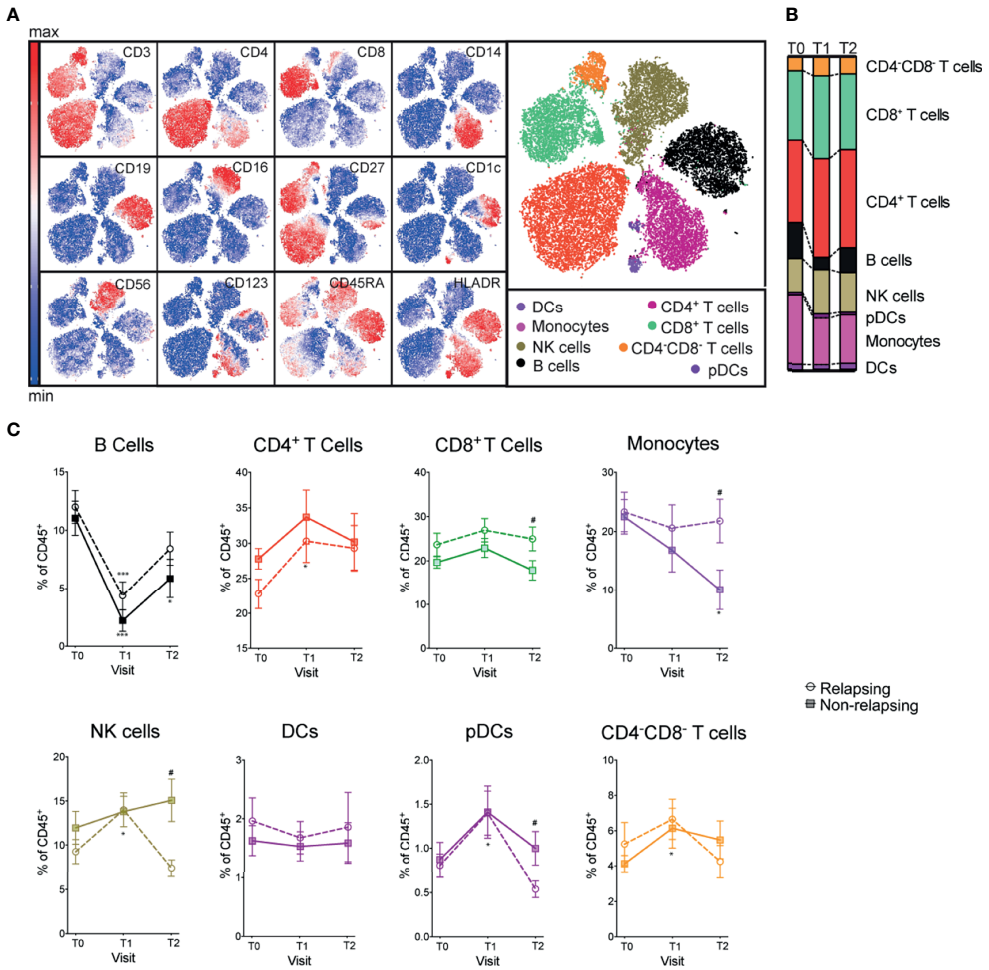

FIGURE 2 | Frequencies of major immune compartments in INS patients receiving OFA or RTX therapy. **(A)** viSNE analysis of peripheral blood mononuclear cells (PBMC) from a representative patient including 3 time points (during immunosuppressive treatment, after immunosuppressive treatment withdrawal and at the time of relapse) colored by the relative expression of CyTOF markers to designate major immune clusters [populations defined in **(B)**]. **(C)** Percentages of major immune compartments once demultiplexed and based on the summation of Phenograph clusters before (T0) and serially after (T1 and T2) anti-CD20 therapy. Comparisons between relapsing and non-relapsing at the same timepoint $^{\#}p < 0.05$. Comparisons between different timepoints $^{*}p < 0.05$, $^{***}p < 0.001$. Two-way ANOVA corrected for multiple comparisons. Data points depict mean ± SEM.

Proportion of Switched B Cell Subsets Is Higher in Relapsing Patients

Magnitude of B cells ($CD19^+$) decrease was similar between relapsing and non-relapsing patients (**Figure 2C**). Nadir B cell percentage in OFA-treated patients was significantly lower than in RXT-treated ones, but there was no significant difference between OFA and RTX in post-treatment B cell percentages or absolute numbers (**Supplementary Figures 1A, B**). We next compared kinetics and makeup of B cell subsets between relapsing and non-relapsing patients by performing a second level of unbiased clustering on B cells using 8 subset markers (CD127, CD21, IgM, IgD, CD27, CD95, CD38, CD25). B cells from all patients and all visits were pooled together, and clustered using B cell specific markers into subsets based on relative expression defining markers. Subsets were then demultiplexed to extract the subpopulation frequencies over time. We explored the different clusters using a heatmap displaying the relative expression of each marker per cluster and grouped them based on similarity (**Figure 3A**). Within the B-cell compartment we identified 10 distinct B-cell subpopulations, including B cells with a naïve (IgD^+CD27^-) (24), regulatory ($CD25^{hi}$) (25, 26), antibody secreting (IgD^-,$CD27^+$,$CD38^+$) (24), and memory phenotype ($IgD^+CD27^+CD38^-$) (24). Frequencies of transitional B cells, B regulatory cells (B_{REG}), and class-switched memory (CSM) resting B cells significantly increased after therapy (**Supplementary Figure 2**) despite global decreases in absolute numbers (**Figure 3B**). While we did not observe differences in single subset frequencies between the two groups at analyzed timepoints (**Supplementary Figure 2**), we found that frequencies of switched B cell subsets altogether (B_{REG}, CSM resting, CSM

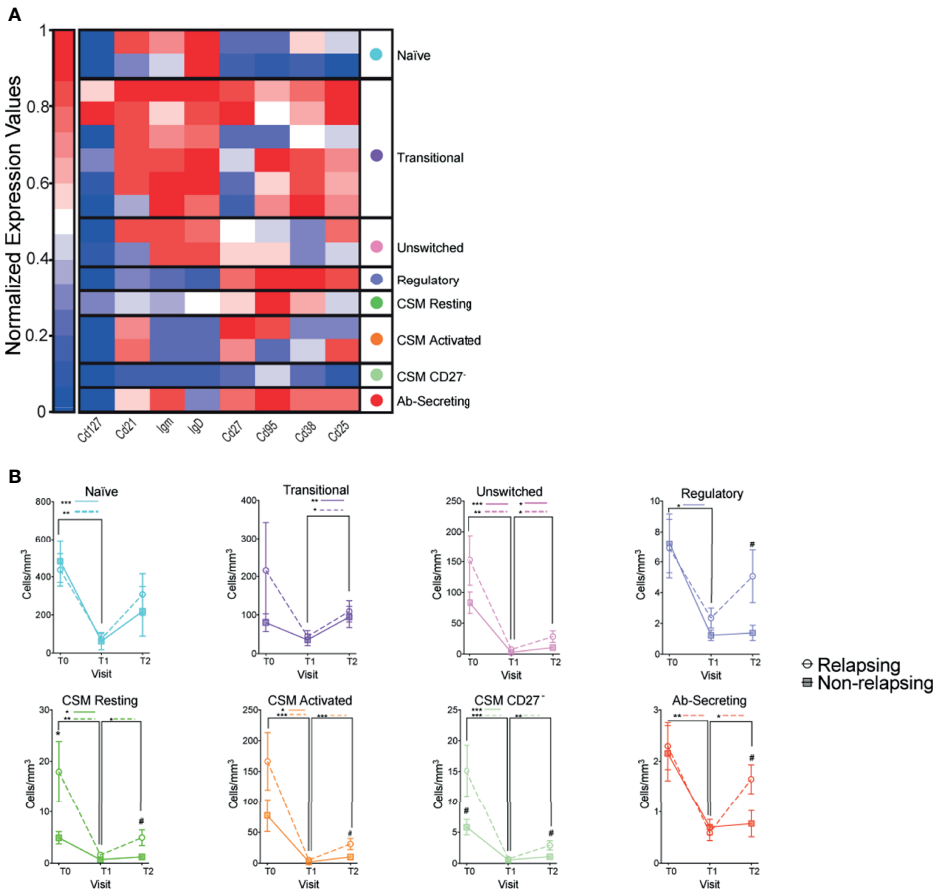

FIGURE 3 | Changes in B cell subsets after anti-CD20 Ab therapy. **(A)** Heatmap of $CD19^+$ immune cells colored and labeled by Phenograph cluster for all subjects and time points combined (see Methods). Rows represent different clusters identified and columns the relative expression of each marker in that particular cluster **(B)** Absolute numbers of $CD19^+$ B cell subsets once demultiplexed and based on the summation of Phenograph clusters before (T0) and serially after (T1 and T2) anti-CD20 therapy. Comparisons between relapsing and non-relapsing at the same timepoint $^{\#}p < 0.05$. Comparisons between different timepoints *p < 0.05, **p < 0.01, ***p < 0.001. Two-way ANOVA corrected for multiple comparisons. Data points depict mean ± SEM.

activated, CSM $CD27^-$, and Ab-secreting B cells) were significantly higher in relapsing patients than in non-relapsing individuals, both at T0 and T2 (**Figures 4A, B**, p<0.05). We also quantified composite absolute numbers of switched B cells and observed significantly higher numbers at T2 amongst relapsing patients (**Figure 4C**). At T1, there were no significant difference in switched B cells between individuals who fully withdrew immunosuppression and those who were on minimal residual immunosuppression (not shown).

T Cell Subsets Are Not Different Between Relapsing and Non-Relapsing Patients

We next quantified and compared T cell subset frequencies over time between relapsing and non-relapsing patients. Several investigators have observed differences in T cell subpopulations after B cell depleting therapies that implicate a role in treatment effect (27, 28). We performed a second level of unbiased clustering within $CD4^+$ and $CD8^+$ T cell compartments using 18 ($CD4^+$) and 17 ($CD8^+$) subgroup markers, respectively (PD-1, TIGIT, ICOS, CD57, TIM3, Foxp3 only for CD4+, 2b4, CTLA4, OX40, CCR6, CXCR5, CD45RO, CD95, CD127, CCR7, CD25, CXCR3, CD45RA) quantifying subpopulation differences between study groups (**Supplementary Figure 3A** and **Supplementary Figure 4A**).

At T1, absolute numbers of $CD4^+$ T cells, as well as central and effector memory subgroups, were lower in relapsing patients, but their frequencies were not different (**Supplementary Figures 3B, C**). Percentages of regulatory T cells (T_{REG}) – a $CD4^+$ T cell subset whose reduction others associated with relapse risk (29, 30) - did not significantly differ between groups. $CD4^+$ T cells with a T-follicular helper-like (T_{FH}) phenotype (see **Supplementary Figure 3** clusters for markers), a population of crucial importance in antibody production, were also similar between groups.

Unfractionated $CD8^+$ T cell absolute numbers over time were similar between relapsing and non-relapsing patients (**Supplementary Figure 4C**). At T2, absolute numbers (but not

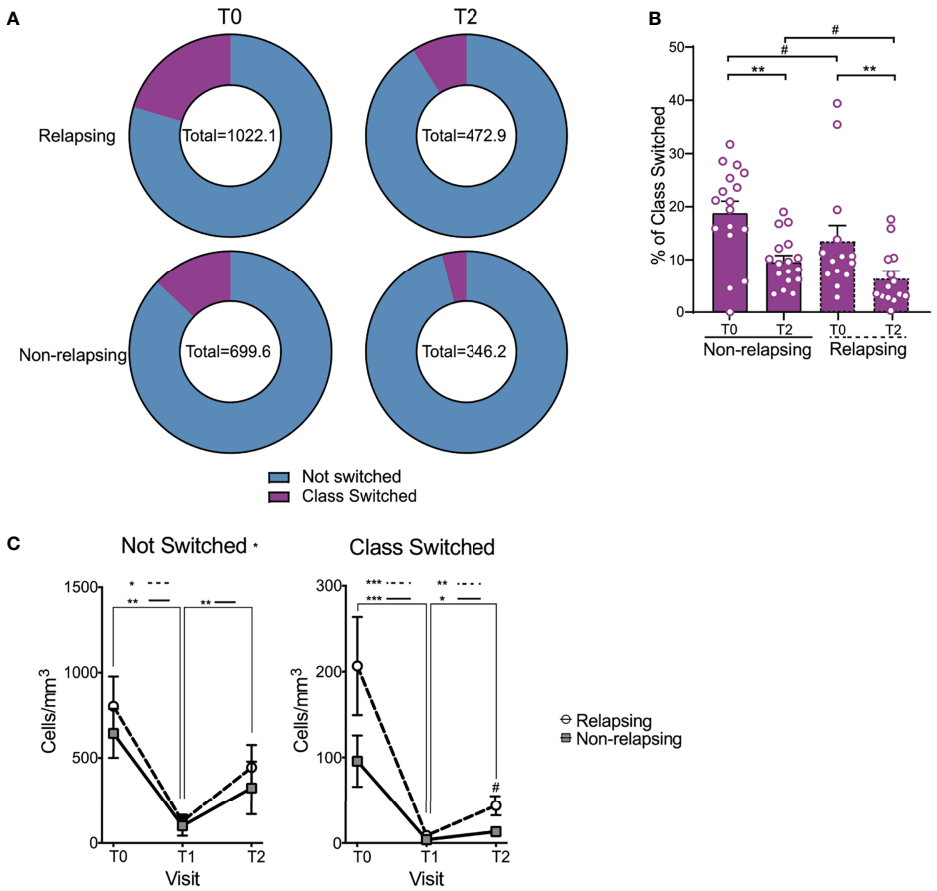

FIGURE 4 | Changes in not switched and class switched B cells after anti-CD20 Ab therapy. **(A)** Doughnut pie graph of the number of class switched B cells (total = average number of class switched and not-switched cells per sample) before anti-CD20 treatment (T0) and at relapse (or at the same time point after anti-CD-20 therapy in non-relapsing patients) (T2). **(B)** Bar graph illustrating the percentage of class switched B cells. **(C)** Changes in absolute numbers of not switched and class switched B cells in relapse and non-relapsing patients at T0, T1 and T2. Comparisons between the relapsing and non-relapsing groups at the same timepoint $^{\#}p < 0.05$. Comparisons between different timepoints $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Two-way ANOVA corrected for multiple comparisons. Bar plots and data points depict mean ± SEM.

percentages) of exhausted and effector memory (EM) $CD8^+$ T cells were significantly higher in relapsing patients (**Supplementary Figures 4B, C**). At T1, there were no significant difference in CD4+ nor in CD8+ T cells between individuals who fully withdrew immunosuppression and those who were on minimal residual immunosuppression (not shown).

DISCUSSION

Our work is the first CyTOF-enabled immune compartment survey of INS patients receiving B cell depleting therapies. Standard flow cytometry is limited in the number of markers that can be probed in a single experiment due to autofluorescence and spectral spillover associated with fluorophores. CyTOF utilizes metal isotopes that possess unique mass spectrometry signatures enabling the analysis of over 40 cellular markers at the same time. Furthermore, CyTOF reduces experimental variability as metal isotopes can be used to tag samples with barcodes, allowing simultaneous sample analysis. Even with limited samples, CyTOF increases the likelihood that our results are reproducible, making them more clinically relevant.

A previous study in INS patients showed that accelerated reconstitution of $CD19^+CD27^+IgM^-IgD^-$ B cells (defined as switched-memory) identifies patients at higher risk of disease recurrence (31). Our data corroborates this specific finding in memory cells and extends such results using a more comprehensive characterization of B cell clusters. B cell depletion strategies are widely used in treating other autoimmune diseases. The present data in INS patients add to several published studies showing that recovery of class-switched memory B cells heralds relapses of other autoimmune diseases such as myasthenia gravis, neuromyelitis optica, and rheumatoid arthritis (32, 33). Therefore, monitoring relevant B cell subsets could guide timing of repeat treatment with depleting therapies or use of alternative immunosuppression regimens. This provides advantages over

current clinical management that relies on markers of end organ damage – proteinuria and serum creatinine.

Intriguingly, switched B cells were significantly higher in relapsing patients despite the fact that T2 samples were taken after the initiation of steroid therapy, which has been shown to reduce this cell population (34, 35), a phenomenon that has been observed prior in INS (35) and other autoimmune conditions (36).

Five subsets of switched B cells recovered early in patients with disease relapse. In addition to class-switched memory cells, $IgD^-CD27^+CD38^+CD95^+$ Ab-secreting cells represent the subset most strongly associated with disease recurrence (37). This subpopulation lacks IgD expression and is CD27 positive, signifying mature B cells (38), and additionally express CD38. CD38 is a highly conserved type II glycoprotein that possesses pleiotropic effects on B-cell function and maturation (39–41). CD38 induces apoptosis in early B cells but promotes survival in germinal center B cells (42). Antibody-secreting cells, made up of plasma cells and immature plasmablasts (43), express high levels of CD38, whereas memory B cells, also IgD^- and $CD27^+$ (44–46), lack CD38 expression (47, 48).

We also found that patients with disease relapse had a faster recovery of $CD25^+CD127^-IgD^-IgM^-$ B cells, a phenotype compatible with B_{REG}. These cells inhibit T cells through the release of IL-10, IL-35, and transforming growth factor β (TGF-β) and their importance is known for several autoimmune conditions (49–51). Originally identified as a transitional B cell subset, it is now known that B_{REG} can acquire suppressive functions at different stages of development in response to environmental cues (52). Possibly, B_{REG} expansion in relapsing patients reflects a compensatory mechanism to active antibody production, similar to expansion of regulatory T cells (T_{REG}) in kidney transplant recipients with acute rejection (53). However, more studies are needed to determine whether their presence is directly or indirectly related to INS pathogenesis. We analyzed T cell subpopulations because antibody mediated diseases (*e.g.* membranous nephropathy) depend on cognate B cell-T cell interactions. Previous studies showed that RTX affects homing of T_{REG} and T follicular helper cells (T_{FH}) (54), possibly as a consequence of reduced interaction with B cells (55).

Most data on the efficacy of B cell depleting therapies in INS patients have been generated with rituximab, a chimeric anti-CD20 monoclonal antibody. Ofatumumab is a fully humanized anti-CD20 monoclonal antibody of last generation. OFA binds CD20 with more affinity, potentially leading to more efficient complement-dependent cytotoxicity (56, 57). In a small series, OFA-induced remission in children with SR-INS who did not respond to RTX (31). Our data confirm the higher peripheral B cell depleting efficacy of OFA compared to RTX. Despite this, kinetics of B cell reconstitution did not differ significantly between the two treatment arms suggesting that, at the doses used in our study, their effects on B cells in secondary lymphoid organs are similar. Because B cell reconstitution portended disease relapse, we hypothesized that differences in B cells subsets exist amongst patient groups.

B cell depleting agents have variable penetrance into secondary lymphoid structures. Relapse in patients with disproportionate persistence of antigen experienced B cells may represent preferential survival of autoreactive B cells. Consistent with this hypothesis, our data showed trends for increased frequency of Ab-secreting B cells that first occurred when B cells were depleted (T1, **Supplementary Figure 2**).

Our data provide basis to pursue critical questions regarding antigen specificity of surviving/reconstituting cell populations. The present findings are also consistent with murine data showing that podocyte targeting antibodies are sufficient to change the glomerular filtration barrier and are the "permeability factor" in some patients with INS due to FSGS (58). Clinically, our findings suggest that combined B cell therapies (*e.g.* RTX + proteasomal inhibitor) could provide enhanced treatment efficacy by targeting all class-switched and Ab-secreting cells.

B cells, especially the ones expressing activation marker CD25, can act as antigen presenting cells to activate T cells (59). However, in contrast to B cell subsets, our comprehensive immune phenotypic analysis identified few significant differences between relapsing and non-relapsing patients in $CD4^+$ and $CD8^+$ T cell clusters at T2. An exception to this were exhausted $CD8^+$ T cells that were higher in relapsing patients. T cell exhaustion arises from prolonged antigen exposure (60) and is observed in patients with chronic autoimmune diseases (61) or renal transplant recipients (62). Higher frequencies of exhausted $CD8^+$ T cells in relapsing patients at T2 raises the intriguing hypothesis that T cell exhaustion is a marker of disease chronicity/activity, indicative of increased B cell activation.

The main immune cell compartments were largely comparable between relapsing and non-relapsing patients with the exception of monocytes and NK T cells which underwent reciprocal changes. Changes we observed are likely related to resumption of high-dose corticosteroids in relapsing patients (63). Absence of differences in major immune compartments is an important negative finding with respect to B cell depleting therapies and emphasizes how CyTOF powered analysis provides uniquely in-depth and broad analysis.

Limitations

Our study main limitation is the relatively limited sample size and the absence of prospective validation. Although the limitations associated with a small number of patients cannot be entirely overcome, CyTOF allowed comprehensive immune cell phenotyping with increased sensitivity for low-expressed markers. Therefore, we extracted a significant amount of information from a reduced number of samples. Additionally, we resampled and reanalyzed B-cell clusters 3 times to further ensure our findings were statistically robust and valid. Lastly, samples were run simultaneously, further decreasing variation due to technical issues.

Another intrinsic limitation of the study is the non-standardized time for blood sampling, as the time of relapses could not be planned in advance. However, the frequent sampling allowed us an excellent matching in the sample timing between relapsing and non-relapsing patients.

Previous studies have shown that RTX may affect not only B-cell composition, but also their function (64). Lack of functional studies on reconstituted switched B cells prevents any definitive conclusion on the effect of these cells in relapsing and non-relapsing patients. Future studies will be required to address this relevant issue as well as antigen specificity of

reconstituted populations in relapsed patients. Our work provides the most solid basis, to date, for interrogating class-switched B cells in these patients.

CONCLUSIONS

Overall, the present study demonstrates that 5 subsets of circulating switched B cells are preferentially increased in INS patients who relapsed after CD20-depleting therapies, suggesting a pathogenic link and potential for use as a biomarker. Our work provides focus and a strong rationale for future studies testing the hypothesis that monitoring the number and, potentially, function of switched B cells identifies patients at higher risk of relapse instead of using total B cells or clinical indicators, alone. Transcriptional and/or proteomic analyses of switched B cells in relapsing or non-relapsing patients may provide important mechanistic insights on INS pathogenesis.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

PC, GG, MCo, and MF conceived and designed the study. MCi collected the samples. CC, JL, KB, and SB were involved in the preparation of samples for CyTOF analyses. MF performed all the statistical analyses. PC and MF critically interpreted the data and wrote the first draft of the manuscript together with LP. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.726428/full#supplementary-material

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Rituximab Immunomonitoring Predicts Remission in Membranous Nephropathy

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Primary membranous nephropathy (pMN) is an autoimmune kidney disease and a common cause of nephrotic syndrome in adults. Rituximab is becoming a first line therapy for patients with persistent nephrotic syndrome with proven safety and efficacy, achieving remission in 60%–80% of cases. For the remaining 20%–40% of patients there is an urgent need to identify early biomarkers of resistance to rituximab to adapt therapeutic management. In nephrotic patients, rituximab is found in the blood more transiently than in other autoimmune diseases without proteinuria, due to rituximab wasting in the urine. However, rituximab immunomonitoring is not routinely performed. We evaluated the predictive value of serum rituximab levels in patients with pMN three months after rituximab injection (month-3) on clinical remission rates six months (month-6) and 12 months (month-12) after injection and investigated predictive factors for serum rituximab levels at month-3. Sixty-eight patients treated with rituximab between July 2015 and January 2020 from two French nephrology centers were included. We identified residual rituximab levels at month-3 as a novel early predictor of remission at month-6 (p <0.0001) and month-12 (p = 0.001). Reduced likelihood of remission in patients with undetectable rituximab at month-3 was associated with lower serum albumin and higher anti-PLA2R1 titers at baseline and with lower serum albumin, higher proteinuria, higher $CD19^+$ counts and higher anti-PLA2R1 titers during follow-up. In multivariate analysis, high baseline proteinuria and undetectable rituximab levels at month-3 were independent risk factors for treatment failure at month-6 and high baseline weight and undetectable rituximab levels at month-3 were independent risk factors for treatment failure at month-12. We identified serum albumin at baseline as a predictive factor for serum rituximab levels at month-3. Patients with serum albumin below 22.5 g/L at baseline had an 8.66-fold higher risk of having undetectable rituximab levels at month-3. Therefore, rituximab immunomonitoring in pMN patients treated with rituximab would allow the detection of patients at risk of treatment failure as early as month-3. Studies are needed to

assess whether patients with low residual rituximab levels at month-3 may benefit from an early additional course of rituximab.

Keywords: membranous nephropathy, nephrotic syndrome, autoimmunity, rituximab, chronic kidney disease rituximab immunomonitoring in membranous nephropathy

INTRODUCTION

Primary membranous nephropathy (pMN) is an autoimmune disease affecting kidney glomerulus and the most common cause of nephrotic syndrome (NS) in non-diabetic adults. The course of the disease is highly variable, ranging from spontaneous remission to progressive chronic kidney disease. Histologically, pMN is characterized by subepithelial immune deposits containing immunoglobulins G (IgG) and complement fractions resulting in thickening of the glomerular basement membrane and the formation of spikes (1). The pathophysiology of pMN involves autoantibodies targeting podocyte proteins such as M-type phospholipase A2 receptor 1 (PLA2R1) and thrombospondin type-1 domain-containing 7A (THSD7A) in 70%–80% and 3%–5% of patients, respectively (2, 3). Immune complex deposits are responsible for the activation of the complement cascade and podocyte damage (4–6). The pathogenicity of anti-PLA2R1 and anti-THSD7A autoantibodies has been demonstrated *in vitro* and *in vivo* (4, 7, 8). The recognition of pMN as an autoantibody-mediated disease has promoted the use of immunosuppressive drugs. Rituximab – a chimeric monoclonal antibody targeting CD20 – can trigger B cell death by apoptosis, complement-mediated cytotoxicity and antibody-dependent cellular cytotoxicity leading to an elimination of autoantibodies (9–11). Rituximab was first developed for the treatment of hematological malignancies, but is now used to treat many immune-mediated diseases (12). Rituximab is progressively becoming a first line therapy for pMN patients with proven safety and efficacy, achieving remission in 60%–80% of patients (13–15). However, for the remaining 20%–40% of patients there is an urgent need to identify early biomarkers of resistance to rituximab in order to adapt therapeutic management. Some patients with pMN may develop anti-rituximab antibodies that may decrease the effectiveness of the treatment (16). In these cases obinutuzumab and ofatumumab have been shown to be effective (17–20). Other patients are undertreated because of the highly variable bioavailability of rituximab in nephrotic patients (21). In nephrotic patients, rituximab – which binds to albumin – can be eliminated in the urine, thus rituximab is found in the blood more transiently than in other autoimmune diseases treated with rituximab without proteinuria (21, 22). There is still uncertainty about which rituximab protocol to use in nephrotic patients. Patients with the shortest exposure to rituximab could benefit from additional courses of rituximab to increase their likelihood of clinical remission. However, rituximab immunomonitoring is not yet routinely performed in patients with pMN.

The aims of this study were: (i) to evaluate the predictive value of serum rituximab levels in patients with pMN three months after rituximab injection (month-3) on clinical remission rates six months (month-6) and 12 months (month-12) after rituximab injection and (ii) to establish predictive factors for undetectable serum rituximab levels at month-3.

MATERIAL AND METHODS

Study Participants

Sixty-eight patients with pMN from a prospective cohort were included (NCT02199145). Patients were enrolled from two French nephrology centers from July 2015 to January 2020. The inclusion criteria were (1) biopsy-proven diagnosis of membranous nephropathy; (2) primary membranous nephropathy defined by the absence of concomitant autoimmune disease, negative hepatitis B and C serologies, and negative cancer workup; (3) rituximab treatment with two 1 g infusions two weeks apart; and (4) serum samples available at month-3. Patients should not receive any other immunosuppressive therapy at the same time as rituximab. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the appropriate institutional review committee. Written informed consent was obtained from participants prior to inclusion in the study.

Outcome

Clinical remission was evaluated at month-6 and month-12. Clinical remission was defined according to the 2012 Kidney Disease: Improving Global Outcomes (KDIGO) guidelines (23). Complete remission was defined by a urinary protein-to-creatinine ratio < 0.3 g/d, accompanied by a normal serum albumin concentration and a preserved kidney function. Partial remission was defined by urinary protein-to-creatinine ratio < 3.5 g/d with 50% reduction of proteinuria, accompanied by an improvement or normalization of the serum albumin concentration and preserved kidney function. Treatment failure was defined by the lack of clinical remission.

Detection of Anti-PLA2R1 and Anti-THSD7A Antibodies

Serum levels of total IgG anti-PLA2R1 antibodies were measured by the ELISA test developed by EUROIMMUN (Medizinische Labordiagnostika AG, Lübeck, Germany). Participants were considered as anti-PLA2R1–positive when levels were >14 RU/mL.

Total IgG anti-THSD7A antibodies were measured by the indirect immunofluorescence test developed by EUROIMMUN (Medizinische Labordiagnostika AG, Lübeck, Germany).

Rituximab Immunomonitoring by ELISA

Serum levels of rituximab were measured by ELISA according to the manufacturer's instructions (LISA-TRACKER Duo Rituximab; Theradiag, Croissy Beaubourg, France). This assay measures only free rituximab. The limit of detection defined by

the manufacturer was 2 μg/mL. Rituximab levels were measured 3 months after the last infusion. This time interval was chosen based on a previous bioavailability study (21).

Anti-Rituximab Antibodies Detection

Anti-rituximab antibodies were detected by ELISA (LISA-TRACKER, Theradiag, Croissy Beaubourg, France). The limit of detection for anti-rituximab antibodies defined by the manufacturer was 5 ng/mL.

Statistical Analyses

Categorical data were described as frequency and percentage and were compared using Fisher's exact test. Quantitative data were described as median and interquartile range and were compared using Wilcoxon-Mann-Whitney test. Survival curves were assessed by the Kaplan-Meier method and compared with the log-rank test. A *P*-value < 0.1 was used to select variables that were entered into a single multivariate logistic model with backward selection (threshold = 0.05). We performed a receiver operating characteristic curve (ROC) analysis to assess the optimal threshold of predictive factors identified in multivariate analysis that could predict the risk of undetectable rituximab levels at month-3. A *P*-value < 0.05 indicated statistical significance. Statistical analyses were performed using GraphPad Prism 8.4.3 (GraphPad Software Inc., San Diego, CA) and SAS Enterprise Guide 7.1 (SAS Institute Inc., Cary, NC).

RESULTS

Population Characteristics

Sixty-eight patients with pMN treated with rituximab (two 1 g infusions two weeks apart) were included. A supportive therapy with angiotensin-converting enzyme inhibitor or angiotensin receptor blocker was consistently associated with rituximab. **Table 1** shows the characteristics of the study cohort at baseline (i.e. the day of rituximab injection) and during follow-up.

Serum rituximab levels were measured at month-3. In 38 patients (56%), rituximab was undetectable in the serum (i.e. < 2 μg/mL) at month-3. In 30 patients (44%), serum rituximab was over 2 μg/mL at month-3. At baseline, in patients with undetectable serum rituximab levels at month-3, anti-PLA2R1 titers were higher and serum albumin levels were lower than in patients with serum rituximab levels > 2 μg/mL at month-3 (**Table 1**). Proteinuria tended to be higher in patients with undetectable serum rituximab levels at month-3, although this did not reach statistical significance (**Table 1**). All other baseline characteristics were similar, including age, gender, weight, type of autoantibodies, serum creatinine levels and $CD19^+$ cell counts.

During follow-up in patients with undetectable serum rituximab levels at month-3, anti-PLA2R1 titers were higher at month-3 and month-6; proteinuria was higher at month-3 and month-6; and serum albumin levels were lower at month-3 and month-6 than in patients with serum rituximab levels > 2 μg/mL at month-3 (**Table 1**). B cells re-emerged more quickly in patients with undetectable serum rituximab levels at month-3 than in patients with serum rituximab levels > 2 μg/mL at month-3 (**Table 1**).

Anti-rituximab antibodies were never detected at month-3, and were detected at month-6 in 4 of 38 patients (11%) with undetectable serum rituximab levels at month-3 and 2 of 30 patients (7%) with serum rituximab levels > 2 μg/mL at month-3 (**Table 1**).

Outcome

Clinical remission was analyzed at month-6 and month-12. Clinical remission (partial or complete) was obtained in 36 of 68 patients (53%) at month-6 and in 41 of 68 patients (60%) at month-12 (**Table 1**). Serum rituximab levels at month-3 were significantly lower in patients who failed to achieve clinical remission at month-6 (0.06 μg/mL [IQR, 0.1–1.1] *versus* 3.2 μg/mL [IQR, 0.9–11.0]; $p < 0.0001$) and at month-12 (0.1 μg/mL [IQR, 0.0–1.2] *versus* 2.3 μg/mL [IQR 0.4–9.0]; $p = 0.0004$) (**Figures 1A, B**). Patients with serum rituximab levels > 2 μg/mL at month-3 achieved clinical remission more frequently at month-6 and month-12 than patients with undetectable serum rituximab levels at month-3 (**Table 1**). Kaplan–Meier analysis demonstrates faster time to clinical remission when serum rituximab levels are over 2 μg/mL at month-3 (**Figure 1C**). After pairing data for age, gender, weight and anti-PLA2R1 titers at month-3, with propensity score matching using logit model, serum rituximab levels at month-3 predicted remission at month-6 ($p = 0.02$ using McNemar's test). In multivariate analysis, an undetectable serum rituximab level at month-3 and high proteinuria at baseline were independent risk factors for treatment failure at month-6 (hazard ratio (HR), 12.00; 95% confidence interval (CI), 2.77–52.99; $p = 0.007$ and HR, 1.37; 95% CI, 1.08–1.73; $p = 0.02$, respectively) (**Table 2**); and an undetectable serum rituximab level at month-3 and high weight at baseline were independent risk factors for treatment failure at month-12 (HR, 10.98; 95% CI, 2.58–46.72; $p = 0.01$ and HR, 1.05; 95 % CI, 1.01–1.09; $p = 0.02$, respectively) (**Table 3**).

For patients with undetectable serum rituximab levels at month-3: (i) clinical remission was never achieved at month-6 if proteinuria was greater than 5.5 g/d at month-3; (ii) clinical remission was achieved at month-6 in 33% of cases if proteinuria was between 3.5 g/d and 5.5 g/d at month-3; and (iii) clinical remission was achieved at month-6 in 77% of cases if proteinuria was less than 3.5 g/d at month-3 (**Figure 2**).

Regarding cases who developed anti-rituximab antibodies, 4 of 6 patients (67%) did not achieve clinical remission at either month-6 or month-12. Two patients with anti-rituximab antibodies (1 with undetectable serum rituximab at month-3 and 1 with serum rituximab > 2 μg/mL at month-3) achieved partial clinical remission at month-6.

We then investigated the impact of rituximab levels on clinical outcome in patients with severe nephrotic syndrome, i.e. with baseline proteinuria exceeding the median value (6 g/d). Among these patients, 20 patients had undetectable rituximab levels at month-3 and 14 patients had rituximab levels > 2 μg/ml at month-3. Patients with serum rituximab levels > 2 μg/ml at month-3 achieved clinical remission more frequently at month-6 and month-12 than patients with undetectable serum rituximab

TABLE 1 | Characterization of patients at baseline (i.e. the day of rituximab injection) and during follow-up.

Variables	All patients (n = 68)	Patients with serum rituximab level < 2µg/mL at month-3 (n = 38)	Patients with serum rituximab level > 2µg/mL at month-3 (n = 30)	*P*-value
Characteristics at baseline				
Age (years)	58.5 [49.0–67.2]	57.5 [44.0–67.5]	60.5 [50.5–68.0]	0.3
Gender (Female/Male)	19/49	14/24	5/25	0.1
Weight (kg)	76.6 [67.4-84.0]	76.3 [66.6-83.6]	77.0 [67.9-86.0]	1
UP (g/d)	6.0 [4.3–8.9]	7.0 [4.9–10.1]	5.5 [3.9–7.1]	0.07
Serum creatinine (µmol/L)	120 [87–151]	119 [83–138]	137 [90–183]	0.1
Serum albumin (g/L)	22.6 [16.0–29.0]	20.2 [14.1–24.6]	26.6 [22.0–31.7]	0.001
$CD19^+$ count (cell/µL)	186.5 [123.5–270.0]	208.0 [138.0–280.0]	142.5 [77.2–215.5]	0.2
Etiology				0.2
Anti-PLA2R1-associated pMN	62 (91%)	35 (92%)	27 (90%)	
Anti-THSD7A-associated pMN	2 (3%)	0 (0%)	2 (7%)	
Double negative patients	4 (6%)	3 (8%)	1 (3%)	
Anti-PLA2R1 Ab titer (RU/mL)	149 [57–303]	184 [71–489]	89 [20–173]	0.01
Anti-proteinuric treatment	68 (100%)	38 (100%)	30 (100%)	1
Characteristics at month-3				
UP (g/d)	3.0 [1.5–5.9]	5.4 [2.4–8.1]	1.8 [1.1–3.2]	<0.0001
Serum creatinine (µmol/L)	110 [92–142]	110 [87–139]	105 [92–166]	0.7
Serum albumin (g/L)	29.0 [23.0–35.0]	24.6 [17.7–31.3]	35.0 [29.5–37.3]	0.0002
$CD19^+$ count (cell/µL)	2.0 [0.0–7.0]	2.5 [0.9–18.5]	0.0 [0.0–2.0]	0.005
Anti-PLA2R1 Ab titer (RU/mL)	5 [0–23]	19 [5–63]	0 [0–4]	<0.0001
Patients with anti-RTX Ab	0 (0%)	0 (0%)	0 (0%)	1
Characteristics at month-6				
UP (g/d)	1.7 [1.0–4.7]	4.5 [1.7–8.0]	1.2 [0.6–1.7]	<0.0001
Serum creatinine (µmol/L)	107 [89–137]	110 [90–139]	104 [85–142]	0.7
Serum albumin (g/L)	32.5 [25.5–37.0]	29.0 [22.2–33.6]	37.0 [32.8–39.2]	<0.0001
$CD19^+$ count (cell/µL)	22.5 [3.0–52.2]	41.0 [19.0–127.0]	2.0 [1.0–33.0]	0.0009
Anti-PLA2R1 Ab titer (RU/mL)	3 [0–24]	15 [2–80]	0 [0–4]	<0.0001
Patients with anti-RTX Ab	6 (9%)	4 (11%)	2 (7%)	0.7
Clinical outcome				
Remission at month-6	36 (53%)	12 (32%)	24 (80%)	<0.0001
Remission at month-12	41 (60%)	16 (42%)	25 (83%)	0.001

Anti-PLA2R1 Ab, anti-Phospholipase A2 Receptor 1 antibody; Anti-THSD7A Ab, anti-Thrombospondin Type 1 Domain Containing 7A antibody; Anti-RTX Ab, anti-rituximab antibody; pMN, primary membranous nephropathy; UP, 24-hour urinary protein excretion.

levels at month-3 (at month-6: 64% *versus* 10%, $p = 0.002$ and at month-12: 71% *versus* 30%, $p = 0.03$).

Factors Associated With Undetectable Serum Rituximab Levels at Month-3

In multivariate analysis, only baseline serum albumin level was associated with the risk of having an undetectable serum rituximab level at month-3 (HR, 0.89; 95% CI, 0.82 –0.97; $p = 0.005$) (**Table 4**). Using ROC curves, we determined a threshold of albuminemia at baseline of 22.5 g/L that was associated with 8.66-fold (95% CI, 2.90–25.87) higher risk of undetectable serum rituximab levels at month-3 with a sensitivity of 77% (95% CI, 59–88%) and a specificity of 71% (95% CI, 55–83%) (air under curve (AUC) = 0.72; $p = 0.002$).

DISCUSSION

The first aim of this study was to evaluate the predictive value of serum rituximab levels at month-3 in patients with pMN on clinical remission rates at month-6 and month-12 after rituximab injection. We observed that rituximab underdosing is not uncommon, with 56% of patients having undetectable serum rituximab levels at month-3. Undetectable serum rituximab levels at month-3 were associated with lower clinical remission rates at month-6 and month-12 and with a longer time to achieve clinical remission. Undetectable serum rituximab levels at month-3 were also associated with higher PLA2R1 titers and lower serum albumin levels at baseline and with higher PLA2R1 titers, higher proteinuria, higher $CD19^+$ counts and lower serum albumin levels at month-3 and month-6. These results suggest that patients with severe NS have a shorter exposure to rituximab – likely due to rituximab wasting in the urine – compared to patients with a mild NS or without NS, leading to a decrease in the effectiveness of B cell depletion and therefore to a higher rate of treatment failure. This is consistent with our previous results showing that higher residual serum rituximab concentrations at month-3 were significantly correlated with higher B cell depletion and lower requirement for repeat doses of rituximab (21).

The second aim of this study was to identify factors associated with undetectable serum rituximab levels at month-3. Little attention has been paid to rituximab pharmacokinetics in nephrotic patients. Antibodies – including rituximab – interact with the neonatal Fc receptor for IgG (FcRn) present on epithelial cells, endothelial cells, monocytes and/or macrophages, and dendritic cells (24). FcRn protects antibodies from degradation by lysosomes and thus reduces antibody clearance by allowing recycling into the cellular environment (25, 26). However, despite

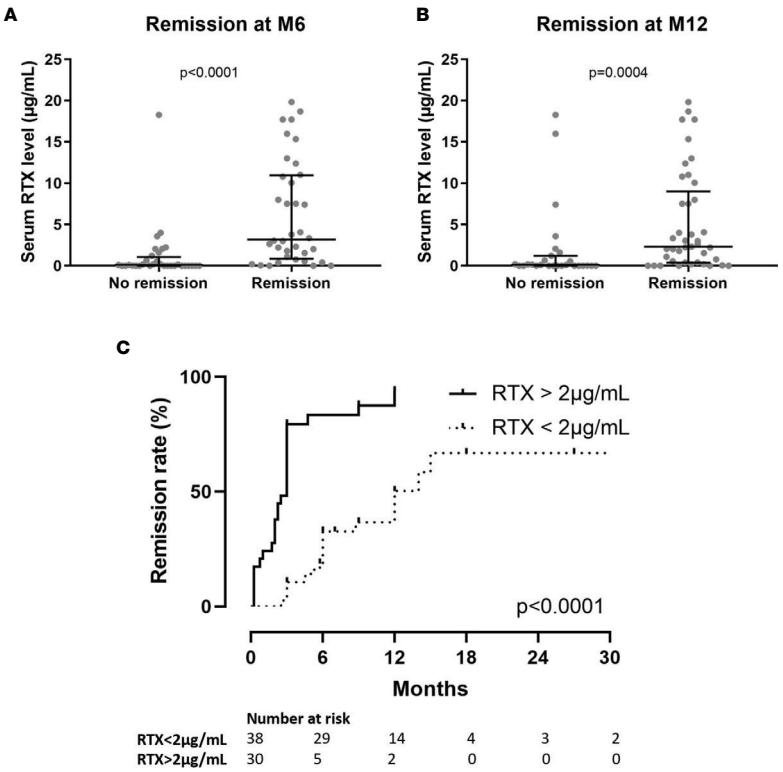

FIGURE 1 | **(A)** Serum rituximab (RTX) levels at month-3 (M3) according to clinical status at month-6 (M6). **(B)** Serum rituximab (RTX) levels at month-3 (M3) according to clinical status at month-12 (M12). **(C)** Time from initiation of rituximab treatment to remission of nephrotic syndrome according to serum rituximab levels at month-3. M3, month-3 after rituximab injection; M6, month-6 after rituximab injection; M12, month-12 after rituximab injection; RTX, rituximab; UP, 24-hour urinary protein excretion.

rituximab recycling, nephrotic patients have a shorter exposure to rituximab compared to a population without proteinuria, due to the clearance of rituximab in the urine (22). Fervenza *et al.* have demonstrated a shorter rituximab half-life in patients with pMN compared to patients with rheumatoid arthritis (11.5 days *versus* 18.0 days) treated with four doses of 375 mg/m^2 (day 1, day 8, day 15, and day 22) (15). More recently, we have shown that residual rituximab levels at month-3 were significantly lower in patients with pMN compared to myasthenia gravis patients without proteinuria matched for age, gender and weight and treated

TABLE 2 | Univariate and multivariate analysis of variables associated with clinical remission at month-6.

Variables	Remission at month-6 (n=36)	No remission at month-6 (n=32)	Univariate *P*-value	Multivariate *P*-value
Age (years)	58.0 [46.0–67.7]	58.5 [49.2–67.7]	0.7	
Gender (Female/Male)	9/27	10/22	0.6	
Weight (kg) at baseline*	74.3 [66.0–82.0]	77.3 [70.7-89.6]	0.1	0.1
UP (g/d) at baseline*	5.0 [4.0–7.0]	7.8 [5.8–12.2]	0.0001	0.02
Serum creatinine (µmol/L) at baseline	119 [86–151]	121 [87–163]	0.7	
Serum albumin (g/L) at baseline*	25.5 [20.7–30.2]	16.6 [12.5–23.0]	<0.0001	0.8
CD19$^+$ count (cell/µL) at baseline	158.5 [112.8–277.5]	208.0 [122.0–291.0]	0.3	
CD19$^+$ count (cell/µL) at month-3*	0.0 [0.0–2.7]	3.0 [1.0–25.0]	0.002	0.3
Etiology			0.2	
Anti-PLA2R1-associated pMN	33 (92%)	29 (91%)		
Anti-THSD7A-associated pMN	0 (0%)	2 (6%)		
Double negative patients	3 (8%)	1 (3%)		
Anti-PLA2R1 Ab titer (RU/mL) at baseline*	89 [21–173]	218 [76–561]	0.002	0.9
Patient with RTX <2µg/mL at month-3*	12 (33%)	26 (81%)	<0.0001	0.007

**Variables with p < 0.1 in univariate analysis tested into the multivariate logistic regression model.*

Anti-PLA2R1 Ab, anti-Phospholipase A2 Receptor 1 antibody; Anti-THSD7A Ab, anti-Thrombospondin Type 1 Domain Containing 7A antibody; pMN, primary membranous nephropathy; RTX, rituximab; UP, 24-hour urinary protein excretion.

TABLE 3 | Univariate and multivariate analysis of variables associated with clinical remission at month-12.

Variables	Remission at month-12 (n=41)	No remission at month-12 (n=27)	Univariate *P*-value	Multivariate *P*-value
Age (years)	60.0 [50.0–68.0]	56.0 [42.0–67.0]	0.3	
Gender (Female/Male)	11/30	8/19	1	
Weight (kg) at baseline*	71.5 [66.0–82.0]	78.0 [74.0–89.2]	0.06	0.02
UP (g/d) at baseline*	5.2 [4.0–7.1]	7.4 [5.6–11.9]	0.005	0.2
Serum creatinine (µmol/L) at baseline	118 [87–149]	123 [87–184]	0.4	
Serum albumin (g/L) at baseline*	24.8 [20.2–29.4]	16.0 [13.0–22.1]	0.001	0.7
$CD19^+$ count (cell/µL) at baseline	186.0 [125.0–285.0]	185.0 [88.7–263.8]	0.9	
$CD19^+$ count (cell/µL) at month-3*	0.0 [0.0–2.7]	3.0 [1.0–16.0]	0.005	0.9
Etiology			0.8	
Anti-PLA2R1-associated pMN	37 (90%)	25 (93%)		
Anti-THSD7A-associated pMN	1 (2%)	1 (3%)		
Double negative patients	3 (8%)	1 (3%)		
Anti-PLA2R1 Ab titer (RU/mL) at baseline*	86 [21–188]	226 [110–561]	0.001	0.5
Patient with RTX <2µg/mL at month-3*	16 (26%)	22 (85%)	0.001	0.01

**Variables with p < 0.1 in univariate analysis tested into the multivariate logistic regression model.*
Anti-PLA2R1 Ab, anti-Phospholipase A2 Receptor 1 antibody; Anti-THSD7A Ab, anti-Thrombospondin Type 1 Domain Containing 7A antibody; pMN, primary membranous nephropathy; RTX, rituximab; UP, 24-hour urinary protein excretion.

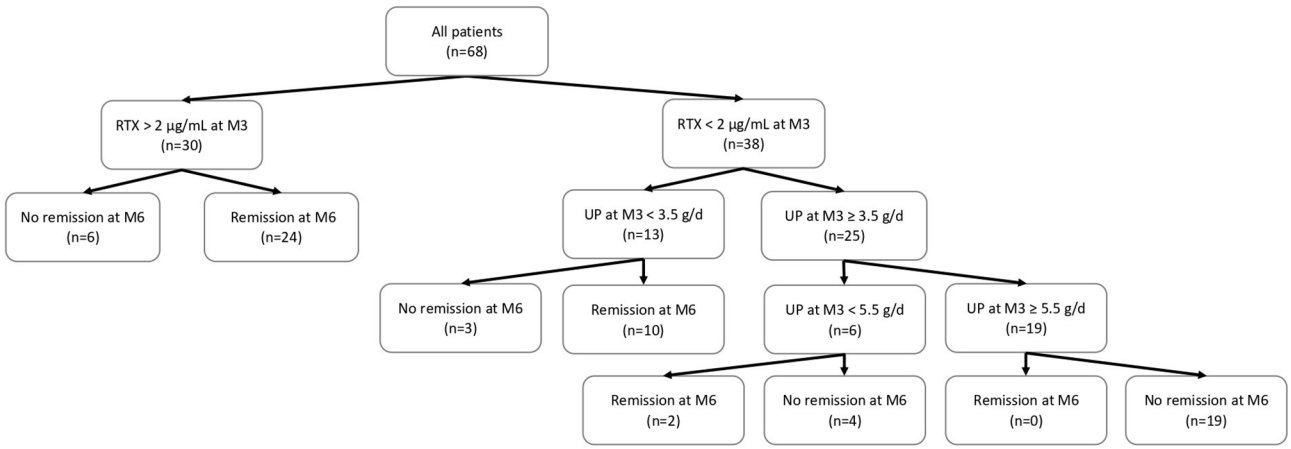

FIGURE 2 | Flow chart of patients included in this study and outcome according to serum rituximab levels at month-3. M3, month-3 after rituximab injection; M6, month-6 after rituximab injection; RTX, rituximab; UP, 24-hour urinary protein excretion.

TABLE 4 | Univariate and multivariate analysis of variables associated with serum rituximab levels at month-3.

Variables	Patients with serum rituximab level < 2µg/mL at month-3 (n = 38)	Patients with serum rituximab level > 2µg/mL at month-3 (n = 30)	Univariate *P*-value	Multivariate *P*-value
Characteristics at baseline				
Age (years)	57.5 [44.0–67.5]	60.5 [50.5–68.0]	0.3	
Gender (Female/Male)*	14/24	5/25	0.1	0.3
Weight (kg)	76.3 [66.6-83.6]	77.0 [67.9-86.0]	1	
UP (g/d)*	7.0 [4.9–10.1]	5.5 [3.9–7.1]	0.07	0.2
Serum creatinine (µmol/L)*	119 [83–138]	137 [90–183]	0.1	0.3
Serum albumin (g/L)*	20.2 [14.1–24.6]	26.6 [22.0–31.7]	0.001	0.005
$CD19^+$ count (cell/µL)	208.0 [138.0–280.0]	142.5 [77.2–215.5]	0.2	
Etiology			0.2	
Anti-PLA2R1-associated pMN	35 (92.%)	27 (90%)		
Anti-THSD7A-associated pMN	0 (0%)	2 (7%)		
Double negative patients	3 (8%)	1 (3%)		
Anti-PLA2R1 Ab titer (RU/mL)*	184 [71–489]	89 [20–173]	0.01	0.5

**Variables with p < 0.1 in univariate analysis tested into the multivariate logistic regression model.*
Anti-PLA2R1 Ab, anti-Phospholipase A2 Receptor 1 antibody; Anti-THSD7A Ab, anti-Thrombospondin Type 1 Domain Containing 7A antibody; pMN, primary membranous nephropathy; UP, 24-hour urinary protein excretion.

with a similar treatment regimen (21). In addition to urinary excretion of rituximab, internalization and destruction of rituximab by target B cells (27, 28) and anti-rituximab antibodies (16, 20) may also contribute to decreased residual rituximab levels. Here, we identified baseline serum albumin level as a predictive factor for residual serum rituximab levels at month 3. Patients with severe NS and especially with serum albumin below 22.5 g/L at baseline were more likely to have an undetectable serum rituximab level at month-3. Previously, we have shown that a high-dose rituximab protocol (two perfusions of 1 g two weeks apart) was more effective than a low-dose rituximab protocol (two perfusions of 375 mg/m^2 one week apart) in achieving clinical remission at month-6 in patients with pMN (29). The median residual rituximab level at month-3 was also higher with the high-dose rituximab protocol (29). Our present results suggest that this regimen of rituximab may still be insufficient in patients with severe NS. It has recently been shown that reinfusion of rituximab can induce remission in patients considered refractory to rituximab (20, 30). Therefore, the management of pMN patients treated with rituximab should be individually adjusted by rituximab immunomonitoring. For patients with undetectable serum rituximab levels at month-3 and active NS, additional rituximab injections should be planned and supportive therapy optimized to obtain higher serum rituximab levels and thereby increase the likelihood of remission and reduce time with active NS. Additional doses of rituximab should be considered as early as month-3 in patients with proteinuria greater than 5.5 g/d at month-3 as we have shown that these patients never achieve remission at month-6. However, it seems acceptable to wait until month-6 for patients with proteinuria between 3.5 g/d and 5.5 g/d at month-3 as we have shown that 33% of these patients achieve remission at month-6. The addition of a calcineurin inhibitor (CNI) to rituximab could also be discussed (31). In addition to their immunosuppressive effect, CNIs may decrease the urinary loss of rituximab by causing afferent and efferent glomerular arteriolar vasoconstriction. However, this combination is likely to result in enhanced immunosuppression with concomitant adverse events.

Recently, a study has shown that rituximab was less effective than cyclophosphamide in inducing immunological remission in patients with the highest anti-PLA2R1 titers (32). However, patients with the highest anti-PLA2R1 titers were more likely to have severe NS and thus more likely to be underdosed in rituximab (33–35). In these patients, a personalized management based on the rituximab immunomonitoring at month-3 may increase the effectiveness of rituximab and thus be as effective as cyclophosphamide but with less toxicity.

This study has several limitations. First, it is a retrospective analysis of a prospective cohort but we used systematically collected prospective data and samples. Second, the number of participants is relatively small. Third, patients with undetectable serum rituximab levels at month-3 had more severe disease at baseline, which may suggest that initial disease severity may contribute to their worse outcome. However, in multivariate analysis, an undetectable serum rituximab level at month-3 was an independent risk factor for treatment failure at months 6 and 12. Furthermore, by comparing the outcome of patients with detectable or undetectable rituximab levels in the group of patients with baseline proteinuria > 6.0 g/d – these patients are expected to have a similarly severe nephrotic syndrome – we demonstrated that rituximab levels were still predictive of clinical remission at months 6 and 12. Thus, these results show that rituximab levels at month-3 are predictive of clinical remission, regardless of the initial severity of the disease.

To conclude, in pMN patients treated with rituximab, rituximab immunomonitoring at month-3 might be a useful biomarker to adapt therapeutic management in addition to CD19$^+$ cell count monitoring, anti-rituximab antibodies monitoring (16, 20), anti-PLA2R1 antibody titer (36) and PLA2R1 epitope spreading (37). Personalized management may increase the efficacy of rituximab in patients with severe NS and may dispense the use of cyclophosphamide. Prospective studies are needed to compare the personalized management of patients with pMN based on rituximab immunomonitoring to conventional management.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by NCT02199145. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

BS-P and VB provided the research idea and study design. MT, VB, BS-P, and KZ analyzed and interpreted the data. BS-P, VE, TC, MC, and SB-S provided medical oversight. VB and SB performed rituximab measurement and anti-rituximab antibody detection. MT, BS-P, and VB drafted and revised the manuscript. BS-P and VB contributed equally to this work. All authors contributed to the article and approved the submitted version.

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REVIEW
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How Times Have Changed! A Cornucopia of Antigens for Membranous Nephropathy

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The identification of the major target antigen phospholipase A2 receptor (PLA2R) in the majority of primary (idiopathic) cases of membranous nephropathy (MN) has been followed by the rapid identification of numerous minor antigens that appear to define phenotypically distinct forms of disease. This article serves to review all the known antigens that have been shown to localize to subepithelial deposits in MN, as well as the distinctive characteristics associated with each subtype of MN. We will also shed light on the novel proteomic approaches that have allowed identification of the most recent antigens. The paradigm of an antigen normally expressed on the podocyte cell surface leading to *in-situ* immune complex formation, complement activation, and subsequent podocyte injury will be discussed and challenged in light of the current repertoire of multiple MN antigens. Since disease phenotypes associated with each individual target antigens can often blur the distinction between primary and secondary disease, we encourage the use of antigen-based classification of membranous nephropathy.

Keywords: membranous nephropathy, membranous lupus nephritis, antigen, epitope spreading, serologic testing, autoimmune profiling, mass spectrometry

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INTRODUCTION

Membranous nephropathy (MN) is an autoimmune kidney disease that is the second leading cause of nephrotic syndrome. The pathological hallmark of MN is the deposition of immunoglobulin G (IgG) and variable amounts of complement proteins within the subepithelial space. Immune complexes can form *in situ* due to circulating antibodies targeting an intrinsic or planted antigen within the glomeruli or from deposition of immune complexes that form in the circulation and then become trapped in the subepithelial space (1, 2). The specific antigens within these immune complexes have been progressively identified through technological advancements.

Analogous to Moore's Law which predicted an exponential increase in computing power as technology allowed more transistors per chip, MN research has recently been marked by an accelerated pace in the discovery and identification of antigens, more so than at any time since the first delineation of MN as a distinct clinicopathologic entity in 1957 (3). The research journey to uncover the underlying mysteries of MN is best described by Couser in the title of a 2005 review article entitled "Membranous Nephropathy: A Long Road But Well Traveled (4)". It is worthwhile to review this journey within the context of a wave of recent advances in MN research, which has identified new antigens and highlighted new methodologies for such discoveries.

Early studies questioned whether the source of the subepithelial immune deposits in MN was from immune complexes that formed in the circulation and then deposited in the glomeruli (which was the prevailing view) or if, instead, they formed *in situ* (5). Similarly, whether these antigens were normally expressed in the glomerulus, and by which cell type, or were extrinsic proteins that became implanted in the glomerular basement membrane (GBM) (6, 7) or beneath the podocyte was not clear. An animal model known as Heymann nephritis provided an excellent investigatory platform and early efforts focused on replicating features of the disease process in this experimental rat model to better understand human MN pathogenesis (8). This particular model involved immunizing rats with a fraction of proximal tubular brush border, which resulted in a histopathological pattern of MN nearly identical to what was seen in human glomeruli. Two groups independently disproved the reigning hypothesis that circulating immune complexes were responsible for the glomerular deposits and showed evidence for *in situ* formation of the deposits, with circulating antibodies targeting an intrinsic antigen of the glomerular filtration barrier (9, 10). While these data support *in situ* immune complex formation/planted antigens, the existence of circulating complexes has not been disproved. It has been proposed that circulating immune complexes can occur in the setting of membranous nephropathy related to autoimmune disease, such as membranous lupus nephritis, but has not been proven in humans or animal models. The brush border component that triggered immunogenicity in the Heymann nephritis model was ultimately identified as megalin, also known as low density lipoprotein-related protein 2 (LRP2) (11). Despite the similarities between Heymann nephritis and human MN, megalin could not be shown to represent the target antigen in human disease (12). Of note, LRP2 was later identified to be the main antigen in the tubular basement membrane immune deposits in anti-brush border antibody disease (anti-LRP2 nephropathy), which is associated with MN features (13).

Other animal models of MN had been developed, including those utilizing exogenous cationic bovine serum albumin (BSA) as an antigen that traversed the GBM and become planted in a subepithelial space. Mice injected with cationic BSA developed features of MN with proteinuria developing within two weeks (14). This modified protein was later identified to represent an actual antigen in a minority of human cases of childhood MN associated with antibodies to cationic BSA (6). Other murine models implicated antibodies to dipeptidyl peptidase IV (15), aminopeptidase A (APA) (16), α3NC1 (17), and annexin 3 (18), but corresponding antigens in humans have not been identified to date.

The first evidence of an intrinsic glomerular antigen in human MN came through experiments of nature. Ronco and colleagues documented rare cases of antenatal MN occurring in newborns of mothers deficient in neutral endopeptidase (NEP), a known glomerular protein (1). These mothers developed alloantibodies to NEP which led to subepithelial deposits in the fetal kidney (which expressed NEP due to paternal inheritance); the alloantibodies disappeared several months after birth and were associated with clinical resolution of disease in the infant (1). This finding confirmed that circulating antibodies directed against a podocyte antigen could lead to MN in humans. Yet the target antigen in adult disease, representing the vast majority of MN, remained unknown.

It was not until 2009 that a target antigen was identified in adult MN. Using patient serum to probe immunoblots of human glomerular proteins, Beck and Salant were able to find evidence of a common 185 kDa protein identified by the serum from a proportion of idiopathic MN cases. Mass spectrometric analysis of the gel band revealed this protein as the M-type (muscle derived) phospholipase A2 receptor (PLA2R) (19). PLA2R was thus the first identified target antigen in adult MN, and is now known to be the most commonly-targeted antigen, associated with 70-80% of MN cases (19). A second minor antigen, thrombospondin type 1 domain-containing 7A (THSD7A) was identified several years later using similar methodology (20).

Despite the identification of THSD7A and PLA2R, the specific target antigens in approximately 30% of primary MN cases remained unknown. There was a need to further characterize the antibody repertoire in MN for patients who were PLA2R and THSD7A-negative. However, for rare antigens, it was becoming increasingly difficult to identify common pattern of reactivity by Western blotting, confounded by a proportion of patients who are in immunologic remission at the time of serum sampling and therefore would not have these rare circulating autoantibodies. Therefore, new approaches were necessary that instead focused on the kidney biopsy as a tissue source to identify the antigen, under the assumption that the targeted antigen would be enriched in the subepithelial immune complexes relative to normal glomerular proteins (21, 22).

TECHNOLOGIES FOR ANTIGEN IDENTIFICATION

Western blotting for the detection of glomerular antigens remains an effective method by which to identify additional candidate antigens and to further validate tissue-based discovery. Moreover, analysis of bands differentially recognized by serial serum samples under different clinical states (nephrosis *vs.* remission *vs.* relapse) (19, 22, 23), followed by immunoprecipitation and analysis by MS remains a viable technique for antigen identification (**Figure 1A**).

Recent advances in proteomic technologies have enabled large-scale profiling of proteins in tissue and sera from patients, with the potential to better diagnose and classify autoimmune diseases such as MN (24). The powerful method of laser capture microdissection (LCM) of glomeruli from formalin-fixed paraffin-embedded biopsy tissue, followed by mass spectrometric (MS) proteomic analysis, capitalizes on enrichment of glomerular proteins or antigens in certain disease states (25, 26) (**Figure 1B**). Multiple kidney diseases have been better defined through LCM-MS, including membranoproliferative glomerulonephritis (GN), amyloidosis, cryoglobulinemic GN, fibrillary GN, and membranous-like

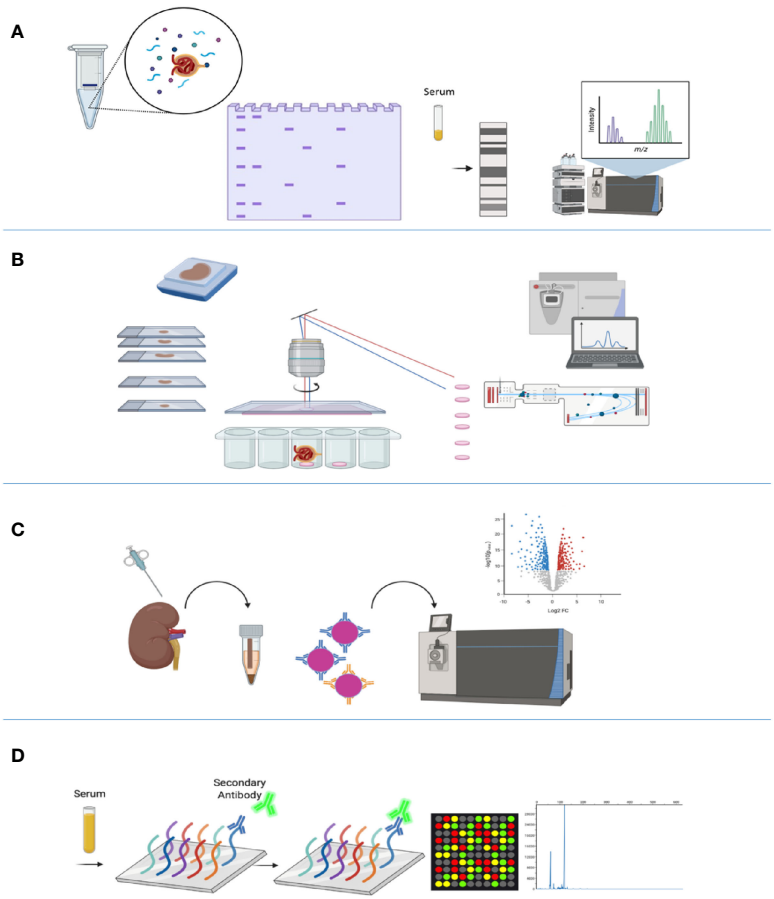

FIGURE 1 | Technologies used in the identification of membranous nephropathy antigens. **(A)** Western blotting evaluates seroreactivity against human glomerular extract to identify podocyte antigens by mass spectrometry. **(B)** Laser capture microdissection (LCMD) from kidney biopsy specimens enriches for glomerular proteins, which is followed by mass spectrometry for protein identification. **(C)** Protein G immunoprecipitation (tissue IP) from frozen tissue enriches for immune complexes in kidney biopsy tissue for protein identification by mass spectrometry. **(D)** Autoimmune profiling evaluates seroreactivity against an array of peptides to identify potential autoantigens. Created with BioRender.com.

glomerulopathy with masked IgG kappa deposits (MGMID) (25–29).

In the field of MN, the use of sequential methods of LCM followed by MS has allowed the discovery of multiple new autoantigens including the exostosin 1/2 complex (EXT1/2) (21), neural epidermal growth factor-like 1 (NELL1) (22), semaphorin 3B (SEMA3B) (30), and protocadherin 7 (PCDH7) (31). It was also utilized as an ancillary technique in confirmation of the new autoantigens serine protease HTRA1 (HTRA1) (23), neural cell adhesion molecule 1 (NCAM1) (32), and type III transforming growth factor-beta receptor (TGFBR3) (33). Additionally, the LCM-MS methodology confirmed the appropriate target antigen in cases of PLA2R- and THSD7A-associated MN cases, validating its utility for all known subtypes of MN.

This technique was subsequently followed by protein G immunoprecipitation (IP) from biopsy tissue, specifically focusing on immune complexes eluted from kidney tissue, followed by MS for identification of antigenic targets (**Figure 1C**). This tissue IP was the main method of identification of NCAM1 and TGFBR3, and additionally supported the discovery of HTRA1 (23). Functional validation of targeted antigens was performed by co-localization of each target antigen with IgG within the glomerular immune deposits.

Autoimmune profiling, based on serum reactivity with whole proteome arrays, has emerged as a promising methodology to complement the previously mentioned modalities (**Figure 1D**). Autoimmune profiling, a new technology based on peptide or protein fragment microarrays for the analysis of the antibody repertoire in various autoimmune conditions, has only been implemented in a few studies (34–36). This technique has been applied to MN as an ancillary technique for the discovery of autoantibodies against a novel MN antigen serine protease HTRA1 (23), demonstrating proof-of-concept for its use in this disease.

An overview of these methods is shown in **Figure 1**.

AUTOANTIGENS IN PRIMARY MN

Traditionally, MN has been classified as a primary (previously 'idiopathic') or secondary disease. The presence of humoral autoreactivity against a known antigen (*e.g.*, PLA2R or THSD7A) has been considered to represent primary MN (37) while the association of MN with various concurrent conditions such as malignancy, systemic autoimmune diseases, infections, or medications has been considered secondary, especially in the absence of staining for discrete MN antigens (38). While many of these secondary causes have consistently been associated with MN, in some cases it is difficult to exclude the coincidental occurrence of primary MN and another condition (39).

Features on kidney biopsy can often point to primary or secondary disease. The histopathology of primary MN, typically evident with PLA2R- and THSD7A-associated disease, demonstrates diffuse and global granular capillary loop staining for IgG with C3, but not C1q or other immunoglobulin heavy chains, within glomeruli. Ultrastructurally, subepithelial and intramembranous electron-dense deposits are present, with the absence of mesangial or subendothelial deposits (40) (**Figure 2**). IgG subclass staining, when performed, demonstrates a predominance of IgG4 in primary MN (41–44).

While the field may be moving away from a categorization of primary *vs*. secondary MN and more towards an antigen-based classification system (45, 46), we will continue to use the terms 'primary' and 'secondary' in this review when applicable. We will describe both podocyte antigens, including PLA2R, THSD7A, HTRA1, and SEMA3B as well as non-podocyte antigens including NELL1 and PCDH7, in what we still call 'primary' membranous nephropathy. This will be followed by antigens commonly encountered in membranous lupus nephritis, including the podocyte-expressed antigens NCAM1, TGFBR3, and CNTN1 as well as the more ubiquitously-expressed proteins EXT1 and EXT2.

PRIMARY MN ANTIGENS EXPRESSED WITHIN PODOCYTES

Phospholipase A2 Receptor

PLA2R is a transmembrane glycoprotein and member of the small mannose-receptor family that is expressed in podocytes and in other tissues (47). PLA2R is comprised of multiple domains, and the conformation of many of these domains is maintained by regular patterns of disulfide bonding (48). The extracellular region comprises an N-terminal cysteine-rich (CysR or ricin B) domain, a single fibronectin type-2 (FnII) domain, and eight C-type lectin-like domains (CTLD). Motifs present in the short cytoplasmic domain enable constitutive endocytic recycling in clathrin-coated pits. PLA2R undergoes pH-dependent conformational changes that are necessary for ligand binding and subsequent release of the ligand in the more acidic pH of endosomes and lysosomes (48, 49). Patients often have a genetic predisposition with risk alleles in both MHC class II genes and in the *PLA2R1* gene itself (50). Patients with PLA2R-positive MN had a mean age of 58 years and had male predominance (19). Kidney biopsies of patients with PLA2R-positive MN showed diffuse and global granular capillary loop staining for IgG, C3, and light chains and did not show significant expression for other immune reactants.

Autoantibodies are reactive against the protein in its native and non-reduced conformation, with seroreactivity lost under reducing conditions (19). PLA2R antibodies, predominantly of the IgG4 subclass (19), correlate with disease activity and remission and are useful to evaluate treatment decisions and evaluate prognosis (51–53). Epitope spreading from the N-terminal cysteine-rich (CysR) domain to C-type lectin-like domains (CTLD1-8) has been identified in several studies and is associated with a poor prognosis (54, 55). Dependence of autoantibody binding to PLA2R and other proteins under native conditions with pH and/or disulfide bond dependence has been based on immunoblotting experiments under reducing and non-reducing conditions. For PLA2R, this was supported by further epitope identification requiring non-reducing conditions, however work towards epitope identification has not been extensively studied for most autoantigens. However, this was investigated with similar requirements for native conditions found for the second identified primary MN autoantigen, THSD7A.

Thrombospondin Type 1 Domain-Containing 7A

THSD7A was the second autoantigen discovered in primary MN, identified after recognition that MN sera uncommonly showed reactivity by immunoblotting with a 250 kDa antigen present in human glomerular protein extracts that was subsequently identified as THSD7A (20). THSD7A is also a large glycoprotein expressed by the podocyte, and stimulates an IgG4-predominant autoantibody response in 3%–5% of patients with primary MN (20). Similar to PLA2R, the epitopes within THSD7A are sensitive to reducing agents (20). Autoantibodies against THSD7A recognize multiple protein domains within the protein, with the N-terminal end of the protein being the predominant region (56).

Patients with THSD7A-associated MN had a mean age of 62 years and had a slight male predominance (57). Similar to PLA2R-associated MN, there was diffuse and global granular capillary loop staining for IgG, C3, and light chains without significant staining for other immune reactants. THSD7A-positive MN is not strongly associated with underlying autoimmune diseases (such as systemic lupus erythematosus) but is seen in some patients with malignancy, where there is corresponding increased expression in tumor tissue (58, 59). Anti-THSD7A antibodies correlate with disease activity (60, 61) and serologic testing is routinely used in clinical practice (56). Further details regarding PLA2R and THSD7A MN have been discussed in a prior review (62).

Serine Protease HTRA1

HTRA1 was identified by an inter-institutional collaborative group including these three authors, utilizing four independent but adjunctive methodologies (23). Western blotting of human glomerular proteins with serum from the index patient identified a

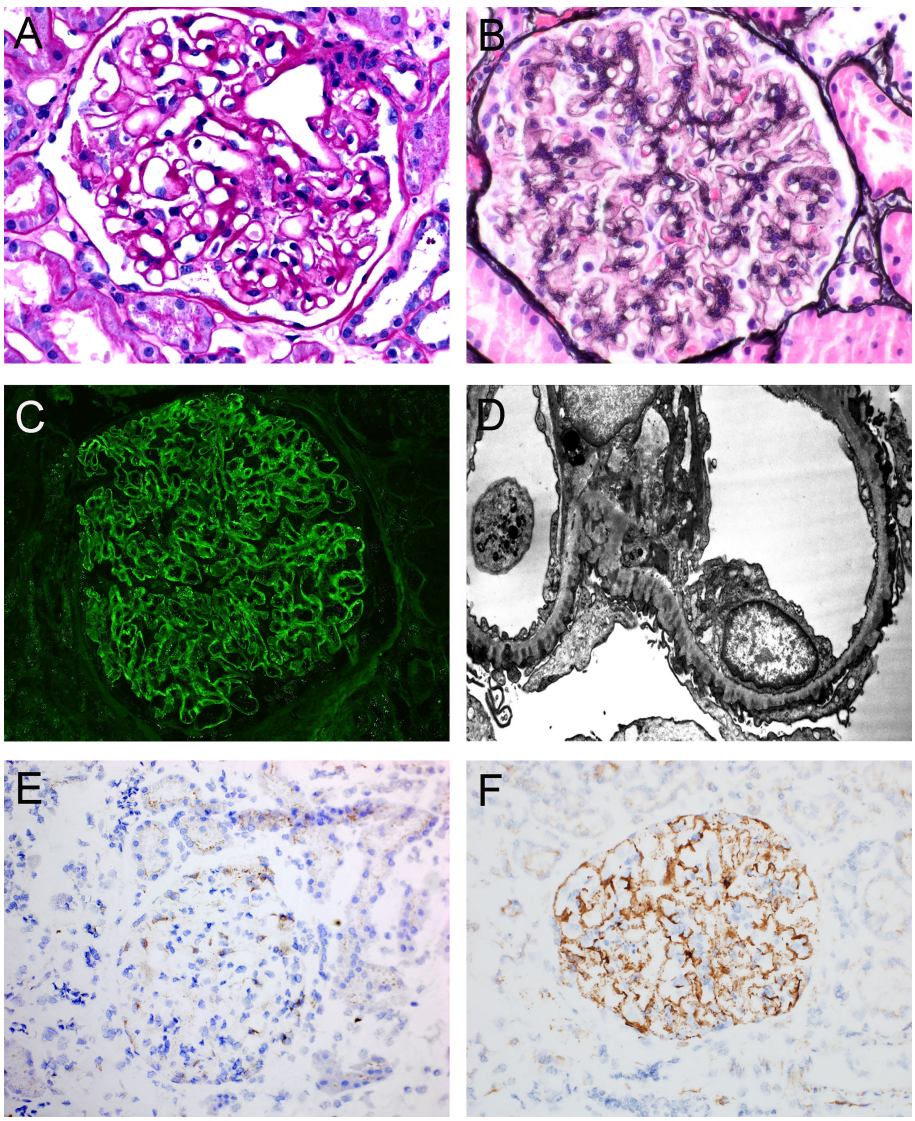

FIGURE 2 | Representative histopathologic features of membranous nephropathy. **(A)** Glomerulus with prominent glomerular capillary loops, PAS, 400x. **(B)** Glomerulus with capillary loop holes/lucencies, Jones Methenamine Silver, 400x. **(C)** Global granular capillary loop staining, IgG immunofluorescence, 400x. **(D)** Electron photomicrograph of subepithelial and intramembranous electron-dense deposits. **(E)** Immunohistochemistry of a case of PLA2R-negative MN, demonstrating staining within podocyte cell bodies consistent with inherent low-level PLA2R expression, 200x. **(F)** Immunohistochemistry of a case of PLA2R-positive MN, demonstrating global granular capillary loop staining (positive result), 200x. Created with BioRender.com.

50 kDa candidate antigen and mass spectrometry of immunoprecipitates from serum at the time of nephrosis but not remission revealed the identity of this protein as the serine protease HTRA1. This finding was independently confirmed by tissue-based studies with LCM-MS and tissue IP-MS, as well as by autoimmune profiling of nephrotic *versus* remission sera from the index patient. HTRA1 is a secreted trypsin-like serine protease that is involved in the homeostasis of the extracellular matrix.

HTRA1-associated MN comprises a total of 4.2% of PLA2R-, THSD7A-, NELL1-, and EXT2-negative cases, with the caveat that it represents a much smaller proportion of the entire primary MN spectrum. It also appears to be a disease of elderly, with a mean age of 67 years in this US-based cohort. Similar to PLA2R- and THSD7A-associated MN, there is diffuse and global capillary loop IgG staining on biopsy, with expression of IgG and C3, but not other immune reactants. IgG4 was the dominant IgG subclass. HTRA1 was specifically detected within immune deposits of HTRA1-associated MN but not in other types of MN. HTRA1-associated MN is favored to be mostly primary, based on the lack of a clinical history of autoimmune or infectious disease and negative staining in MLN biopsies. Circulating anti-HTRA1 antibodies recognized native and recombinant protein under both reducing and non-reducing conditions, suggesting a linear epitope and lack of disulfide

bond dependence. Longitudinal measurements of anti-HTRA1 antibodies by immunoblotting showed an apparent correlation with disease course, suggesting serial monitoring might assist in therapeutic decision making.

Semaphorin 3B

SEMA3B was also identified in primary MN by LCM-MS and is enriched within cohorts of pediatric MN patients, some of which were less than 2 years of age (30). SEMA3B is part of the semaphorin family of proteins, which have high expression in the nervous system and, similar to NELL1, is highly expressed during development (63). It is expressed in podocytes, as demonstrated from protein expression in the Human Protein Atlas (64, 65) and single cell RNA sequencing analysis data (66). A related protein, semaphorin 3A, has been associated with podocyte foot process effacement and proteinuria (67), although the function of the related SEMA3B in the kidney is unknown.

In the discovery cohort by Sethi et al., eight of 11 cases of SEMA3B-associated MN were in pediatric patients (30), making up approximately 10% of MN biopsies in this population, while this form of MN was found to occur rarely in adult MN (<1%). Nearly all patients in this report had achieved partial or complete clinical remission at last follow-up (30). Unlike PLA2R-, THSD7A-, and NELL1-associated MN, IgA and/or C1q staining of the glomerular deposits has been demonstrated in some patients. Approximately one-third of the pediatric cases also had tubular basement membrane (TBM) deposits. IgG1 is the predominant IgG subclass (30). A possibility of familial predilection was discussed in this report, based on two cases.

The presence of SEMA3B autoantibodies in the circulation was demonstrated by serum reactivity against the recombinant protein. Interestingly, and unlike the case for autoantibodies against PLA2R and THSD7A, the serum from these cases reacted only with the reduced form of SEMA3B and not the non-reduced protein (30). This raises the question of how the autoantibody would react with the protein in its native configuration and whether another mechanism (or genetic variant) would be required to expose this cryptic epitope.

PRIMARY MN ANTIGENS NOT EXPRESSED WITHIN PODOCYTES

Neural Epidermal Growth Factor-Like 1

NELL1 was identified as the first antigen in non-lupus related MN initially using LCM-MS (22) and later by tissue IP (68). NELL1 is a non-membrane bound glycoprotein that is expressed during development and primarily expressed within the nervous system (69). Unlike the case for the target antigens in PLA2R and THSD7A-positive MN, NELL1 is not routinely expressed by podocytes under normal conditions. NELL1 associated MN comprises 3.8-16% of all PLA2R-negative MN cases in the United States and may be more frequent among Chinese patients, making up 35% of PLA2R-negative MN cases in these cohorts (22, 68, 70).

NELL1-associated MN has unique histopathologic features, including a segmental or incomplete global pattern of immune complex deposition (71) and IgG1 subclass predominance (22, 68, 71). The presence of NELL1 autoantibodies in the circulation was demonstrated by seroreactivity against recombinant protein under non-reducing conditions and has been independently confirmed by three groups (22, 68, 70, 72). In a single patient where serial serum samples were available, immunological remission (*i.e.*, disappearance of anti-NELL1 antibodies) preceded clinical remission (22), consistent with what is seen in PLA2R-associated MN (19).

While initially felt to represent a mainly primary type of MN, NELL1-associated MN has been found to be associated with malignancy in 11.7-35% of cases (22). NELL1-associated MN was also reported in the setting of graft-*versus*-host disease (73) and in post-transplant nephrotic syndrome (72). A temporal association of lipoic acid use with NELL1-associated MN has also been shown in data from clinical trials, where remission of proteinuria occurred upon drug cessation (74).

Protocadherin 7

A small subset of primary MN is associated with autoantibodies to protocadherin 7, and can be identified through immunohistochemical staining of kidney biopsies (31). PCDH7 is a transmembrane glycoprotein that is expressed at high levels in the nervous system (75), similar to THSD7A, NELL1, and SEMA3B, but has not been shown to be expressed by normal podocytes. Its presence in this form of MN was suggested based on LCM-MS. Similar to what had been shown for anti-PLA2R and anti-THSD7A, IgG reactive with recombinant PCDH7 could be eluted from the frozen tissue under non-reducing conditions demonstrating the presence of antibodies within the tissue in addition to enrichment of the antigen within glomerular immune deposits (31). Unique to this form of MN, PCDH7-associated MN has only low levels of complement staining on biopsy, a feature which might prompt the renal pathologist to further evaluate such a biopsy for PCDH7 positivity (31). The minimal C3 staining on biopsy was corroborated by MS, as spectral counts for many complement proteins (C3, C4A, C4B, C5 and C9) were reduced in PCDH7-associated MN when compared to a historical cohort of PLA2R-positive MN cases (76). IgG subclasses in PCDH7-associated MN were shown to be predominantly IgG1 and IgG4.

PCDH7-associated MN appears to be a disease of older adults (mean age = 61-66 years) and often presents with sub-nephrotic range proteinuria. PCDH7-associated MN was reported to account for 5.7-10.5% of PLA2R-, THSD7A-, EXT1/2-, SEMA3B-, and NELL1-negative MN cases (31). There are no known clinical associations to date and PCDH7 was not found to be localized to immune deposits in any cases of membranous lupus nephritis.

Further evidence of the rapidly developing field and consistent with Moore's law are two new antigens identified and presented at abstracts at the American Society of Nephrology Kidney Week meeting in 2021. One antigen, protocadherin FAT1, may unravel the dilemma of *de novo* membranous nephropathy in the setting of stem cell

transplantation or kidney transplantation. It was discovered by LCMD, followed by mass spectrometry. Similar to the other protocadherin protein associated with MN, PCDH7, there was minimal complement deposition see by immunofluorescence (77). The other newly identified autoantigen was Netrin G1 (NTNG1), identified by seroreactivity against human glomerular extract and subsequent western blotting (78).

We would like to emphasize that it is been becoming exceedingly difficult to extrapolate percentages to the larger MN population, since some studies have reported percentages of PLA2R-negative cases or triple/quadruple antigen-negative cases, while others have reported percentages of antigen-specific MN subclasses in their whole cohort. Therefore, we tried to provide our best estimates of the frequencies of each antigen in MN through adjusting to reported percentages of PLA2R MN (**Table 1**).

CONSIDERATIONS FOR MN IN PEDIATRIC PATIENTS

Compared to adult MN, identifying specific antigens has been more of an exception than the rule in children. Childhood MN has a lower overall rate of PLA2R-positivity of 45%, compared to greater than 70% in adults (79, 80). This frequency is quite low below the age of 10 but is significantly higher in adolescence. SEMA3B comprises approximately 10% of pediatric MN cases (30). Rarer considerations include MN in the setting of primary immunodeficiencies (81), antibodies to cationic bovine serum albumin (6), and anti-neutral endopeptidase in newborns (1). In children with immunodeficiency, MN is interestingly the leading cause of nephrotic syndrome. It is unclear why patients with impaired immunoglobulin production/hypogammaglobulinemia develop MN and the inciting autoantigen is unknown in most cases. PLA2R was found to be the culprit antigen in a neonate with immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome (82).

In cationic BSA-induced MN, the dietary antigen cationic BSA (presumed to have arisen from modifications to bovine albumin present in infant formula) interacts with the anionic glomerular basement membrane and, with its corresponding antibodies, forms *in situ* immune complexes resulting in a loss of charge and size barrier, resulting in proteinuria (6). Complement pathway activation may also play a role in pathogenesis, resulting in the formation of membrane attack complex C5b-9 that localizes to subepithelial deposits. Infectious triggers should additionally be considered as potential etiologies in children, especially hepatitis B in areas with low rates of vaccination. Regardless, there is a large knowledge gap in our understanding of pediatric MN.

AUTOANTIGENS IN MEMBRANOUS LUPUS NEPHRITIS

Membranous lupus nephritis (MLN, or class V LN) is the most common secondary etiology of MN and disproportionately affects young females of African or Hispanic descent, as well as other minority populations. There are some histopathologic findings on kidney biopsy that increase suspicion for MLN over primary MN (**Figure 3**). These include "full house" staining on biopsy (all three immunoglobulin heavy chains - IgA, IgG, IgM, and two complement components - C3, C1q), extraglomerular immune deposits (TBM staining, vascular deposits, or a 'tissue ANA' pattern of nuclear staining), subendothelial electron-dense deposits, and the presence of endothelial tubuloreticular inclusions. These findings have variable sensitivity and specificity alone, however when three or more of these five are present there is an 80% sensitivity and 95% specificity for a diagnosis of MLN over MN (83). Mesangial electron-dense deposits are also common and nearly always present in MLN, however, this finding has poor specificity as they can also be seen in primary MN cases. Additional, although non-specific, features suggestive of MLN are C1q positivity in conjunction with the presence of more than one IgG subclass

TABLE 1 | Characteristics of the target antigens and biomarkers identified in membranous nephropathy.

Antigen	Size (kDa)	% Positivity (adjusted)	Compartment (transmembrane, secreted)	Podocyte expression	Circulating antibodies	Mean age (years)	Sex (M:F)	Distinctive associations
PLA2R	180	65-70%	Transmembrane	Yes	Yes	58	2-2.5:1	No distinctive associations
THSD7A	250	1.3-2.6%	Transmembrane	Yes	Yes	62	1.6:1	Malignancy 10%
NELL1	89	1.8-2%	Secreted	No	Yes	67	1.6:1	Malignancy 33%
HTRA1	51	1.4%	Secreted	Yes	Yes	67.3	1.3:1	No distinctive associations
PCDH7	116	1.0%	Transmembrane	No	Yes	61	1.3:1	Autoimmune disease 14%, Malignancy 21%
SEMA3B	83	<1%	Secreted	Yes	Yes	6.9 pediatrics, 36 adults	1.7:1	Pediatrics, Potential familial
EXT1/2	86/82	2.3 -3.4% (primary) and 17 -38.4% (MLN)	ER transmembrane	No/Yes	No	39.6	0.19:1	Membranous lupus nephritis
NCAM1	95	0.3-2% (primary) and 6.6% (MLN)	Transmembrane	Yes	Yes	34	0.4:1	Membranous lupus nephritis
TGFBR3	94	0 % (primary) and 5.5% (MLN)	Transmembrane	Yes	No	39.6	0.06:1	Membranous lupus nephritis

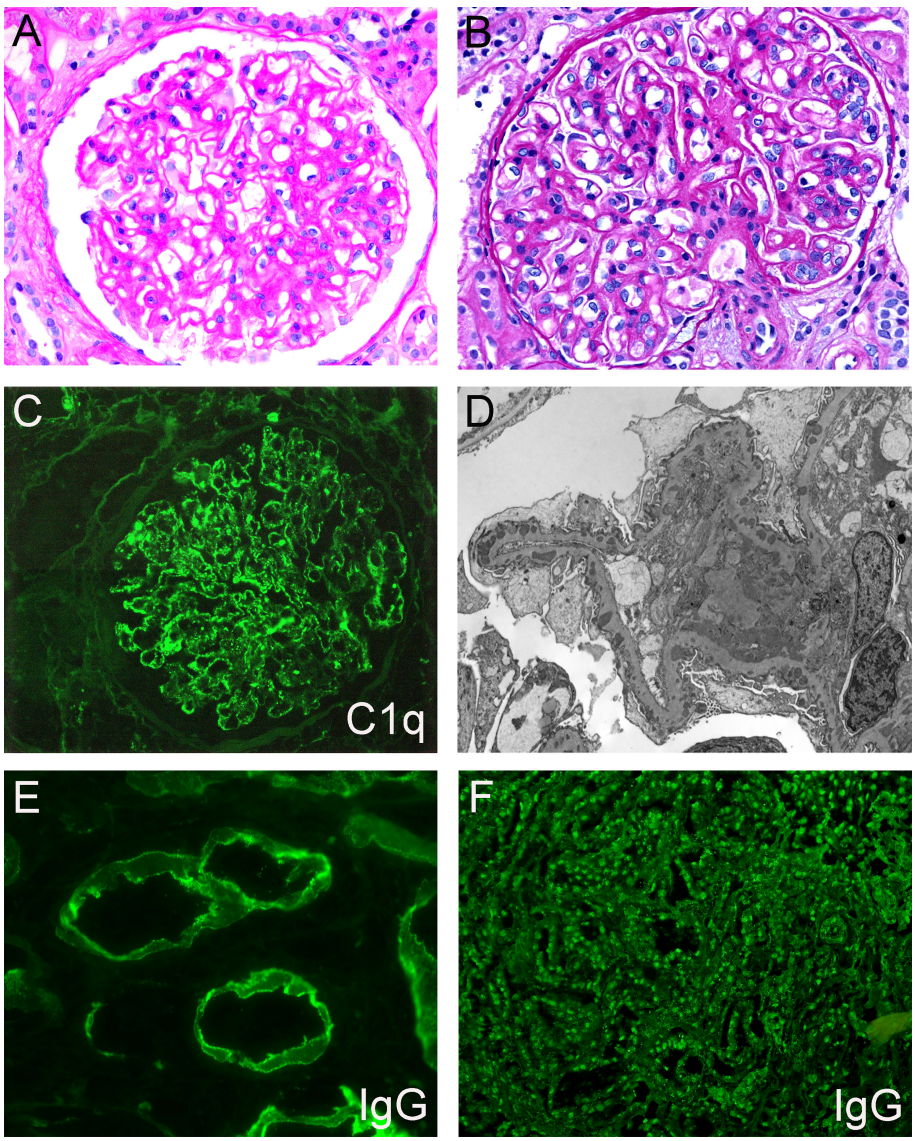

FIGURE 3 | Representative histopathologic features of membranous lupus nephritis. **(A)** Glomerulus with mesangial expansion and prominent capillary loops, PAS, 400x. **(B)** Glomerulus with prominent capillary loops and endocapillary proliferation, PAS, 400x. **(C)** Granular mesangial and capillary loop staining, C1q immunofluorescence, 400x. **(D)** Electron photograph of subepithelial, intramembranous, and mesangial electron-dense deposits. **(E)** IgG immunofluorescence showing granular staining along tubular basement membranes, 600x. **(F)** IgG immunofluorescence demonstrating staining of tubular epithelial cell nuclei ('tissue ANA'), 200x. Created with BioRender.com.

(particularly IgG1 and IgG3) within glomerular immune deposits, consistent with activation of the classical complement pathway. In primary MN, IgG4 often predominates, which is uncommon in MLN. These histopathologic features are also shared in patients with MN secondary to autoimmune diseases other than systemic lupus erythematosus (SLE). Histopathologic features described in primary or MLN are shown in **Table 2**.

While the antigenic targets involved in the majority of primary MN cases have been discovered, the majority of autoantigens in MLN are unknown. To date, three autoantigens have been described in membranous lupus nephritis, which together comprise approximately 1/3 of all MLN cases. These include the exostosin 1/exostosin 2 complex (EXT1/2) (21), neural cell adhesion-molecule 1 (NCAM1) (32), and transforming growth factor β receptor 3 (TGFBR3) (33). PLA2R positivity can be rarely seen in MLN (in up to 5.3% of cases) (84). Contactin 1 (CNTN1) is also described in the setting of autoimmunity, although not specifically within membranous lupus nephritis.

Exostosin 1/2 (EXT1/EXT2)

The first biomarker to be described in MLN is the EXT1/EXT2 complex, identified by LCM-MS of kidney biopsies. The EXT1/EXT2 complex represents a hetero-oligomeric glycosyltransferase that requires both proteins for enzymatic function. It participates

TABLE 2 | Typical histopathologic features associated with each subtype of membranous nephropathy.

Antigen	Global or segmental	Proliferative changes	Predominant IgG subclass	IgA	IgM	C3	C1q	Mesangial	Subendothelial	TBM deposits
PLA2R	Global	No	IgG4	10%	15%	91%	2%	10%	1%	Absent
THSD7A	Global	No	IgG4	40%	10%	80%	10%	40%	10%	Absent
NELL1	Segmental or Global	No	IgG1	8%	10%	78%	0%	24%	0%	Rare
HTRA1	Segmental or Global	No	IgG4	0%	0%	100%	7%	14%	0%	Absent
PCDH7	Global	No	IgG4/IgG1	7%	0%	43%	29%	0%	0%	Absent
SEMA3B	Global	No	IgG1	9%	18%	91%	45%	0%	0%	Present
EXT1/2	Segmental or Global	Yes (27%)	IgG1>IgG2>IgG3>IgG4	49%	46%	94%	48%	99%	20%	Present
NCAM1	Segmental or Global	Yes (25%)	IgG1>IgG2>IgG3-IgG4	65%	68%	85%	55%	95%	55%	Present
TGFBR3	Segmental or Global	Yes (29%)	All IgG subclasses	71%	88%	88%	59%	94%	35%	Present

in GBM homeostasis through regulation of heparan sulfate (85). EXT1 mutant alleles have been previously described in familial kidney disease, with loss of function mutations leading to proteinuria (86).

Both the EXT1 and EXT2 components of the heterodimer are present within deposits in all cases and therefore staining for either component can be performed to identify positive cases. EXT1/EXT2-positive MN appears tightly associated with autoimmunity, with 70% of patients having a positive ANA, 35% having a clinical diagnosis of SLE and 12% of patients having mixed connective tissue disease at the time of biopsy (21). The frequency of EXT1/EXT2 positivity in MLN is 17.0-38.4% (33, 87, 88). From the largest cohort of 374 patients with MLN, 32.6% had EXT1/EXT2-positivity. Of these patients, 75% were 'pure' MLN cases (ISN/RPS class V) and 25% had a concurrent proliferative lupus nephritis component in addition to MLN (ISN/RPS class III/IV + V) (87). Confocal microscopy studies have confirmed the presence of EXT1/EXT2 immune complexes along the subepithelial surface of the GBM and have demonstrated co-localization with IgG. While accumulation of this protein occurs in the subepithelial space, no autoantibodies were identified within serum under reducing or non-reducing conditions, questioning whether this represented a true autoantigen or may merely represent a biomarker of disease (21).

Although unclear at this time whether the EXT1/EXT2 complex is a putative autoantigen or a biomarker, the finding has prognostic significance. Two independent investigations indicated its value as a biomarker of favorable outcome with less progression of kidney disease in patients with MLN, when compared to EXT1/EXT2-negative MLN (87, 88). Remarkably, these studies also included patients with a concurrent proliferative component and showed a similar prognostic significance to EXT1/EXT2 status.

Neural Cell Adhesion Molecule 1

NCAM1 is an immunoglobulin superfamily cell surface glycoprotein that is expressed within the central nervous system, immune system, and within podocytes (89, 90). It is a negative regulator of the expansion of T cells and dendritic cells in the adaptive immune response (91). NCAM1 was identified to be an autoantigen in membranous lupus nephritis through tissue-based proteomic studies, utilizing both LCM and tissue IP (32). NCAM1 co-localizes with IgG within glomerular immune deposits and is present in 6.6% of all MLN biopsies and 0.4-2.0% of primary MN cases (45). Of patients with MLN, 25% had concurrent class III or class IV lupus nephritis (32). Seroreactivity was identified by immunoblotting with NCAM1 recombinant protein exclusively under non-reducing conditions and by indirect immunofluorescence (IFA) using a NCAM1-overexpressing cell line.

Patients with NCAM1-associated MN were predominantly female (70%), with a mean age of 34 ± 12.1 years. Interestingly, 40% of NCAM1-associated MN patients with SLE had an increased frequency of neuropsychiatric manifestations at the time of biopsy (8/20 patients), a rate 4-5 times higher than reported in SLE overall (9% prevalence). There are limited data to conclude whether NCAM1 may be a link between nephritis and neuropsychiatric disease in patients with SLE, but the question warrants further investigation to determine if such a connection exists. Manifestations of SLE vary in time and it could be helpful to determine if patients with NCAM1 autoantibodies are at future risk for neuropsychiatric disease and therefore might benefit from closer monitoring.

TGFBR3

The type III transforming growth factor-beta receptor (TGFBR3) was identified as a putative target in MLN by tissue-based proteomics utilizing LCM and tissue IP (33). TGFBR3 is an accessory receptor for TGF-β signaling and is involved in negative regulation of T-cell dependent antibody responses through reducing $CD4^+$ T-cell specification to the Th1 lineage (92). TGFBR3 is expressed within podocytes.

TGFBR3 is positive within 5.5% of MLN biopsies and is not identified in primary MN cases (33). Patients with TGFBR3-associated MN had a mean age of 39.6 ± 16.1 years and were predominantly female. Nearly all patients had a history of autoimmune disease with 82% having a diagnosis of systemic lupus erythematosus (SLE) at the time of MN biopsy (33). A concurrent proliferative (class III or IV) component was identified in 29.4% of cases. Several different methods were tried but failed to show seroreactivity against TGFBR3, including immunoblotting patient sera against the recombinant protein, use of a TGFBR3-overexpressing cell line in a cell-based indirect immunofluorescence assay, and immunoprecipitation with human glomerular extract. As circulating antibodies have not yet been identified, we cannot determine whether TGFBR3 represents an autoantigen or a biomarker for MLN, similar to the situation with EXT1/EXT2 (21, 33).

Moreover, it conceivable that, despite the paradigm that immune complexes in lupus form extra-renally in the circulation, the pathogenesis of pure lupus nephritis may be more similar to that of primary MN with antibodies targeting intrinsic, induced, or planted podocyte antigens to form immune complexes *in situ*, an argument that is supported by the lack of mesangial or subendothelial deposits in some cases of MLN.

ADDITIONAL AUTOIMMUNE DISEASES ASSOCIATED WITH MN

In addition to SLE, other autoimmune diseases have been associated with MN, including sarcoidosis (38, 93, 94), urticarial vasculitis (38, 95), ANCA-associated glomerulonephritis (38, 96), rheumatoid arthritis (38, 97), Sjogren syndrome (38, 98, 99), systemic sclerosis (38, 100, 101), thyroiditis (38, 102, 103), chronic inflammatory demyelinating polyneuropathy (CIDP) (104–106), autoimmune myositis (99, 106), and ankylosing spondylitis (38). Therefore, patients with a positive ANA, systemic manifestations, and MN should undergo a thorough workup to exclude autoimmune disease.

Myeloperoxidase (MPO) is a target autoantigen associated with MN in the setting of ANCA-associated disease. A concurrent crescentic glomerulonephritis is frequent, but MN can occur in its absence. These cases are PLA2R-negative and MPO has been demonstrated in the epimembranous immune deposits in glomeruli (96, 107, 108).

Membranous-like glomerulopathy with masked IgG kappa deposits (MGMID) is an autoimmune disease that can exhibit a membranous pattern on kidney biopsy and affects young women, often with positive antinuclear antibodies (109). In most cases, IgG deposits along the glomerular capillary loops are 'masked' meaning that they are not identified by routine immunofluorescence but are seen by paraffin immunofluorescence after pronase digestion of FFPE tissue. Evaluation by immunofluorescence after pronase digestion is triggered when there are subepithelial electron-dense deposits by electron microscopy, but no IgG staining on routine immunofluorescence microscopy (109, 110). This glomerular disease looks like membranous nephropathy on a kidney biopsy, but is a separate disease entity, of which can be identified by staining for the biomarker serum amyloid P (SAP) (29). It should be considered in the differential diagnosis when MN has kappa restriction (more specifically, IgG1-kappa restriction). Although light chain restricted, it is not thought to be a paraprotein-mediated disease and instead the antigen SAP interacts with IgG1-kappa (111). Conversely, other forms of MN that are light chain restricted should be investigated for an underlying lymphoproliferative disorder, particularly if PLA2R negative (in 75% of cases) (112).

CNTN1 was recently identified as a target antigen in MN with chronic inflammatory demyelinating polyneuropathy (CIDP) and autoimmune myositis (105). It is a cell adhesion molecule near the node of Ranvier of neurons. Anti-CNTN1 autoantibodies can be monitored by ELISA-based testing (104). Patients with autoimmune neuropathy that is CNTN1-associated have an increased prevalence of nephrotic syndrome, which is often found to be due to MN. Antibodies were IgG4-subclass predominant (106). LCMD of glomeruli was performed and confirmed the presence of CNTN1 in these patients. Therefore, CNTN1 may represent a link between autoimmune neuropathy and nephrotic syndrome. Very rare cases of CNTN1-positive MN have also been identified in patients without neurological symptoms (106).

MEMBRANOUS ANTIGENS ARE PROTEINS SHARED BY THE CENTRAL NERVOUS SYSTEM

Perhaps relevant to these links to neuropsychiatric disease or autoimmune neuropathies, several MN autoantigens are proteins expressed in both neurons and podocytes. There are similarities in signaling between podocytes and neurons, both of which are specialized cells with arborized processes supported by robust cytoskeletal dynamics and an intercellular communication utilizing similar proteins expressed at synapses and foot processes, such as synaptopodin and dendrin (113, 114). Other proteins with restricted expression within podocytes and neurons include neurexin 1, NPHS1, SYNPO, and KHDRBS3, among others (113, 114). Proteins required for axonal extension and survival in neurons, polarity, and preservation of synaptic connections are also expressed within podocytes and are crucial to preserve the glomerular filtration barrier. This link has recently culminated in identifying CNTN1 as the main antigen involved in nephrotic syndrome in those with demyelinating neuropathy (106). An additional potential target for CIDP-related MN is neurofascin 155 (NF155) (104), a similar paranodal protein. Other target antigens in MN expressed in neurons include THSD7A (115), NCAM1 (116, 117), NELL1 (118), HTRA1 (119), PCDH7 (120), and SEMA3B (121).

SOURCES OF POTENTIAL MEMBRANOUS AUTOANTIGENS

Antigens targeted in MN can be intrinsic podocyte proteins, podocyte proteins that may be induced by certain stressors, extrinsic non-podocyte proteins that become planted in a subepithelial position, or those that precipitate from circulating immune complexes. The concept that target antigens in MN are often podocyte-expressed proteins is relatively recent. The antigenic component of rat proximal tubular brush border is megalin which, in a not-well-understood quirk of nature, is universally expressed by the podocyte in the rat, but not so in other species. PLA2R and THSD7A were identified as target antigens in MN before they were found to be podocyte proteins. Detailed scrutiny of the more-recently identified antigens may reveal that they are expressed by podocytes in some cases, including HTRA1, SEMA3B, NCAM1, and TGFBR3, but not in others, including EXT1/2, NELL1, PCDH7, and CNTN1.

However, our knowledge of what is or is not expressed by normal human podocytes in the resting state is not comprehensive, and new studies consistently reveal novel podocyte proteins. There may also be age-related developmental changes in expression, or induced expression by disease or environmental factors. Such an induced expression may, for instance, explain the segmental pattern of NELL1-associated MN, since NELL1 does not seem to be normally expressed by the podocyte. In challenging the prevailing paradigm in understanding MN, the presence of antigens not inherently expressed in glomeruli, such as EXT1/2, NELL1, and PCDH7, may suggest more than one pathogenic pathway can lead to similar pathologic manifestations.

MEDICATIONS INDUCING MEMBRANOUS NEPHROPATHY

Multiple medications are described to cause MN, comprising 6.6-14% of total MN in prior studies (122–124). Drug-induced MN was reported with therapy with gold salts, penicillamine, bucillamine, captopril, non-steroidal anti-inflammatory drugs (NSAIDs), selective COX-2 inhibitors, tiopronin, trimethadione, and lipoic acid (74, 123). NSAIDs are currently the leading offender and this form of MN is often reversible, remitting with discontinuation of the offending drug (125). Some medications associated with development of MN may act as weak reducing agents and potentially modify protein folding. Alternatively, they may serve as haptens.

INFECTIOUS TRIGGERS OF MEMBRANOUS NEPHROPATHY

Some infections have been identified in association with MN and in certain cases the microbial protein has been identified within immune complexes. It is unclear whether microbial proteins are true antigenic targets or are passively trapped within the subepithelial space. Hepatitis B virus (HBV) infection can induce MN with the hepatitis B e antigen (HBeAg) identified within subepithelial deposits. Proteinuria persists with HBV antigenemia and one-third develop progressive disease, despite treatment (126, 127). In a recent study, the prognosis of HBV-associated MN was similar to that of PLA2R-positive MN (128). The incidence of HBV-associated MN may be even higher in children, as children with nephrotic syndrome in endemic regions commonly have HBV-associated MN (129). HBV-associated MN has been described in the setting of PLA2R and THSD7A positivity as well, which could be coincidental or instead represent a viral trigger for autoimmunity (130).

Hepatitis C virus (HCV) infection has also been reported as a secondary cause of MN which can remit with antiviral therapy (131, 132). Membranous nephropathy can also be associated with human immunodeficiency virus (HIV) infection; in one study, 9% of HIV-infected patients with proteinuria had MN (133, 134). Syphilis, which can be co-morbid with HCV or HIV infection, is also associated with MN. Treponemal antigens have been identified within glomerular immune complexes (135) with resolution of nephrotic syndrome following treatment of the syphilis (136). Schistosomiasis can also trigger MN in endemic regions (137, 138). Elution of immune complexes from kidneys of patients with schistosomiasis identified reactivity against *Schistosoma mansoni* by indirect immunofluorescence (139). Recently in the COVID-19 pandemic, cases of MN have been reported in patients with SARS-CoV-2 infection, or post-SARS-CoV2 vaccination (140–146). There is an increased frequency of PLA2R-negative MN than expected for primary MN, however it is unclear whether this is due to molecular mimicry with a viral component, such as the spike protein. It is possible that PLA2R-positive MN may be more likely to be empirically treated without a biopsy in those with positive serology, particularly during the COVID-19 pandemic where elective procedures which include kidney biopsies have been halted during disease surges.

It is intriguing that only a few infections trigger MN. This could be due to molecular mimicry with a limited subset of microbial proteins found in these associated infections that might induce autoreactivity to host proteins. A peptide contained within the dominant epitope in the N-terminal cysteine-rich domain of PLA2R also exists in D-alanyl-D alanine carboxypeptidase, a cell wall enzyme in several bacterial strains (147). No clinical evidence has yet been produced to support this potential connection. Molecular mimicry may also play a role in the case of HTRA1-associated MN, in which bacterial form of the HTRA protein found in the cell wall of *Orientia tsutsugamushi* is known to be antigenic and has been used in a vaccine developed against scrub typhus, demonstrating immunogenicity (148).

Toll-like receptor responses may also be involved through interacting with microbial nucleic acids. Interferon regulatory factor 4 (IRF4), a negative regulator of TLR signaling, was identified in a large genome wide association study for MN as a highly significant risk locus (149). IRF4 binds MyD88 (a common signal transduction protein critical to TLR signaling) and competes with IRF5. In murine lupus models, induction of ligands to active TLR3, TLR7, and TLR9 (which interact with dsRNA, ssRNA, and CpG DNA respectively) increase severity of nephritis (150).

MALIGNANCY-ASSOCIATED MEMBRANOUS NEPHROPATHY

Another secondary etiology of MN is hematologic or solid organ malignancy, comprising approximately 10% of MN cases. MN is the leading cause of nephrotic syndrome in patients with malignancy and patients with MN have a three-fold increase in the incidence of cancer compared to the general population (151, 152). Therefore, cancer screening is an initial step after MN diagnosis (153). Concurrent MN and malignancy confer a poor clinical outcome with a reduced rate of remission and a four-fold increased risk of thromboembolic disease (153). When MN is malignancy-associated, proteinuria can respond dramatically to resection, chemoradiation, or other treatments. Likewise, proteinuria may worsen with recurrence or metastasis.

Because both MN and malignancies tend to occur in older individuals, it can often be difficult to determine if the cancer is causal or coincidental (39). To classify the MN as malignancy-associated, three key factors may be present, although all may not necessarily be known at the time of diagnosis. The patient's diagnosis of MN and cancer should be temporally associated, proteinuria should resolve with cancer remission, and/or relapse of the malignancy causes recurrence of the MN. Multiple tumor types were identified in temporal association with membranous nephropathy, including carcinomas (58), soft tissue tumors (154), melanoma (155), thymoma (154), and lymphoma (68, 156, 157). Histopathologic clues for malignancy-associated MN include PLA2R-negativity, a segmental pattern on IgG staining, endocapillary hypercellularity (154), and IgG1 and IgG2-predominant immune deposits (as most cases of primary MN have IgG4-predominant immune deposits) (158). NELL1 is the autoantigen in approximately one-third of malignancy-associated MN cases while THSD7A is found in approximately 10% (68). Both NELL1 and THSD7A have been shown to exhibit increased protein expression in certain human cancers (59, 64, 159).

The mechanisms underlying malignancy-associated MN are largely unknown. It is possible that dysregulated expression of immunogenic proteins by the tumor incites autoimmunity with subsequent deposition of immune complexes within the subepithelial space. Genetic mutations within the tumor may alter the amino acid sequence to create neo-epitopes that are immunogenic. Alternatively, there could be molecular mimicry of a tumor antigen that shows sequence similarity to a podocyte protein (39).

Increased expression within the tumor due to copy number increases, as has been seen with THSD7A due to polysomy of chromosome 7 (58), may also occur for other autoantigens. Many cancers carry a high mutational burden (160), particularly those with mismatch repair defects, of which could create neo-epitopes that may elicit an autoimmune response. Increased RNA transcript stability, protein stability, or epigenetic modifications are other possibilities. Some tumors have a high frequency of over-expression of a target protein, as seen in both THSD7A and NELL1, yet a majority of patients with malignancy do not develop kidney disease. It is possible that a second hit may be required, which warrants further investigation.

ANTIGEN-BASED CLASSIFICATION OF MN – ABANDONING PRIMARY *VERSUS* SECONDARY

The categorization of MN as either primary or secondary was based on the absence or presence of a detectable underlying cause, such as systemic autoimmune disease, cancer, or infection, as discussed above. Subsequently, after the identification of PLA2R as target antigen in the majority of primary MN, PLA2R-negative cases elicited an extensive workup to identify a possible secondary etiology (37). Recently identified autoantigens have been detected, commonly or more rarely, in settings that would more typically be associated with a potential secondary etiology of the MN, calling into question whether MN associated with a known antigen is necessarily primary. Due to the inability to assign a particular target antigen as exclusively representative of primary or secondary disease, the field has been moving toward a more antigen-based classification system (45).

An overview of demographic, clinical, and histopathologic characteristics for the MN subtypes associated with each of these various antigens in MN is provided in **Tables 1**, **2**. Given the large number of autoantigens identified to date and extrapolating from clinical experiences with PLA2R and THSD7A, such an antigen-based classification system could result in development of precision diagnostics for monitoring disease and has been advocated by others (45, 161). The large number of MN antigens thus far identified has also made it impractical to perform antigen subtyping by immunostaining of the kidney biopsy for each one. Instead, a mass spectrometric approach might instead be considered in cases of PLA2R-negative MN, similar to what is done for the typing of amyloidosis cases that are found to be non-AL amyloid-associated (161).

However, as such an MS-based strategy for the typing of MN is currently impractical for routine clinical practice, we propose a staining algorithm for antigen subtyping based on clinical and pathologic features (**Figure 4**). As PLA2R-positive MN comprises the majority of MN cases, PLA2R staining should be performed first as a high-yield diagnostic assay. For PLA2R-negative cases, clinical considerations should be utilized to inform further staining. For patients with known autoimmune disease, or those with positive antinuclear autoantibodies that lack a diagnosis of a particular rheumatologic condition, EXT1/2, NCAM1, and TGFBR3 staining can identify approximately 40% of cases, although our current knowledge gap of the remaining antigens still leaves the majority of SLE patients untypeable. Age can be a useful factor, as elderly individuals may have an increased frequency of HTRA1- or PCDH7-associated MN (23) and young children have an enrichment in SEMA3B positivity (30). Histopathologic variables can be informative, including low complement staining enriching cases of PCDH7-associated MN, segmental or incomplete capillary loop staining in NELL1-positive cases or patients with concurrent ANCA-associated glomerulonephritis (71).

HOW DO ANTIBODIES CAUSE PROTEINURIA?

Subepithelial immune complex formation is the hallmark of MN. Complement pathway activation has been considered as the paradigmatic mechanism leading to podocyte injury and failure of the glomerular filtration barrier, resulting in the nephrotic syndrome. However, other mechanisms inducing podocyte injury have been postulated and the primacy of complement as a pathomechanism in MN has been challenged. In the passive Heymann nephritis model, proteinuria depends on activation of the complement system with subsequent formation of the membrane attack complex (C5b-9) (162, 163). Other

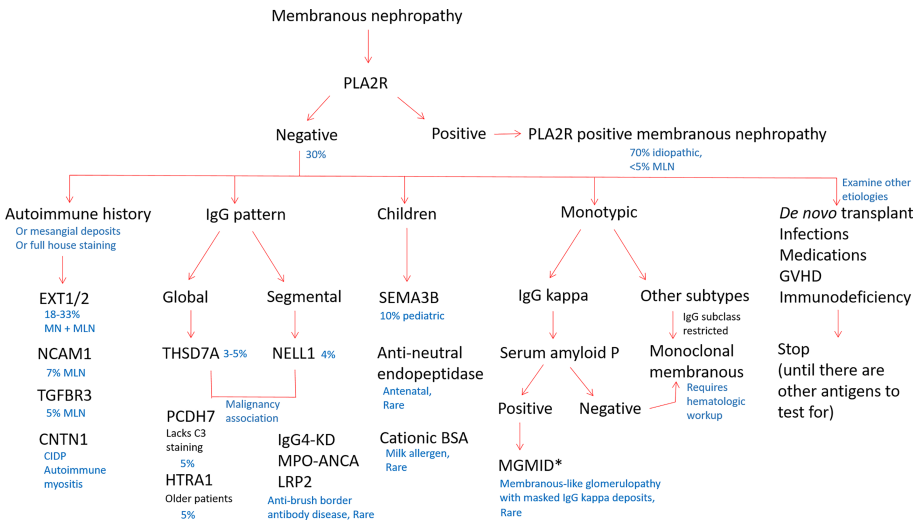

FIGURE 4 | Proposed staining algorithm for phenotyping of membranous nephropathy cases. For 'all comers' of MN cases without a known history of systemic lupus erythematosus, we suggest staining for PLA2R, as it will identify the majority of cases (approximately 70%). For biopsies that are PLA2R negative, the clinical history, demographics, and pattern of immune reactants on biopsy could guide which antigens to evaluate. For patients with an autoimmune history (positive ANA or history of autoimmune disease), staining for EXT1/2, TGFBR3, NCAM1, and CNTN1 can together identify approximately 40% of MN cases secondary to autoimmune disease or membranous lupus nephritis. Children with MN are most commonly PLA2R-positive as adults, although SEMA3B staining will pick up approximately 10% of pediatric MN cases. In neonates, anti-neutral endopeptidase and cationic BSA are additional considerations. NELL1 and THSD7A may be enriched in patients with malignancy. The IgG pattern on biopsy is useful to choose additional antigens for staining, as THSD7A, PCDH7, and HTRA1 typically have a diffuse and global granular capillary loop pattern and NELL1 MN often shows segmental IgG staining. IgG4-related kidney disease, ANCA-associated glomerulonephritis (p-ANCA/MPO antibodies), and LRP2-associated nephropathy also often show a segmental IgG pattern along capillary loops. When MN is restricted to one light chain, cases with IgG kappa can be evaluated for SAP to identify membranous-like glomerulopathy with masked IgG kappa deposits (MGMID)*. If lambda light chain restricted or negative for SAP, IgG subclasses are helpful to identify if the MN is restricted to one subtype, for which a hematologic workup can be indicated to evaluate for an underlying lymphoproliferative disorder as a driver of disease. In patients with *de novo* MN following transplantation, infections, medications, graft-*versus* host disease, or immunodeficiency, it is common to not identify a known autoantigen at this time. *In the setting of subepithelial electron-dense deposits by electron microscopy, but no IgG staining by routine immunofluorescence microscopy, pronase digestion of FFPE tissue can 'unmask' immune deposits in MGMID and is required in the majority of cases. Created with BioRender.com.

studies, however, provide evidence that proteinuria can develop in the absence of certain complement components (164, 165). For example, in a mouse model of MN with passive transfer of human anti-THSD7A antibodies, proteinuria developed without C3 or C5b-9 deposits.

It is possible that any antigen-antibody complex at the appropriate location can activate complement and thereby induce MN. The immune deposits in human MN contain substantial amounts of complement, including C3 and C5 cleavage products and the terminal C5b-9 complex, yet the predominant IgG subclass in primary MN is IgG4, which is incapable of activating complement *via* the classical pathway (166). In addition, mass spectrometry of LCM-dissected glomeruli revealed increased protein expression of complement components C3, C4, C5, C6, C7, C8, and C9, as well as regulators of complement pathway activation (76). The absence of C1q in subepithelial deposits suggested that either the alternative pathway or mannose binding lectin (MBL) pathway are involved in disease pathogenesis. The presence of C4 in the absence of C1q suggests a role for MBL pathway activation. However, reported cases of MN in patients deficient in MBL (167) and ficolin 3 (168) suggest involvement of alternative complement pathway. Recent studies using the IgG4 subclass of anti-PLA2R have provided *in vitro* evidence of direct cytotoxic effects on podocytes *via* the MBL pathway of complement activation (169).

Additionally, antibodies may induce proteinuria by functional interference with proteins critically involved with maintaining the glomerular filtration barrier or podocyte health. Such a mechanism was suggested by the impairment of cellular adhesion to type IV collagen, a key matrix molecule in the GBM, by PLA2R autoantibodies (170), although other studies did not confirm this (171). Further evidence gleaned from a mouse model of MN suggested that THSD7A autoantibodies may directly lead to cytoskeletal structural alterations that result in proteinuria (172) possibly related to the fact that THSD7A localizes directly beneath the slit diaphragm (173). HTRA1 is involved in extracellular matrix (ECM) homeostasis (90), but whether anti-HTRA1 antibodies interfere with podocyte-ECM cross-talk has yet to be determined.

A prerequisite for circulating antibodies to interact with a target podocyte antigen is the presence of one or more exposed humoral epitope in an extracellular location, either within the extracellular region of transmembrane protein or on a secreted protein that associates with the podocyte basal surface or the GBM. While many epitopes likely exist in accessible sites dictated

by the native conformation of the protein, others may be cryptic and require additional exposures or modifications, as has been suggested for SEMA3B. Both THSD7A and PLA2R proteins are transmembrane proteins with extracellular domains that are exposed and accessible to the humoral immune system. HTRA1 is a matrix modifying enzyme that is secreted in conjunction with extracellular matrix molecules that serve a substrate for its protease activity and thus can localize to the GBM and be targeted by autoantibodies in this environment (174–177).

Certain intracellular proteins, such as superoxide dismutase, can be induced by oxidative stress by podocytes or other nearby cells such as glomerular endothelial cells and may be accessible as target antigens at the cell surface, subepithelial space, or associated with the GBM (41, 178, 179). Prunotto et al. showed the presence of IgG4-dominant antibodies against endothelial proteins superoxide dismutase 2 (SOD2), aldose reductase, and enolase in MN (179). The recent discovery of non-podocyte proteins as MN antigens, including PCDH7, NELL1, and EXT1/2, further established that non-podocyte proteins can indeed be MN antigens. Moreover, the identification of microbial proteins and tumor proteins within immune complexes supports the concept that non-native podocyte proteins can be targeted antigens. The pathophysiologic mechanisms through which antibodies targeting antigens not expressed in podocytes remain to be fully elucidated. One may argue that antigens that become planted in a subepithelial position by virtue of charge or other characteristics are also considered *in situ* immune complex formation and does not necessarily indicate that the antigen is an intrinsic podocyte protein.

Understanding how these non-native proteins become planted can help us understand disease pathogenesis. Treatment of podocytes, which have low baseline expression of these intracellular proteins, with hydrogen peroxide to induce oxidative stress resulted in SOD2 expression along the outer plasma membranes of podocytes (179). This suggests that some proteins may be induced as neoantigens under oxidative stress. It was postulated the antigenicity starts at the podocyte level with neo-epitope exposure triggering autoimmunity, with subsequent *in situ* immune complex formation and nephrotic syndrome (179–182).

Some environmental factors may also be involved in triggering autoimmune responses that may occur outside of the kidney. MN is more common in areas with high levels of microparticulate (PM2.5) air pollution, potentially implicating this environmental exposure as a risk factor for disease initiation (183). This has shed light on a potential extrarenal site of antigenicity, as PLA2R is known to be expressed within alveolar macrophages of the lung and may be more highly expressed in the setting of airway inflammation (184). It is possible that such induced protein overexpression overwhelms the cell's capacity to properly translate and fold these highly-disulfide bonded proteins, resulting in misfolding, perturbed protein trafficking, or endoplasmic reticulum stress that could ultimately trigger an immune response in genetically susceptible individuals. Extrinsic protein overexpression as a trigger for antigenicity is also suggested by malignancy-associated THSD7A-positive MN in which THSD7A is abnormally expressed in tumor tissue due to polysomy of chromosome 7, providing a potential source of antigen that can overwhelm host tolerance mechanisms (59).

GENETIC FACTORS PREDISPOSING TO MEMBRANOUS NEPHROPATHY

Genetic predilection has also been implicated in MN pathogenesis. Genome-wide association studies (GWAS) and whole exome sequencing (WES) have revealed highly significant risk alleles predisposing to MN. A single-nucleotide polymorphism (SNP) at the *HLA-DQA1* locus has been associated with higher risk of developing PLA2R-associated MN (185), more so if concurrent with a SNP in *PLA2R1* (185). Most SNPs in *PLA2R1* that associate with disease risk are common in the normal population (186, 187), so it is unclear whether there is multi-hit hypothesis leading to development of MN in only those with particular HLA haplotypes, other genetic aberrations, or exposure to environmental factors. It is possible that the *PLA2R1* SNP drives abnormal expression of PLA2R, while the HLA SNP more easily allows presentation of PLA2R-derived peptides in the binding grooves of major histocompatibility complex class II molecules. The most significant SNPs in *PLA2R1* are intronic and thus are not expected to change protein structure (50, 188, 189), but rather may alter protein expression level with consequent misfolding and disturbed trafficking leading to antibody formation. The presence of different transcript isoforms or altered expression levels corresponding to the specific antigen remains to be investigated.

EPITOPE SPREADING

Epitope spreading is an immunologic phenomenon whereby an initial antibody response to a given antigen may extend from one particular location (epitope) on the antigen to involve other region(s) of the same antigen (intramolecular spreading) or nearby or related antigens (intermolecular spreading) (6). Epitope spreading is a common phenomenon in autoimmune disease (190–193). Initial experimental evidence that epitope spreading occurs in MN and affects its severity was demonstrated in Heymann nephritis using only N-terminal domains of megalin to trigger subsequent humoral responses to more distal portions of the molecule (7). Epitope spreading was subsequently described in humans with PLA2R-associated MN. In PLA2R-associated MN, patients first appear to develop autoreactivity against an epitope within the cysteine-rich domain (CysR). Epitope spreading to include the CTLD1 and CTLD7 domains (in addition to the CysR) is associated with poor prognosis (55). Some studies suggested that in patients with PLA2R-positive MN, epitope spreading during follow-up associated with disease progression, whereas reverse spreading back to only a CysR profile associated with favorable outcomes (174, 194). However, this finding has been debated by other

studies, which suggest that the correlation with epitope spreading and worse prognosis is more related to overall PLA2R antibody titers (195–197) and it remains unclear whether this truly reflected spreading or concurrently target epitopes from disease initiation.

A better understanding of the evolution and repertoire involved in epitope spreading to any MN target antigen may help to guide prognosis, to understand the initial triggering events in disease pathogenesis, and to allow development of antigen-targeted therapies. While epitope spreading is important in the initiation and progression of MN and other autoimmune diseases (198–201) and carries potential prognostic value, it is not yet utilized in routine clinical practice.

Intramolecular epitope spreading, as described above, may be more common than intermolecular epitope spreading. Rare reports of dual positivity for antigenic targets has been observed with concurrent PLA2R and THSD7A-positive cases (202), PLA2R and EXT1/2 co-positivity (203), THSD7A and EXT1/2 co-positivity (203), and dual NCAM1 and EXT1/2 staining (32). However, no study has shown that one autoimmune reaction predated the other, so the existence of intermolecular epitope spreading (*i.e.*, antibody-mediated cytotoxicity due to one antigen resulting in exposure of the second protein to the immune system) is only speculative. The incidence of PLA2R and THSD7A dual positivity is approximately 1%, shown in a study where 2/258 cases of primary MN shown dual positivity by immunostaining and serologic testing (202). Insufficient data exists to determine whether dual positive cases are associated with a worse prognosis.

In contrast to these infrequently-encountered dual responses, most antigen-based MN subtypes are mutually exclusive. It is unclear why, in the majority of cases, there is an autoimmune response to only a single antigen. One possibility is related to an individual's class II HLA repertoire and the decreased likelihood of possessing two or more HLA risk alleles that could support initiation and propagation of an autoimmune response to both antigens. If intermolecular epitope spreading were related only to cell damage, we would expect more cases of dual reactivity due to the universal expression of PLA2R and THSD7A by the human podocyte.

SEROLOGIC MONITORING OF DISEASE

One of the most evident benefits of knowing the antigenic target of a humoral immune response is the ability to detect and measure circulating autoantibodies. Serologic testing (or "immune surveillance") offers the ability to monitor immunologic disease activity and response to therapy in a non-invasive manner. As demonstrated with anti-PLA2R, autoantibody levels correlate with disease activity but with a lag time, decreasing and often disappearing prior to a full proteinuric response with treatment, or increasing after remission to signal oncoming relapse of disease. As serologic remission precedes clinical remission, it can be useful to adjust immunosuppressive therapy accordingly and can inform prognosis. Serologic diagnostic tests for PLA2R-associated MN (51) and THSD7A antibodies (61) show a high sensitivity (70.6-78%) and specificity (91-94.6%) (52, 53), although are subject to false negatives or false positives if used to screen all patients with nephrotic syndrome.

As a proof of concept and extrapolating from the PLA2R experience, a combination of IFA and ELISA-based testing provided high specificity for disease. This has allowed providers in some situations to avoid the need to perform a kidney biopsy in patients who have with anti-PLA2R antibodies and rather to monitor them according to anti-PLA2R titers (204, 205). This diagnostic strategy is now supported in the 2021 KDIGO Clinical Practice Guideline for the Management of Glomerular Diseases in not mandating a kidney biopsy to confirm PLA2R-associated MN in a seropositive patient (206). This is supported by our experience in which we see a lower frequency of PLA2R-positive kidney biopsies in our recent cohort, compared to historical studies suggesting that clinicians are already deciding not to biopsy patients diagnosed by the non-invasive serological test (33).

The identification of new antigens in MN may also provide a means for clinical monitoring when circulating antibodies are detectable, yet there is a need for development of validated and reproducible immunoassays directed towards these antigens for use in the clinical setting. With such advances in serologic testing, the diagnosis and monitoring of the recently identified MN subtypes may be possible and more widely available. However, some subtypes of MN, including EXT1/2- and TGFBR3-associated disease, have yet to have autoantibodies identified, creating a challenge for development of these assays. Identifying autoantibodies directed against all distinct MN types could alter treatment paradigms for MN by adding to the list of treatment-specific biomarkers, identifying new therapeutic targets, and providing tools to forecast impending MN flares. This would allow for patients to be pre-emptively treated, perhaps without the need for biopsy, shorten exposure to toxic drugs by treating only to the point of immunologic remission and limiting permanent kidney damage.

TREATMENT

Treatment of MN has so far focused on non-specific immunosuppressive therapy, similar to most other autoimmune diseases. Treatment for six months alternating cyclophosphamide with high-dose corticosteroids is still recommended for MN patients that are at very high risk of progression. This 'gold standard' in the treatment of MN of cyclic treatment with alkylating agents (cyclophosphamide or chlorambucil) and methyprednisolone, is described in the Ponticelli and 'modified Ponticelli' protocols (207) and to date, is the most efficacious at inducing remission. While effective, these cytotoxic drugs have multiple off-target side effects. Rituximab, an anti-CD20 monoclonal antibody for B-cell depletion, has been utilized to induce remission in place of the Ponticelli regimen, with variable efficacy, assessed in various studies comparing with other agents (208–211) and is now recommended for moderate and high risk patients. Calcineurin inhibitors are still a viable alternative to MN patients at moderate

risk of progression, but are associated with an increased risk of relapse (212) and dependence.

Corticosteroids, cytotoxic medications, B-cell depletion, and calcineurin inhibitors lack specificity and are potently immunosuppressive. Unraveling the complexity of this disease might lead to more targeted therapy tailored to specific patient needs. Moreover, therapies targeting specific antigens through blockade with mimotopes or interference with synthesis can prove to be beneficial. This may be accomplished through targeting a certain antigen or targeting the epitope *versus* addressing a stressed organelle such as endoplasmic reticulum, or disturbed cytoskeleton, or other methods. Ideally, specific depletion of the responsible clones may result in individualized medicine with improved efficacy and reduced off-target toxicities.

DISCUSSION

Future Directions and Unanswered Questions

One challenge to clinically identifying and characterizing MN is the invasive nature of diagnosis by kidney biopsies. The current practice of collecting a renal biopsy is invasive and expensive, and it is impractical for physicians to closely monitor patients using serial biopsies. Future studies will build upon serum and tissue-based proteomic approaches to identify remaining specific biomarkers, potentially enabling development of non-invasive diagnostics and/or prognostic assays. Further characterization of temporal changes in autoantibodies and reactive epitope profiles throughout the different stages of MN is warranted.

Additionally, for some types of MN (EXT1/2 or TGFBR3), it is not yet known whether autoantibodies exist or are these proteins simply biomarkers of disease. The identification of immune complexes eluted from frozen kidney biopsy specimens by tissue IP suggests that a true antigen-antibody interaction exists. Therefore, why are we unable to detect autoantibodies in sera? Antibodies could have very low avidity binding, may circulate in multi-molecular immune complexes where epitopes are opsonized by complement and unavailable for binding, or post-translational modifications *in vivo* are not recapitulated in *in vitro* assays. There may be a mutation resulting in altered protein structure inducing antigenicity, which would not be re-capitulated through use of commercial recombinant proteins. Further work and novel approaches may yield autoantibody detection.

Further understanding of the etiopathogenesis of MN may elucidate further treatment targets. Currently, there is a lack of reliable preclinical models that recapitulate human pathology. To address this issue, future studies will likely introduce mouse models that might allow further investigation of the underlying molecular events of pathological development in this disease.

Ideal therapy in MN should be more specific and tailored to induce tolerance or deplete cells producing the pathogenic antibodies. It is anticipated that future interventional trials will employ two different approaches to target pathological mechanisms in MN. For example, chimeric-antigen-receptor-T-cell (CAR-T) technology will be leveraged to generate specific T cell clones that are able to recognize and destroy B cells expressing antigenic receptors with epitope specificity for target MN antigens (213, 214). Based on proteomic data instigating complement in the pathogenesis of MN (76) and with advances in the therapeutic use of complement inhibitors, it is likely complement inhibition will play a role in the future targeted therapy for MN (215). Peptide-based therapy could also generate mimetics that are able to induce immunological tolerance (216, 217). In this regard, *in vitro* studies of tolerogenic peptides suggested several mechanisms of tolerance induction, such as blocking of MHC class II presentation of the pathogenic peptides by using excess amount of the peptide mimetics or antagonism/partial agonism of TCR signaling and subsequent inhibition of T cell activation (216, 218). This could lead to dramatic improvements in the treatment of MN and reduce drug toxicities.

CONCLUSION

In summary, multiple advances have been made in MN over little more than a decade, with the discovery of PLA2R providing the ability to monitor disease. Within only the past three years, a plethora of autoantigens have been identified, reducing our knowledge gap for the protein targets of MN. This could open the door to precision classification through use of algorithmic and multiplex immunostaining, or alternatively, a mass spectrometry-based classification system. Knowledge of autoantigens also opens the door to develop assays for non-invasive monitoring through a serologic-based approach. Additionally, clinical associations of specific antigens with secondary manifestations (malignancy, lupus, and medications) can guide the clinician to evaluate for underlying triggers of the disease. Our understanding of MN pathogenesis is incomplete and further studies will be informative to identify new potential treatment targets through precision medicine. The journey of membranous nephropathy has already reached a new avenue moving away from primary and secondary MN to an antigen-based classification system, enabling diagnosis and monitoring of all types of MN non-invasively, with novel treatments developed from the discoveries.

AUTHOR CONTRIBUTIONS

All three authors (LA-R, TC, and LB) contributed equally to the writing of this article, and all authors are accountable for the content of the work.

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Collapsing Focal Segmental Glomerulosclerosis in Viral Infections

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Collapsing glomerulopathy represents a special variant of the proteinuric kidney disease focal segmental glomerulosclerosis (FSGS). Histologically, the collapsing form of FSGS (cFSGS) is characterized by segmental or global condensation and obliteration of glomerular capillaries, the appearance of hyperplastic and hypertrophic podocytes and severe tubulointerstitial damage. Clinically, cFSGS patients present with acute kidney injury, nephrotic-range proteinuria and are at a high risk of rapid progression to irreversible kidney failure. cFSGS can be attributed to numerous etiologies, namely, viral infections like HIV, cytomegalovirus, Epstein–Barr-Virus, and parvovirus B19 and also drugs and severe ischemia. Risk variants of the APOL1 gene, predominantly found in people of African descent, increase the risk of developing cFSGS. Patients infected with the new Corona-Virus SARS-CoV-2 display an increased rate of acute kidney injury (AKI) in severe cases of COVID-19. Besides hemodynamic instability, cytokine mediated injury and direct viral entry and infection of renal epithelial cells contributing to AKI, there are emerging reports of cFSGS associated with SARS-CoV-2 infection in patients of mainly African ethnicity. The pathogenesis of cFSGS is proposed to be linked with direct viral infection of podocytes, as described for HIV-associated glomerulopathy. Nevertheless, there is growing evidence that the systemic inflammatory cascade, activated in acute viral infections like COVID-19, is a major contributor to the impairment of basic cellular functions in podocytes. This mini review will summarize the current knowledge on cFSGS associated with viral infections with a special focus on the influence of systemic immune responses and potential mechanisms propagating the development of cFSGS.

Keywords: podocyte, APOL 1, HIVAN, COVAN, Immune response

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INTRODUCTION

Focal segmental glomerulosclerosis (FSGS) is a major glomerular cause of end stage renal disease. The definition of FSGS is derived from its histopathological picture—the focal appearance of segmental scarring in some glomeruli. Before sclerosis ensues, podocytes show foot process effacement, leading to the manifestation of proteinuria. Since FSGS represents a pattern of response to injury, it was recently proposed to group FSGS into primary (immune-mediated) FSGS, adaptive FSGS, FSGS caused by pregnancy-related VEGF-inhibition, genetic, drug- and virus-associated FSGS (1, 2). The Columbia

classification of FSGS differentiates 5 morphologic variants, namely, FSGS not otherwise specified (NOS), perihilar, cellular, tip, and collapsing variant (3).

The collapsing form of FSGS (cFSGS) represents a special form of secondary FSGS. Histopathologically, cFSGS is characterized by segmental or global condensation and obliteration of the capillary tuft associated with wrinkling and collapsing of the glomerular basement membrane. The podocytes display a distinct hyperplastic and hypertrophic phenotype, often containing cytoplasmic protein resorption droplets and pronounced foot process effacement. Severe tubulointerstitial disease with inflammation, edema, interstitial fibrosis and tubular atrophy as well as tubular regenerative changes constitutes an important component of cFSGS (4). cFSGS is associated with different etiologies. One of the best characterized causes is an infection with the human immune deficiency virus (HIV) and the development of HIV-associated nephropathy (HIVAN) (5). Furthermore, cFSGS can also be attributed to other infections, drugs, severe ischemia, autoimmune disease, genetic causes, and idiopathic (6–9). Additionally, an infection with the new Corona-Virus during the pandemic of SARS-CoV2 has been associated with the potential to develop cFSGS.

HIV-ASSOCIATED cFSGS

HIV infection accompanied by acute kidney injury, proteinuria, and a rapid progression to irreversible kidney failure characterizes the course of HIVAN. Tubuloreticular aggregates in endothelial cells and microcystic tubular dilatation in some cases may contribute to differentiate HIVAN from other etiologies of cFSGS in light microscopy (7).

Investigating the interaction of the virus or viral gene products with podocyte signaling pathways that induce massive morphologic alterations in cFSGS, might contribute to our understanding of podocyte biology and find a targeted therapy in (collapsing) FSGS independent of its etiology. It has been shown, even though podocytes do not express CD4-receptors or other known HIV-receptors, that podocytes are directly infected by HIV (10). The virus is known to damage the actin cytoskeleton in any cell type (11). The podocyte cytoskeleton is essential for the maintenance of the glomerular filtration barrier.

Furthermore, it has been shown, that HIV-1 also induces vascular endothelial growth factor (VEGF), leading to proliferation and de-differentiation of podocytes in cFSGS (12). Podocyte VEGF overexpression in a mouse model was able to cause glomerular disease with podocyte foot process effacement (13), while it was also shown that VEGF is crucial for podocyte survival *via* phospatidyl inositol 3 kinase/Akt signaling (14).

HIV associated cFSGS predominantly affects patients of African descent carrying a risk variant of the Apolipoprotein L gene 1 (APOL1), termed G1 and G2. The only known physiological function of APOL1 is its anti-trypanosomal activity (15). Several subspecies of trypanosoma have developed resistency against the "normal" G0 variants of APOL1. Therefore, the presence of one of the APOL1 variants G1 or G2 appears to protect against infection of several subspecies of *Trypanosoma brucei* (16–18). APOL1 is an abundantly secreted protein that circulates and associates with apolipoprotein A-I as a component of high-density lipoprotein (HDL) (19). It is also expressed in the intracellular compartment of various tissues—in the kidney specifically in glomeruli, proximal tubular endothelia and arteriolar endothelium. Within healthy glomeruli, APOL1 is localized exclusively in the podocytes (20). In both FSGS and HIVAN however, APOL1 expressing α-smooth muscle actin-positive cells were detected in the media of medium arteries and arterioles.

The APOL1 risk alleles G1 and G2 increase the risk of chronic kidney disease and are associated with an elevated risk of developing hypertension-associated end stage renal disease, FSGS, Lupus-nephritis, and HIVAN (16, 21–23).

Numerous studies suggested multiple pathways leading to impaired podocyte function and injury in HIVAN. The lack of the APOL1 gene in most model organisms and the absence of tissue specificity constitute barriers in identifying the underlying mechanisms. Conversely, the lack of the APOL1 gene in most mammals and a case report of a healthy APOL1-null patient supports the hypothesis, that APOL1 is not essential for kidney development and homeostasis (24).

The overexpression of APOL1 risk variants in podocytes was associated with increased necrosis and lysosomal permeability with leakage of lysosomal enzymes like cathepsin L into the cytosolic and nuclear compartment. Cathepsin L-induced degradation of the cytoskeletal protein F-actin might contribute to podocyte injury (25). Reduced numbers of autolysosomes led to impaired autophagic flux in APOL1 risk variant expressing HEK293 cells and podocytes (26). APOL1 risk variant dependent on upregulation of miR193a was found to result in the dedifferentiation of podocytes by blocking autophagy (27). Furthermore, APOL1 risk variants seem to downregulate expression levels of nephrin and podocin, key players in the slit diaphragm, and mediators of crucial signaling pathways (28).

These studies indicated the important role of APOL1 in the development of FSGS, as podocyte loss due to cell death occurred in all models of APOL1 risk variant overexpression. Pyroptosis, but not apoptosis was found to be increased in APOL1 risk allele transfected cells and might be a result of elevated levels of cleaved caspase 1 (26).

Scientific efforts could show that expression of human APOL1 risk variants in kidneys, spleens, and macrophages of bacterial artificial chromosome (BAC) transgenic mice promotes cholesterol accumulation (29). BAC/APOL1 transgenic mice express either the G0 allele or the risk alleles G1 and G2 under the endogenous APOL1 promotor. In line with these results, a recent study demonstrated in an APOL1/BAC mouse model that APOL1 risk variant expression drives lipid accumulation in renal cortices but not proteinuria. APOL1 risk variant expression in transgenic mice of a FSGS model (APOL1; Podocin-rtTA; $NFATc1^{nuc}$) were more susceptible to doxycyclin induction as their wildtype littermates (WT; Podocin-rtTA; $NFATc1^{nuc}$) and developed podocyte loss and mesangial matrix expansion. Interestingly, at baseline the human APOL1 transgenic mice did not develop proteinuria. The investigation of urinary podocytes from FSGS patients carrying either the G0/G0 or G1/G2 allele

suggested that the APOL1 risk variants cause mitochondrial dysfunction linked with lipid accumulation and compensatory OXPHOS complexes elevation in podocytes (30).

Since most carriers of two risk alleles do not develop kidney disease spontaneously and the APOL1 variant expression itself was not sufficient to induce podocyte dysfunction, a second hit is postulated essential for the development of kidney disease. Infection with HIV is believed to be the strongest known risk factor for APOL1-associated kidney disease (18) as the innate immune response to HIV upregulates APOL1 gene expression (31).

Several innate immune pathways like interleukin 1ß and Toll-like receptor 3 (TLR3) signaling showed impact on APOL1 expression levels, but the predominant effect upon HIV infection is demonstrated for interferons (INF), especially for type 1 INF (INF-α and -β) (32–34). Type 1 INF represents a part of the innate host response against viruses. It induces the intracellular response to viral infection by orchestrating a signaling cascade through the Janus kinase signal transducer and activator of transcription (JAK-STAT) pathway. The JAK-STAT-pathway regulates the transcription of INF-regulated genes (IRG), that contribute to reduce viral spread *via* distinct mechanism like the inhibition of virus entry, translation, replication and viral egress (35).

Additionally, type 1 INF induces apoptosis of infected cells in an autocrine and paracrine manner. Acute and chronic HIV infections constitute a proinflammatory state with elevated levels of interferon in plasma and tissue (36). It was indicated that type 1 INF-α and -β and also type 2 INF γ is able to upregulate APOL1 gene expression in both endothelial cells and podocytes (34). In addition, case reports describe patients carrying APOL1 risk variants who developed cFSGS after treatment with exogenous INF (34, 37) and INF-γ induced proteinuria in APOL1 G1 transgenic mice (38).

Stimulator of interferon genes (STING) is suggested to be an INF-induced candidate pathway that upregulates APOL1. HIV infection induces cyclic guanosine monophosphate-adenosine (cGAMP) synthase (cGAS) to produce cGAMP, an activator of STING (39). Interferon-inducible protein 16 (IFI16) has also been shown to act as a sensor of HIV infection and activator of STING (40). TANK binding kinase 1 (TBK1) is recruited by activated STING and phosphorylates STING and interferon regulatory factor 3 (IRF3). Phosphorylated IRF3 is then translocated to the nucleus and initiates transcription of INF-β and APOL1 in human podocytes. INF-β can furthermore activate the type I IFN receptor, leading to STAT1 phosphorylation through IFNAR-associated JAK1 kinase and increased upregulation of APOL1 and IFI16, further enhancing upregulation of STING (41). Further, cGAS/STING seem to play a key role in the increased endothelial dysfunction mediated by APOL1 risk variants and might contribute to explain the increased sepsis incidence and severity among patients of African ancestry (42).

Both cGAS and IFI16 might also play a role in the progression to lupus nephritis in SLE patients carrying risk alleles of APOL1 (41, 43).

In line with these results, it was demonstrated that retinoic acid-inducible gene I (RIG-I) also recognizes HIV and enhances APOL1 expression and also activation of nuclear factor kappa B (NFκB). The knockdown of RIG-I in human podocytes resulted in attenuated inflammatory and apoptotic effects of APOL1 (44). Additionally, TLR 3 signaling, that can be activated by double stranded RNAs, can be found in replication cycles of nearly all viruses and was shown to enhance APOL1 expression INF-independent *via* NFκB-signaling (34).

To summarize, the described interactions lead to an enhanced expression of APOL1. As the risk variants seem to exert an endotoxic effect on podocytes in a dose-dependent manner, this overexpression can induce podocyte damage in case of HIV infection, whereas, the toxic influence of the risk variants might not be high enough to induce cell damage and therefore cFSGS, without the "second hit" HIV.

Another recently debated mechanism is the contribution of parietal epithelial cells (PEC) to the cFSGS. PECs are capable of self-renewal and differentiation into various cell types, namely, podocytes (45–47) while the regeneration of podocytes appears limited to non-existent (47, 48). It has been shown *in vitro*, that the induction of APOL1 in PECs leads to the expression of podocyte markers, potentially as a repair mechanism and the effort to replace damaged podocytes (49). Furthermore, analysis of the glomerular extracellular matrix shows differences between non-collapsing and cFSGS, with an altered extracellular matrix remodelling and activation of PECs (50).

PEC activation can be detected as a first sign of ensuing glomerular scarring (51). Podocyte hypertrophy, as present in cFSGS, seems to prevent PEC activation and glomerulosclerosis. In glomerular extracts from biopsies of FSGS and diabetic nephropathy mammalian target of rapamycin (mTOR) and PEC-activation related genes were found to be upregulated (48). mTOR-mediated podocyte hypertrophy during podocyte loss seems to be crucial to maintain glomerular functional integrity, as pharmacological mTOR-inhibition during acute podocyte loss resulted in albuminuria, PEC-activation and glomerulosclerosis in mice (48). Interestingly, exacerbated and persistent podocyte hypertrophy also induced podocyte loss and PEC-activation, indicating a limited beneficial effect (48).

These data suggest the targeting of PECs as a potential therapeutic option in cFSGS. APOL1-risk variant effects on podocytes might impair their capacity to prevent PEC-activation and glomerulosclerosis.

COVID-ASSOCIATED NEPHROPATHY (COVAN)

Renal involvement with acute kidney injury (AKI), proteinuria and hematuria, worsening the overall prognosis, has been shown frequently in COVID-19 patients (52–54). Chronic kidney disease or conditions with increased risk of impaired kidney function represent strong risk factors for a severe clinical course (55). Biopsy and autopsy studies revealed acute tubular necrosis (ATN) in the majority of COVID-19 associated AKI. Nevertheless, glomerular involvement was also reported and should be distinguished from the majority of AKI due to ATN. Beside reports of minimal change disease, membranous nephropathy, anti-glomerular basement membrane glomerulonephritis, IgA-vasculitis, lupus nephritis and crescentic glomerulonephritis (56–58) associated with SARS-CoV-2,

cases of cFSGS displaying similar lesions, observed in HIVAN, have led to the emergence of the SARS-CoV-2 associated entity named "COVAN" (59). COVAN patients mostly present with severe AKI and nephrotic range proteinuria in native kidneys and kidney allografts (60–62).

Like HIVAN, COVAN is mostly reported in patients carrying risk variants of APOL1 supporting the hypothesis of a "second hit" necessary for the onset of APOL1-associated kidney disease. Paying tribute to the short time period of research on COVAN, compared to decades of HIV-research, only few mechanistic data are available for SARS-CoV-2 induced cFSGS. Interestingly, although intracellular and not secreted APOL1 seems to be responsible for kidney injury, a case of SARS-CoV-2 associated cFSGS was reported in a patient carrying 2 APOL1 high risk alleles with a kidney allograft stemming from a low-risk genotype donor (62).

Cytokine induced podocyte damage by the virus and/or direct toxic viral effects on podocytes are suggested to be responsible for SARS-CoV-2 associated cFSGS and might interact with APOL1. The membrane proteins angiotensin converting enzyme II (ACE2) and transmembrane serin protease 2 (TMPRSS2) are used as receptors by SARS-CoV-2 to facilitate cell entry. ACE2 is highly expressed in kidney cells, mainly in the proximal tubule. However, podocytes, parietal epithelial cells, mesangial cells and cells of the collecting duct are found to express ACE2 at lower levels (63). Autopsy studies could detect SARS-CoV-2 RNA and viral proteins in the kidney (64, 65) and indicated, that SARS-CoV-2 renal tropism is associated with the development of acute kidney injury and disease severity (66). In contrast, cases of cFSGS occurred also in patients with mild or even absent respiratory symptoms (67). To our knowledge, all biopsy studies failed to detect SARS-CoV-2 in the kidney. This limits investigating direct toxic viral effects on podocytes and implicates cytokine-mediated effects as shown for HIVAN. Nevertheless, most studies relied on electron microscopy, immunohistochemistry or RNA *in situ* hybridization limiting the conclusions drawn concerning viral detection. PCR based detection methods were regularly able to detect viral RNA within kidney tissue and complimentary RNA and protein detection underline a probable renal SARS-CoV-2 tropism (64, 65, 68–70).

Next to the direct infection of kidney tissue, an injury of the glomerulus induced by the host response might be another plausible mechanism of the development of cFSGS in COVID-19. The often described "cytokine storm" can damage the kidney directly or secondary through the induction of life-threatening circumstances like sepsis, shock, ischemia, hypoxia or rhabdomyolysis (71). Especially patients with severe disease show high plasma levels of cytokines like interleukins, granulocyte cell stimulating factor and tumor necrosis factor α (TNFα) (72). Interferon γ (INF γ) is also upregulated in SARS-CoV-2 infected patients, but a meta-analysis could not show significant differences between the severe and non-severe group of COVID-19 patients (73). Nevertheless, data from subgroups carrying APOL1 risk alleles are not available to our knowledge. A recent study observed that APOL1 risk variants are associated with a higher incidence of sepsis and increased disease severity in patients with COVID-19. Plasma levels of APOL1 were higher in patients with severe sepsis and COVID-19 and correlated with markers of endothelial dysfunction. A mouse model with endothelial cell specific expression of APOL1 risk alleles, developed increased endothelial inflammation, vascular leakage, albuminuria and increased sepsis severity. APOL1 risk variant expression in endothelial cells *in vitro* resulted in mitophagy and leakage of mitochondrial DNA into cytoplasm. Cytosolic DNA is sensed by the NLRP3 inflammasome and by cGAS, an activator of STING, leading to endothelial dysfunction (42). The onset and role of endothelial dysfunction in cFSGS is poorly understood. Although it is commonly assumed that podocyte injury is the first event in the pathogenesis of cFSGS, it was demonstrated, that in Adriamycin-induced nephropathy, endothelial damage occurs prior to podocyte injury (74). Additionally, it was shown that patients with primary FSGS and nephrotic range proteinuria had elevated markers of endothelial dysfunction compared to healthy controls, which were largely related to the activity of the disease (75).

Beyond these recent observations, upregulated cytokines in COVID-19 patients carrying risk alleles of APOL1 could still contribute to the development of cFSGS as a "second hit" as the expression of chemokines (e.g., CCL2 and CXCL10) and Interleukin 6 seems to be elevated within the kidney of COVID 19 patients (76).

It remains elusive, whether podocytes or endothelial cells predominantly orchestrate the histopatholgical changes. All known mechanisms, which lead to kidney damage beyond cFSGS in case of infection with SARS-CoV-2 have been extensively reviewed by Ahmadian et al. (77).

cFSGS and Other Viral Infections

While the association of cFSGS to an infection with HIV is well established and probable for SARS-CoV 2, cases associated with other viral infections are rare and the mechanisms remain incompletely understood due to the limited number of cases. Infections with Parvovirus B19, the cytomegalovirus (CMV), Hepatitis C, simian virus 40 or Epstein–Barr virus are thought to be further potential causes of the development of cFSGS (78). While the CMV seems to be a probable inducer of cFSGS, the association of other viruses and the collapsing variant has still to be proven. Additionally, in 2018 there has been a case series reported the association of dengue virus and zika virus infection and the development of cFSGS (79). In these cases a direct infection of kidney tissue was shown and interestingly, there was no correlation with APOL1 risk alleles. This case study also points to a potential involvement of the complement cascade in the development of the cFSGS. Next to the direct effect of the virus to the podocytes, which has not been shown for these infections, systemic immune response, similar to the response to HIV or SARS-CoV2, might be responsible for upregulation of APOL1 or could directly influence podocyte function (80). A converging mechanism of the innate immune response therefore seems likely, nevertheless has to be further assessed. A summary of these mechanisms is presented in **Figure 1**.

CONCLUSION

Research on molecular changes in podocytes expressing risk variants of APOL1 shed light on multiple pathways relevant for

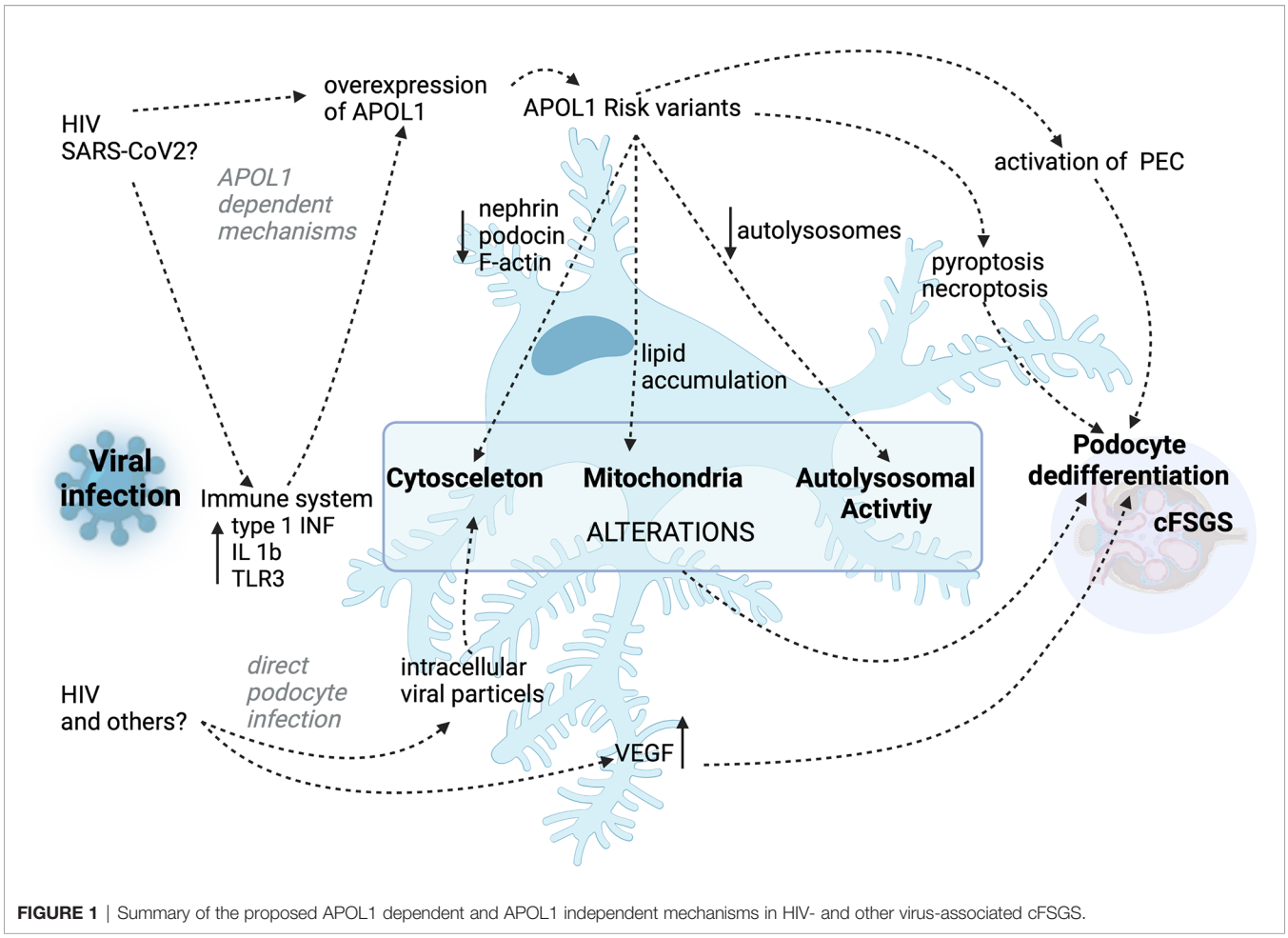

FIGURE 1 | Summary of the proposed APOL1 dependent and APOL1 independent mechanisms in HIV- and other virus-associated cFSGS.

podocyte homeostasis. Although none of the suggested pathways alone could explain the development of cFSGS, the role of a second is evident, at least in APOL1 dependent forms. Limited numbers of patients in case reports hamper the identification of "second hits" like HIV, so the pandemic situation of COVID-19 could help to further scientific effort by providing larger cohorts. Furthermore, the analysis of APOL1-risk factor independent cFSGS forms, the identification of viral etiology and associated damaging mechanisms are crucial to understand the development of the disease. Further investigation of the interaction of viral products and the immune response with podocyte signaling pathways that induce the massive morphologic alterations might contribute to our understanding of podocyte biology and the search for targeted therapies in (collapsing) FSGS independent of its etiology.

AUTHOR CONTRIBUTIONS

AM and SG have performed intensive studies of the current literature and have written main parts of the text and figure. Both first authors contributed equally to this review. TH and FB have performed extensive correction of the text after studying the literature. TH and FB have contributed significantly with the ideas and structure that are found in the review. All authors contributed to the article and approved the submitted version.

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B-Cell Dysregulation in Idiopathic Nephrotic Syndrome: What We Know and What We Need to Discover

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The therapeutic efficacy of B-cell depletion by anti-CD20 treatment in pediatric and, more recently, in adult idiopathic nephrotic syndrome patients suggests a key role of B cells in the pathogenesis of the disease. However, their exact role is still unclear. B cells are able to secrete a large variety of antibodies that can protect against infections. However, B-cell dysregulation is well-established in a variety of autoimmune diseases. In parallel with their ability to produce antibodies, pathogenic B cells display altered effector functions by expressing activating surface molecules, which can strongly modify the immune homeostasis, or by producing specific cytokines, which can directly affect either podocyte structure and functions or modulate T-cell homeostasis. Herein, we report the most relevant clinical and experimental evidences of a pathogenic role of B cells in idiopathic nephrotic syndrome. We further highlight similarities and differences between children and adults affected by non-genetic forms of the disease and discuss what needs to be investigated in order to define the exact mechanisms underlying the pathogenic role of B cells and to identify more tailored therapeutic approaches.

Keywords: Idiopathic Nephrotic syndrome (INS), minimal change disease (MCD), focal segmental glomerulosclerosis (FSGS), B cells, antibodies, cytokines

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INTRODUCTION

Idiopathic nephrotic syndrome (INS), which includes two main histological entities, minimal change disease (MCD) and primary focal segmental glomerulosclerosis (FSGS), is the most frequent glomerular disease in childhood, with an incidence of 1.2-16.9/100,000 children (1). It is characterized by an intense proteinuria associated with hypoalbuminemia and edema due to an alteration of the glomerular permeability barrier leading to podocyte foot-process effacement (1). The response to a standardized steroid-treatment is more predictive of the disease course than histological findings in children, and kidney biopsy is usually not performed in steroid-sensitive INS (SSNS) pediatric patients (1). However, when biopsy is performed, most children present MCD (2).

In contrast, kidney biopsy, which is routinely performed at onset of nephrotic syndrome in adults, shows a very low incidence of INS (0.6–1.8 per 100,000 adults), with the majority of patients presenting other glomerular diseases, mainly membranous nephropathy (2). In adult patients, MCD is less frequent than FSGS (3). Similarly to children, steroid treatment is the first-line therapeutic approach also for adults. However, a more prolonged treatment is necessary to induce remission compared to pediatric forms (2, 4). The pathogenesis of INS is still not well elucidated, but the therapeutic approaches based on immunosuppressive agents largely support a key role of the immune system in steroid-sensitive forms of the disease (5). In these forms, the disruption of the glomerular barrier leading to podocyte cytoskeleton disorganization and subsequent proteinuria seems to be mediated by one or more circulating factors, derived from dysregulated immune cells (6). Nonetheless, until now, the identity of this (these) factor(s) remain(s) undetermined (5, 6). Moreover, both in MCD and in FSGS the renal biopsy shows little or no evidence of glomerular inflammation and of immunoglobulin or complement deposition. For more than 30 years, INS was considered as a T-cell mediated disease (7), and several preclinical and clinical studies largely supported this hypothesis (5). More recently, a potential role of B cells has emerged due to the therapeutic efficacy of the B-cell depleting anti-CD20 antibodies (rituximab and ofatumumab) in inducing and/or maintaining a prolonged remission in children with INS (8–11). Similar results from non-prospective randomized clinical trials suggest that B-cells depleting agents may be useful in adult patients with steroid dependent or frequently relapsing INS to reduce the relapse frequency and to maintain remission despite cessation or tapering of steroids and/or immunosuppressive drugs (3). In addition, previous findings show that INS may occur in association with non-Hodgkin lymphoproliferative disorders or Hodgkin lymphoma where tumor cells derive from mature B cells (12–14) and with Epstein Barr virus infection, which mainly targets B cells (15). Altogether, these findings provide additional evidence implicating B lymphocytes as a key immune cell type involved in INS pathogenesis.

In this review, we describe phenotype and function of B cells and report the most relevant clinical and experimental indications of their potential role in the pathogenesis of INS. Current knowledge regarding potential effects exerted by B lymphocytes in INS pathogenesis is summarized in **Figure 1**.

B-CELL PHENOTYPE AND FUNCTION

B cells are a heterogeneous population composed by different subsets and the physiological distribution of these subpopulations is age-dependent (16). During infancy, the peripheral B-cell pool is predominantly characterized by transitional B cells, which migrate from the bone marrow to the periphery where they acquire a naïve/mature phenotype. Through the circulation into secondary lymphoid organs, they encounter antigens that can induce their activation and differentiation into plasmablasts, which are the precursors of antibody secreting plasma cells, or memory B cells, which can produce IgM or undergo toward an isotype switching to IgG-, IgA-, or IgE-secreting B cells. Circulating plasmablasts and plasma cells generally die after few days, but some long-lived plasma cells return to the bone marrow and maintain antibody production independently of antigen exposure. From infancy to adulthood, the circulating levels of transitional, mature-naïve, memory B cells and plasmablasts/plasma cells progressively decrease, reaching a relatively stable number in adulthood (16).

Beside their ability in producing a large variety of antibodies that can protect from infections - the so called "humoral immunity" - B cells can also modify the homeostasis of specific tissues by producing immunoregulatory or pro-inflammatory cytokines and by expressing surface molecules able to present antigens which can in turn activate circulating T cells (17, 18). In particular, regulatory B cells produce immunomodulatory cytokines such as IL-10 and IL-35, which can dampen immune responses in pathological and transplant settings (18, 19). In parallel, B cells are able to directly or indirectly promote the activation of effector T cells by secreting pro-inflammatory cytokines such as IFN-gamma, TNF-alpha, IL-6 and IL-17 (20). During activation, B cells also express molecules such as HLA class II, CD40, CD80 and CD86, which stabilize the interactions between B and T cells and can sustain T-cell activation in autoimmune and auto-inflammatory diseases (17, 18, 21).

PATHOGENIC ANTIBODIES IN INS

In contrast to the vast majority of the glomerular diseases, INS is usually not considered an antibody-mediated disease due to the lack of immunoglobulin deposits in kidney biopsies (22). Of note, glomerular IgM deposition, which can be observed in some forms of FSGS, seems to derive from a binding of IgM (together with activated complement factors) to epitopes that are exposed after injury of glomerular cells, suggesting that IgM may contribute to disease progression more than representing the causative factor (23, 24). However, a pathogenic role of circulating antibodies in INS has been hypothesized since 1998, when Dantal et al. showed that a permeability factor inducing albuminuria could be or could be bind to an immunoglobulin (25). In addition, an imbalance between serum IgG and serum IgM that sometimes persists during remission of the disease has been reported, suggesting a dysregulated immunoglobulin metabolism in INS (26, 27). More recently, potentially pathogenic IgG directed against surface podocyte proteins have been described in adult and pediatric INS (28–30). In particular, increased serum levels of anti-CD40 IgG were observed in adults with recurrent FSGS. These antibodies were able to cause a disruption of actin cytoskeleton of *in vitro* cultured podocytes and to induce proteinuria (in the presence of recombinant soluble urokinase plasminogen activator receptor) in injected mice (28). Unfortunately, the presence of glomerular IgG deposition following anti-CD40 injection was not evaluated in this study. In 2018, Jamin et colleagues reported increased serum

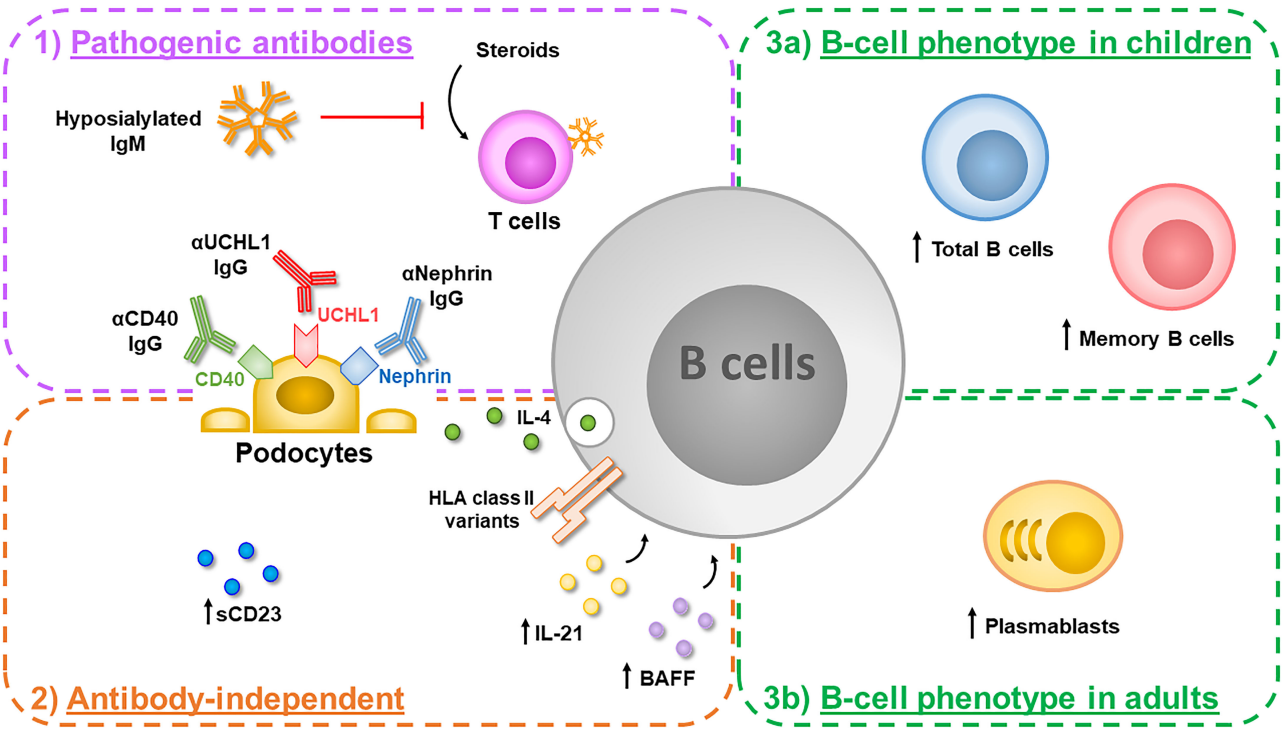

FIGURE 1 | B cells can play a key role in the pathogenesis of idiopathic nephrotic syndrome (INS) thorough different mechanisms. 1) They can produce several potentially pathogenic antibodies targeting proteins constitutively expressed by podocytes such as anti-CD40 IgG, found in adults with recurrent focal segmental glomerulosclerosis (FSGS), anti-UCHL1 IgG, found in children with steroid-sensitive nephrotic syndrome (SSNS) and anti-nephrin IgG, found both in children and adults with minimal change disease (MCD). In addition, B cells produce hyposialylated IgM directed against T cells, which impair T-cell responses to steroid inhibition, in a subgroup of children with SSNS. 2) Activated B cells can also exert antibody-independent detrimental effects: an increased amount of serum CD23, a marker of B-cell activation, has been reported in pediatric SSNS and increased levels of serum B-cell activating factor (BAFF) and IL-21, which can contribute to B-cell activation, have been observed in adult MCD patients. In an experimental mouse model, IL-4 secreting B cells activated locally in the glomerulus induce podocyte effacement and proteinuria. In addition, specific risk alleles in the HLA region, which plays a crucial role in the antigen presentation to T cells, have been described in children with SSNS. 3) Alterations in B-cell homeostasis have been also observed during the active phase of the disease, with increased circulating levels of total B cells and memory B cells in children with SSNS (3a), and increased circulating levels of plasmablasts in adults with MCD (3b).

levels of anti-UCHL1 IgG in children with SSNS (29). Anti-UCHL1 antibodies were able to cause a detachment of *in vitro* cultured podocytes and to induce an intense proteinuria when injected into mice (29). Interestingly, a pathological finding of MCD without glomerular IgG deposits was observed in injected mice and authors hypothesized that the binding of anti-UCHL1 IgG to surface UCHL1 could lead to podocyte detachment, explaining the absence of IgG deposits in INS kidney biopsies (29). Additional studies are needed to verify this intriguing hypothesis and to validate the real pathogenic role of the described anti-podocyte IgG. A more recent study showed that autoantibodies targeting nephrin, the major transmembrane protein of the podocyte slit diaphragm that links the interdigitating foot processes from neighboring podocytes, may be found in 29% of patients (both in children and adults) with biopsy-proven MCD (30). Since IgG deposits in kidney biopsy is an ultra-rare finding in kidney biopsy from patients with INS, the accurate role of these potential pathogenic antibodies remains to be determined. Nevertheless, this finding, highlighting that mechanisms underlying INS pathogenesis may involve autoimmune processes, deeply modifies our knowledge about this glomerular disease. Additional studies investigating the disrupting ability of the identified INS-associated autoantibodies, purified by sera or supernatants of *ex-vivo* isolated B-cell subsets, could be performed using the novel "glomerulus-on-a-chip" technology (31). This artificial platform mimics the glomerular permeability barrier composed of human podocytes, glomerular endothelial cells and extracellular matrix resembling the glomerular basement membrane (31).

In contrast to antibodies directed against podocytes, B cells could also exert detrimental effects by producing antibodies that can indirectly alter the immune homeostasis of INS patients. In this regard, a recent study reports the production of hyposialylated IgM directed against T cells, which inversely correlates with the extent of the response to steroid treatment, in a subgroup of SSNS pediatric patients (32). This study demonstrates that sialylated IgM targeting T cells are rapidly internalized and inhibit T-cell activation *in vitro*. In contrast, hyposialylated IgM, which can also target T cells, remain on the

cell surface after binding but fail to exert inhibitory effects. More importantly, the binding of hyposialylated IgM renders T cells unresponsive to steroid inhibition and favors the secretion of podocyte damaging factors from activated T cells (32). These observations suggest that hyposialylated IgM could sustain T-cell activation induced by external triggers such as infections or atopy and could reduce T-cell response to steroid inhibition. Additional studies are needed to define the mechanism responsible of the production of hyposialylated IgM and to investigate whether immunosuppressive agents other than steroids can revert this phenomenon.

ANTIBODY-INDEPENDENT ROLE OF B CELLS IN INS

The potential role of T-cell derived cytokines in the pathogenesis of INS has been largely investigated, with contrasting results (6, 33). Among them, an increased production of IL-13 and TNF-alpha seems to be the most relevant and reproducible in INS (6, 33). In contrast, the role of B-cell derived cytokines has been poorly investigated. Of note, B cells are able to secrete both IL-13 and TNF-alpha, as well as other potentially pathogenic cytokines such as IL-4, IFN-gamma, IL-6 and IL-17 (20, 34). IL-13 and IL-4 cytokines are usually related to atopy, a condition that is widely associated with first or recurrent episodes of NS (35). In addition, an increased production of IL-4 from mononuclear cells isolated from MCD patients was associated to an increased expression of the B-cell surface CD23, a marker of B-cell activation, in the same patients (36). In 2003, increased levels of soluble CD23, in parallel with increased levels of soluble CD25 (a marker of T-cell activation), were also observed in SSNS pediatric patients in relapse, suggesting a potential role of B cells in sustaining T-cell stimulation (37). However, contrasting results on the amount of serum IL-4 in SSNS patients were reported during years (33). Of note, more than a circulating production of specific cytokines, a local secretion at glomerular level could also be pathogenically relevant in INS, since glomerular infiltration of T and B cells has been reported, especially in FSGS (38). In accordance with this hypothesis, Kim et colleagues elegantly demonstrated that IL-4-secreting B cells activated locally in the glomerulus can induce podocyte effacement and proteinuria in an experimental mouse model (39). The detrimental effects exerted by IL-4 on cultured human podocytes are currently being investigated in order to define its involvement in the pathogenesis of INS (40). Since B cells can secrete several immunoregulatory or pro-inflammatory cytokines, a local production of other B-cell derived cytokines could exert comparable or additional pathogenic effects. A recent report describes how IL-6 can alter the integrity of the glomerular permeability barrier and can derange actin cytoskeleton of *in vitro* treated podocytes, which constitutively express IL-6 receptor, suggesting a potential pathogenic role also for this cytokine (41).

In parallel with the production of specific cytokines, activated B cells could also directly contribute to T-cell stimulation by expressing surface activating molecules. SSNS is a heterogeneous disorder and the different forms of the disease are likely to be mediated by a complex interplay between the environment, the glomerular permeability barrier and the immune system (5). Despite the lack of a monogenic cause of the disease, genetic variants predisposing to develop SSNS following environmental triggers are emerging, as recently reviewed (42). Among all the identified SSNS-associated genetic variants, the strongest association was found in the HLA region, as identified by exome array and transethnic genome-wide association studies in large pediatric cohorts (43–46), supporting the role of an immune dysregulation in the antigen presentation machinery in SSNS forms (44). In agreement with this hypothesis, a recent study showed that INS relapses were associated with a decrease in T regulatory cells and IL-2 expression whereas remission phases under rituximab therapy were associated with a significant decrease in invariant natural killer T cells and $CD4^-$ $CD8^-$ T-cells expressing the invariant Vα24 chain T-cells. These observations suggest that B-cell depleting agents may interfere in T cell-B cell cooperation during the course of the disease (47).

Additional studies should be performed to investigate the B-cell specific expression of activated molecules and their direct interaction with T cells in INS patients.

B-CELL PHENOTYPE IN INS

In 2004, the casual observation of a sustained NS remission in a boy treated with rituximab for his recurrent idiopathic thrombocytopenic purpura proves for the first time the pathogenic role of B cells in INS (8). The successful use of B-cell depleting anti-CD20 monoclonal antibodies in maintaining long-lasting remission and allowing tapering of concomitant immunosuppressive treatment in both pediatric and adults INS patients has subsequently supported the hypothesis of a key role of B lymphocytes in the pathogenesis of INS (3, 9, 10). Interestingly, other immunosuppressive drugs frequently used for the treatment of INS, such as mycophenolate mofetil and calcineurin inhibitors, can target T cells but are also effective in inhibiting B-cell proliferation and immunoglobulin production, and can contribute to maintain remission following anti-CD20 treatment (5, 15, 48, 49). The amount of circulating B cells has been largely investigated in children with INS. **Table 1** summarizes the most relevant studies describing the B-cell phenotype in INS patients. High levels of B cells were already described in SSNS pediatric patients in 2002 (50). However, several subsequent studies reported conflicting results (51–53). Some of the observed discrepancies could be related to the immunosuppression received by most of the described patients before sampling, since the number of total B cells is determined by the proportion of different B-cell subsets that have differential sensitivities to immunosuppressive agents such as steroids, mycophenolate mofetil and calcineurin inhibitors (48). Prednisone treatment, for example, can exert long-lasting effects on different B-cell subsets also several months after interruption, as recently reported in children with SSNS (57). To avoid these confounding effects, the entire circulating B-cell

TABLE 1 | B-cell phenotype in idiopathic nephrotic syndrome patients.

	Type	Status	Treatment	B-cell subsets				
				Total CD19$^+$	Transitional	Mature	Memory	Plasmablasts
Children								
Lama et al. (50)	SSNS	Relapse	Off therapy	↑	ND	ND	ND	ND
		Remission	Off therapy	↑	ND	ND	ND	ND
	SRNS	Relapse	Off therapy	↑	ND	ND	ND	ND
		Remission	Off therapy	↑	ND	ND	ND	ND
Kemper et al. (51)	SSNS	Relapse	Off therapy	↓	ND	ND	ND	ND
			On PDN	↑	ND	ND	ND	ND
		Remission	Off therapy	↓	ND	ND	ND	ND
			On PDN	↑	ND	ND	ND	ND
Lapillonne et al. (52)	SSNS	Relapse	Off therapy	=	ND	ND	ND	ND
		Remission	Off therapy	↓	ND	ND	ND	ND
Printza et al. (53)	SSNS	Onset	Untreated	↑	ND	ND	ND	ND
		Remission	On PDN	=	ND	ND	ND	ND
			After PDN discontinuation	=	ND	ND	ND	ND
Colucci et al. (54)	SSNS	Onset	Untreated	↑	↑	=	↑	ND
		Relapse	On PDN ± MMF ± CNIs	=	=	↓	↑	ND
		Remission	On PDN ± MMF ± CNIs	↓	↓	↓	=	ND
	Genetic SRNS	–	Untreated or on PDN or CNIs	=	=	=	=	ND
Ling et al. (55)	SSNS	Onset	Untreated	↑	↑	↓	↑	ND
		Relapse	On PDN	↑	=	↓	↑	ND
		Remission	On PDN	=	=	=	↑	ND
	SRNS	Onset	Untreated	=	=	↓	↑	ND
Ling et al. (56)	SSNS	Relapsing vs Non-relapsing	Untreated (onset) or on PDN (relapse and remission)	=*	↓*	=*	↑*	ND
Ling et al. (57)	SSNS	Onset	Untreated	↑	↑	=	=	ND
		Remission	On PDN	=	↓	=	↑	ND
			After PDN discontinuation	↓	↓	=	=	ND
Zotta et al. (58)	SSNS	Onset	Untreated	ND	ND	ND	ND	=
		Relapse	On PDN ± MMF ± CNIs	ND	ND	ND	ND	=
		Remission	On PDN ± MMF ± CNIs	ND	ND	ND	ND	=
Adults								
Oniszczuk et al. (59)	MCD	Relapse	Off therapy	=	=	=	=	↑
		Remission	Off therapy	=	=	=	=	=

SSNS, steroid-sensitive nephrotic syndrome; SRNS, steroid-resistant nephrotic syndrome; MCD, Minimal change disease.

Off therapy, patients without treatment for at least 6 months; untreated, patients at onset of the disease before starting prednisone treatment; PDN, prednisone; MMF, mycophenolate mofetil; CNIs, calcineurin inhibitors.

↑, increased values compared to age-matched healthy controls; ↓, reduced values compared to age-matched healthy controls; =, values comparable to those of age-matched healthy controls; ND, not determined.

**, Values compared between relapsing and non-relapsing patients.*

repertoire has been characterized in a large cohort of SSNS pediatric patients at onset of disease before starting any immunosuppressive drug by Colucci et al. (54, 58). Increased levels of transitional and memory B cells, which together contributed to an increased amount of total B cells, were found. However, when SSNS patients treated with prednisone and one or two steroid-sparing agents were analyzed, transitional and mature/naïve B cells were mostly affected by immunosuppression, both in relapse and in remission, whilst memory B cells were found to be less sensitive to treatment (54). Similar results were also obtained by Ling and colleagues, who have recently demonstrated that increased levels of total, transitional and memory B cells can help in discriminating SSNS children from SRNS patients and that a reduced transitional/memory B-cell ratio is significantly predictive of SSNS recurrence (55, 56). Altogether, these results suggest that, among all the B-cell subpopulations, memory B cells play a key role in the pathogenesis of pediatric SSNS. Accordingly, the reappearance of memory B cells is highly effective in predicting relapse following anti-CD20 treatment in SSNS children (60–62).

In contrast to children, data regarding the distribution of circulating B cells in adult patients with INS at disease onset or during B-cell reconstitution after administration of anti-CD20 monoclonal antibodies are scarce. To our knowledge, only one study by Oniszczuk et al. showed that, among all the analyzed circulating B-cell subpopulations, plasmablasts were the only subset present at significantly higher levels during MCD relapses from untreated adult patients in comparison with patients in remission and with healthy controls (59). Of note, plasmablasts were also significantly increased in patients with biopsy-proven

membranous nephropathy (an antibody-mediated glomerular disease characterized by an intense proteinuria, similar to that observed in MCD patients) (59). In this study, plasmablast levels during relapse were correlated with both lower albumin levels and higher proteinuria levels and were significantly associated to higher IgM levels and decreased IgG levels. In addition, an increased production of IL-21, IL-6 and B-cell activating factor (BAFF) was found in MCD patients with nephrotic-range proteinuria. Interestingly, the expansion of the plasmablast population may be partially explained by an increase of BAFF production, providing new evidence for the therapeutic use of anti-BAFF therapy (59). Of note, evaluation of plasmablast levels in children yielded conflicting results. Yang et al. reported higher levels of circulating plasmablasts in 94 children with new onset untreated INS patients, including both steroid-sensitive and steroid-resistant patients, compared to healthy controls (63). However, this increase in circulating plasmablasts was not confirmed in a subsequent study including only children with SSNS (58).

Altogether, these data suggest that alterations of the B-cell homeostasis observed in pediatric and adult INS patients are quite dissimilar, possibly due to the different maturity of the immune system between adults and children (16). This could also in part explain the distinct timing of response to steroid treatment in pediatric and adult patients with INS (2, 4), since prednisone differentially targets each B-cell subset (48).

Additional studies are needed to define the key pathogenic B-cell subset(s) in INS. A French multicenter trial is ongoing to test the efficacy of rituximab to maintain remission of a new-onset MCD episode in 98 adult patients who will either receive two doses of rituximab (375mg/m^2 separated by 1 week) or a progressive tapering of prednisone doses ('RIFIREINS', NCT03970577, Rituximab From the FIRst Episode of Idiopathic Nephrotic Syndrome). The main objective of this study will be to demonstrate that the use of rituximab from the initial episode of MCD in adults may significantly reduce the risk of subsequent relapses and limit prolonged exposure to steroids without serious adverse events. Enrolled patients will be rigorously classified as steroid-sensitive, steroid-dependent or steroid-resistant INS based on their response to a standardized induction therapy with prednisone. In addition, this prospective trial will include an extensive B and T lymphocyte subpopulation monitoring, in order to investigate the potential close relationship between lymphocyte subpopulations and treatment response at different crucial timepoints and in different forms of the disease. The obtained results will help to improve our understanding of the role of lymphocyte subsets in the pathogenesis of INS in adulthood.

CONCLUSION

The accurate molecular mechanisms underlying INS pathogenesis remain to be determined, but compelling data suggest that INS results from immune disorders leading to the release of circulating glomerular permeability factors, possibly T and/or B cell-derived, which in turn alter the glomerular filtration barrier and promote podocyte damages. The recent successful use of anti-CD20 monoclonal antibodies as supportive treatment of some forms of INS in children and adult patients provide additional evidence of a potential key role of B lymphocytes in the pathophysiological processes involved in this quite mysterious glomerular disease. Further studies are needed to elucidate the precise mechanisms by which and which B lymphocytes subpopulation could target the glomerular filtration barrier.

AUTHOR CONTRIBUTIONS

Conception and design: MC, JO, MV, and VA. Analysis of the data and preparation of the figure: MC and JO. Drafting and revision of the manuscript: MC, JO, MV, and VA. All authors contributed to the article and approved the submitted version.

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Strategies Towards Antigen-Specific Treatments for Membranous Nephropathy

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Membranous nephropathy (MN) is a rare but potentially severe autoimmune disease and a major cause of nephrotic syndrome in adults. Traditional treatments for patients with MN include steroids with alkylating agents such as cyclophosphamide or calcineurin inhibitors such as cyclosporine, which have an undesirable side effect profile. Newer therapies like rituximab, although superior to cyclosporine in maintaining disease remission, do not only affect pathogenic B or plasma cells, but also inhibit the production of protective antibodies and therefore the ability to fend off foreign organisms and to respond to vaccination. These are undesired effects of general B or plasma cell-targeted treatments. The discovery of several autoantigens in patients with MN offers the great opportunity for more specific treatment approaches. Indeed, such treatments were recently developed for other autoimmune diseases and tested in different preclinical models, and some are about to jump to clinical practice. As such treatments have enormous potential to enhance specificity, efficacy and compatibility also for MN, we will discuss two promising strategies in this perspective: The elimination of pathogenic antibodies through endogenous degradation systems and the depletion of pathogenic B cells through chimeric autoantibody receptor T cells.

Keywords: membranous nephropathy, antigen-specific antibodies, B cells, chimeric autoantibody receptor, sweeping antibody, autoantibodies

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INTRODUCTION

Membranous nephropathy (MN) is a rare but potentially severe kidney disease and a major cause of nephrotic syndrome in adults. According to the new KDIGO 2021 Clinical Practice Guidelines, a nephrotic syndrome is defined as proteinuria of more than 3.5 grams per 24 hours or a protein-to-creatinine ratio of more than 3 g/g in combination with low plasma albumin, peripheral edema, and hyperlipidemia (1). The descriptive term "membranous" refers to the prominent change that is classically seen in light microscopy: a diffuse thickening of the glomerular basement membrane (2). Additionally, granular depositions of immunoglobulins and complement components can be detected by immunofluorescence microscopy, suggesting a role of both autoantibodies and the complement system in the pathogenesis of MN. The hallmark findings in electron microscopy include electron-dense deposits in a subepithelial localization, i.e. on the outer aspect of the glomerular basement membrane (GBM), and an extensive effacement of podocyte foot processes.

Due to the prominent glomerular IgG positivity in biopsies of affected patients, MN has long been assumed to be an antibody-mediated autoimmune disease. The discoveries of several target antigens for circulating autoantibodies in MN patients in the past decade has corroborated this assumption. These targets include neutral endopeptidase (NEP) (3), M-type phospholipase A2 receptor (PLA2R1) (4), thrombospondin type-1 domain-containing protein 7A (THSD7A) (5), neural epidermal growth factor-like 1 protein (NELL-1) (6), semaphorin 3B (SEMA3B) (7), protocadherin 7 (PCDH7) (8), NCAM1 (9) and HTRA1 (10). PLA2R1-associated MN is diagnosed in around 70% of cases and thus represents the most common MN sub-entity.

The current view on the pathogenesis of MN (**Figure 1**) is that predisposing factors, such as an underlying genetic disposition and/or immune dysregulation, in combination with an initiating trigger such as an infection, a malignancy or environmental factors lead to loss of tolerance for the respective autoantigen with consecutive activation of B cells and production of autoantibodies (11–18). B cells differentiate towards plasma and/or memory B cells, which in turn produce large amounts of autoantibodies. These autoantibodies reach the kidney *via* the circulation and bind to their target antigen, which is assumed to induce damage to podocytes with subsequent loss of plasma proteins to the urine, likely *via* complement-dependent and complement-independent mechanisms (19). Complement-independent mechanisms have been ill-defined so far, but may include modification of cellular signaling, antigen/receptor blocking or stimulation or interference with the antigens' biological function, e.g. enzymatic activity. Importantly, the direct pathogenicity of autoantibodies has been demonstrated by transfer experiments of patient-derived anti-NEP and anti-THSD7A autoantibodies, which cause MN in animals (3, 20). Even though such a mechanism has not been demonstrated for anti-PLA2R1 autoantibodies, the association of high autoantibody levels with an unfavorable clinical outcome in patients with PLA2R1-associated MN and the development of MN transgenic mice expressing the murine PLA2R1 after transfer of rabbit anti-PLA2R1 antibodies strongly argue for a pathogenic role of anti-PLA2R1 autoantibodies (21, 22). Alternative proposed mechanisms of immune deposit formation in MN include glomerular deposition of preformed immune complexes and binding of circulating antibodies to a planted antigen (23, 24), but the relevance of these mechanisms is less clear.

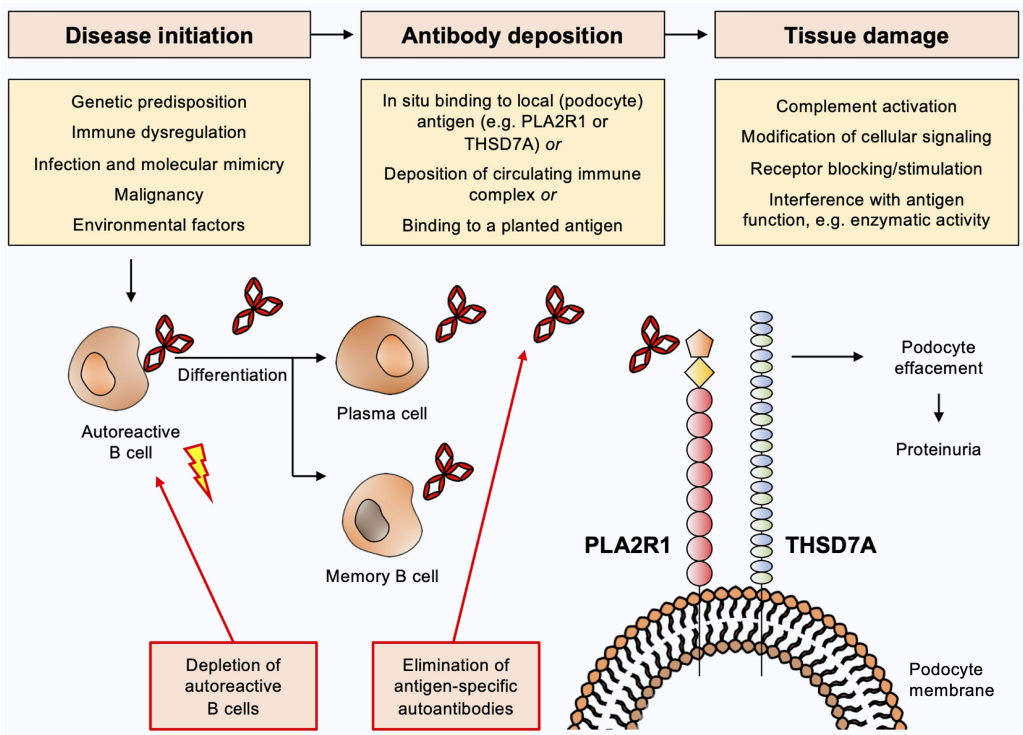

FIGURE 1 | Current view on the pathogenesis of membranous nephropathy and potential targets for antigen-specific treatments. Predisposing factors such as underlying genetic dispositions and/or immune dysregulation in combination with an initiating trigger such as an infection, a malignancy or environmental factors may lead to loss of tolerance for the respective autoantigen with consecutive activation of B cells and production of autoantibodies. B cells differentiate to plasma and/or memory B cells, which in turn produce large amounts of autoantibodies. These autoantibodies reach the kidney *via* the circulation and bind to their target antigen (e.g. PLA2R1 or THSD7A), which induces damage to podocytes with subsequent urinary loss of plasma proteins. Complement-dependent and complement-independent injury mechanisms are likely to be involved. Depletion of autoantibody-producing B cells or elimination of pathogenic autoantibodies constitute potential antigen-specific treatments for MN.

CURRENT TREATMENTS FOR MN

The clinical outcome in patients with MN is highly variable with about one-third of patients experiencing spontaneous remission within one year after diagnosis, whereas another 20-30% develop end-stage renal disease within 10 years (25). The identification of patients with an unfavorable outcome, which would benefit from early immunosuppressive treatment, represents a major clinical challenge. Classical treatment regimens for MN include the use of steroids in combination with alkylating agents or calcineurin inhibitors (26–29). Recently, two large prospective clinical trials have investigated the use of rituximab in the treatment for MN. The MENTOR trial compared rituximab with cyclosporine and found rituximab to be superior to cyclosporine in maintaining remission for up to 24 months (30). The RI-CYCLO trial found comparable remission rates with the use of rituximab and cyclophosphamide and no significant differences in the safety profiles of these medications (31).

The novel KDIGO guidelines define clusters of patients according to the risk of disease progression and loss of renal function. Categories comprise low risk, moderate risk, high risk, and very high risk, depending on proteinuria, serum albumin, estimated GFR (eGFR), and anti-PLA2R1 antibody levels (1). Patients with a low or moderate risk of disease progression are usually treated with optimal supportive care (e.g. antiproteinuric therapy with renin-angiotensin aldosterone system inhibition (RAAS) and blood pressure control) and monitored for 3-6 months. In case of worsening proteinuria, eGFR or antibody levels, patients should be evaluated for treatment with rituximab or calcineurin inhibitors. Patients with a high risk of disease progression should be treated with rituximab or cyclophosphamide plus steroids or a calcineurin inhibitor plus rituximab and patients with a very high risk should receive cyclophosphamide plus steroids (1).

While calcinerin inhibitors and cyclophosphamide are broad immunosuppressants, rituximab is targeting B cells expressing the surface marker CD20. Initially developed as a lymphoma treatment, it acts through different mechanisms of action: i) Antibody-dependent cellular cytotoxicity (ADCC) through either NK cells, monocytes or granulocytes (32–34) (ii) apoptosis of B cells through caspase 3 activation (35) and iii) complement-dependent cytotoxicity (36, 37).

In summary, the targets of autoimmunity in MN have been increasingly understood and therapies have shifted from broad immunosuppression using alkylating agents and calcineurin inhibitors towards a more pathogenesis-based treatment targeting autoantibody-producing B cells using rituximab. However, rituximab treatment may entail resistance towards apoptosis, ADCC, CDC and downregulation or loss of CD20 (38), which can decrease treatment efficacy. It also affects pre-existing protective antibodies, reduces the body's ability to generate an immune response against foreign organisms, and greatly decreases the response to vaccination. These are undesired effects of B cell–targeted treatments, in particular in the setting of a worldwide pandemic (39), and may limit the use of agents such as rituximab in the treatment of antibody-mediated diseases. Additionally, the increasing knowledge on specific antigens in MN principally enables antigen-specific treatment strategies, which would ideally target the immunological disease mechanisms while sparing protective immunity. Such antigen-specific therapies would have enormous potential to enhance specificity, efficacy and compatibility. Hence, there is a considerable gap between the increasing knowledge on the pathogenic role of autoantibodies and autoantigens in MN on the one side and the currently available treatments with limited specificity on the other side.

In the following, we will discuss two promising strategies that could be applied in the field of MN: The elimination of pathogenic antibodies through endogenous degradation systems and the elimination of autoreactive B cells through chimeric autoantibody receptor T cells (**Figure 1**).

NO SWEEPING UNDER THE RUG: THE SWEEPING ANTIBODY TECHNOLOGY

Physiologically, immunoglobulins (IgG) are constantly taken up by endothelial cells and shuttled to the sorting endosome. At pH 6 inside the sorting endosome IgG binds to the neonatal Fc receptor (FcRn) and is transported to the cell surface. Now at pH 7.4 the IgG is released from the FcRn back into the circulation due to a reduced affinity at neutral pH. This system prolongs the half-life of IgG in the circulation to approximately 30 days (40). Consequently, antibodies may also prolong the half-life of their target antigen by constant recirculation from endothelial cells back to the plasma.

Sweeping antibodies as potential therapeutics to remove soluble antigens from plasma were first described by Igawa et al. (41). Sweeping antibodies are engineered IgG that remove soluble antigens from the circulation, which is enabled by two distinct modifications: a pH-dependent binding to the antigen and an increased affinity of the antibody Fc part to an Fc receptor (FcR) (41). A pH-dependent antigen binding can be achieved by histidine mutagenesis, meaning that amino acids in the Fab region of the antibody are substituted for histidines. In the acidic environment of the sorting endosome, the protonated histidines lead to conformational changes in the Fab region of the sweeping antibody, which results in weakening of the binding to the antigen and finally dissociation of antibody and antigen. Enhanced immune complex uptake into the endosome is achieved by mutations in the Fc region, increasing the binding to either FcRn or FcγRIIB.

After injection, a sweeping antibody binds its target molecule in the circulation. The resulting immune complex is then taken up into endothelial cells by pinocytosis or FcR-mediated endocytosis. In the sorting endosome, the pH shifts from neutral to slightly acidic (pH 6) and the antigen is released from the sweeping antibody. While the sweeping antibody – bound by the FcRn – is shuttled to the cell surface and released there, the antigen is degraded in the lysosome. The enhanced affinity to the FcR allows efficient recirculation of the therapeutic sweeping antibody from endothelial cells to the plasma, which in turn leads to further removal of antigens from the circulation, creating the "sweeping" effect. Several different mutations have

been described that enhance active internalization of antigen-antibody complexes *via* FcRn, leading to efficient degradation of the antigen (42–44).

Sweeping can also occur through the FcγRIIB (45). FcγRIIB is the only inhibitory Fc receptor with an immunoreceptor-based inhibitory motif (ITIM) and normally has a very low affinity for IgG monomers (IgG1<IgG2a=IgG2b<IgG3) (46). FcγRIIB is, for example, expressed on B cells regulating their activation (47), and on myeloid-derived cells modulating endocytosis through clathrin-coated pits (48). Liver sinusoidal endothelial cells (LSECs) are scavenger cells specialized to clear blood from smaller immune complexes through pinocytosis (49). In mice, three quarters of all FcγRIIB are expressed in the liver and 90% of liver FcγRIIB is found on the surface of LSECs (50). Interestingly, Ganesan *et al.* could demonstrate that small immune complexes are cleared *via* FcγRIIB-mediated uptake into the liver (50). Binding to FcγRIIB can be dramatically enhanced by mutating the Fc part of the sweeping antibody, leading to efficient clearing of soluble antigens (51).

Recently, it could be demonstrated that the sweeping antibody principle can also be applied for the elimination of antigen-specific antibodies. Devanaboyina et al. (52) used therapeutic antibodies including fragments of either the myelin oligodendrocyte glycoprotein (MOG), an antigen in multiple sclerosis, or HER2, a tumor protein (52). The therapeutic antibodies specifically bound to anti-MOG and anti-HER2 antibodies, which lead to rapid clearance of the resulting immune complex in mice. In a follow-up study, the authors could demonstrate a therapeutic effect using similar constructs in a mouse model of multiple sclerosis (53).

The increasing knowledge on pathogenic autoantibodies in MN and their target antigens offers excellent conditions to apply the sweeping antibody concept also in this disease. Sweeping antibodies with the Fab region substituted for an antigen fragment could be generated and tested for their capacity to bind and eliminate specific autoantibodies. As virtually 100% of patients with PLA2R1-associated MN have antibodies against the most N-terminal domain (the cysteine-rich domain) of PLA2R1 (54–56), a sweeping antibody containing this part of PLA2R1 may be a promising candidate – yet therapeutic antibodies could be engineered for any MN antigen and tailored to the immune and epitope profile of each individual patient. After administration, the therapeutic sweeping antibodies bind their targets, the pathogenic autoantibodies, in the circulation. The resulting immune complex is then taken up by liver LSECs through enhanced affinity for FcγRIIB or by other endothelial cells *via* FcRn, resulting in lysosomal degradation of the pathogenic autoantibodies and potentially recirculation of the therapeutic sweeping antibody (**Figure 2A**). Fewer autoantibodies could bind their target antigen in the kidney, potentially leading to amelioration of disease activity. This can improve the course of MN without affecting parts of the immune system that are essential for the body's defense e.g. against infection. In summary, the elimination of pathogenic autoantibodies through endogenous degradation systems represents a promising therapeutic strategy for patients with MN.

STRIKING THE EVIL AT ITS ROOTS: CHIMERIC AUTOANTIBODY RECEPTOR T CELLS

Chimeric antigen receptor (CAR) T cells are a promising treatment for cancer, used with remarkable success for example in refractory or relapsed B cell lymphoma (57–59). To obtain such CAR T cells, peripheral blood mononuclear cells from the patient's blood are isolated and stimulated with interleukin 2 and anti-CD3 antibodies, leading to their proliferation. The T cells are transduced with a construct encoding for the CAR of interest, e.g. the antigen-binding domain of an anti-CD19 antibody fused to a transmembrane and several intracellular signaling domains such as 4-1BB and CD3ζ (60). Upon transfusion back to the patient, the CAR T cells eliminate cells expressing the target antigen, in this case CD19, which is a marker of B cells. A huge advantage of this treatment is the generation of long-term memory CAR T cells, which offer the opportunity for constant elimination of newly emerging target cells without the need for repetitive dosing.

This strategy can also be applied for the treatment of antibody-mediated autoimmune diseases, with one essential modification: the antigen-binding domain of a conventional CAR is replaced by a part of the autoantigen of interest, resulting in a chimeric autoantibody receptor (CAAR). A T cell expressing this CAAR (CAAR T cell) will bind to and eliminate B cells that express the corresponding B cell receptor (BCR), a membrane-bound immunoglobulin matching the antibody produced by this particular cell clone. This approach was firstly tested in an animal model of pemphigus vulgaris (PV). T cells expressing a CAAR consisting of the PV autoantigen desmoglein 3 fused to CD137-CD3ζ specifically eliminated anti-desmoglein 3-specific autoreactive B cells (61). Moreover, the CAAR T cells erased their targets even in the presence of circulating anti-desmoglein 3 antibodies and did not show significant off target effects. In a follow-up study, the authors preclinically examined the pharmacodynamics and toxicity of this CAAR and showed that the desmoglein 3-CAAR T cells specifically deplete primary human desmoglein 3-specific B cells from PV patients and is effective in an active animal model of PV (62).

Applying the CAAR T cell strategy for the treatment of MN would be a pioneering approach. The antibody binding sites in the antigens PLA2R1 and THSD7A have been already characterized, in parts even down to the level of single antigen domains and smaller epitopes (54–56, 63–65). Given this knowledge, to achieve an optimal intermembrane distance of the immunologic synapse (the space between the CAAR T cell and the target B cell) and minimize potential off-target effects, it appears reasonable to fuse smaller fragments of PLA2R1 or the other MN antigens to the chimeric receptor (**Figure 2B**). In case of PLA2R1 and THSD7A the most N-terminal regions are considered as immunological hot spots, as most reactivity with patient autoantibodies is found in these areas (56, 63, 64). Therefore, a CAAR containing only the N-terminal region of the respective autoantigen might be sufficient to eliminate a number of autoreactive B cells large enough to ameliorate

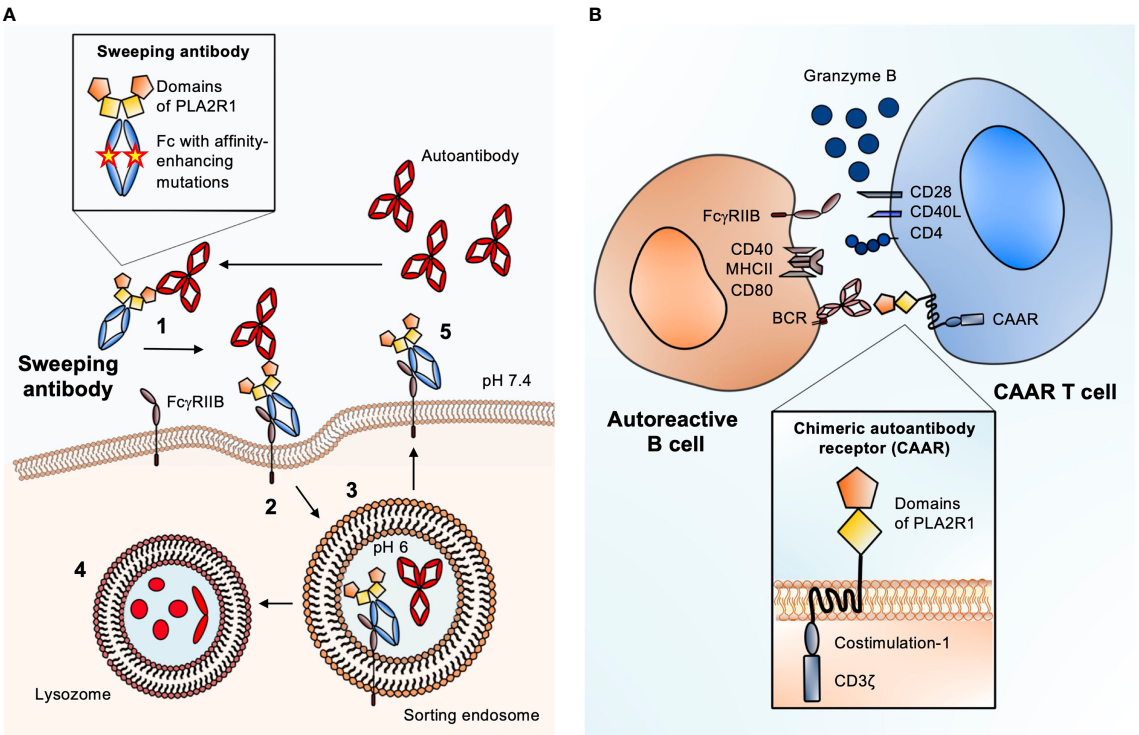

FIGURE 2 | Antigen-specific therapies suited for MN. **(A)** Schematic of the sweeping antibody principle with enhanced FcγRIIB binding. 1. After injection, sweeping antibody and autoantibody bind in the circulation. 2. Scavenger cells like liver sinusoidal endothelial cells (LSECs) that express FcγRIIB bind the circulating immune complex (IC) and internalize it through pinocytosis. 3. Due to a pH shift from neutral to pH 6 inside the sorting endosome, the autoantibody is released from the IC-receptor complex. 4. The autoantibody is degraded inside the lysosome. 5. The Fc receptor-bound sweeping antibody is returned to the surface and can bind new circulating autoantibodies causing the "sweeping" effect. Magnified: structure of a PLA2R1 sweeping antibody: The Fab part is substituted for the most N-terminal domains of PLA2R1 (cysteine-rich and fibronectin type II) to create specificity for anti-PLA2R1 autoantibodies. Mutations in the Fc part of the sweeping antibody enhance the affinity towards FcγRIIB. **(B)** Schematic of chimeric autoantibody receptor (CAAR) T cell principle. The CAAR comprises fragments of the target antigen (in this case the cysteine-rich and fibronectin type II domains of PLA2R1), a transmembrane domain, and several intracellular signaling domains. The CAAR enables binding to a B cell, which expresses the corresponding B cell receptor (BCR), a membrane-anchored IgG corresponding to the autoantibody that is produced by the B cell. CAAR-mediated binding of the T cell to the pathogenic B cell leads to release of granzyme B, which eliminates the target B cell. Magnified: structure of a second generation CAAR. It includes the autoantibody receptor as a ectodomain, here the most N-terminal domains of PLA2R, a transmembrane part and an endodomain with co-stimulation module and the CD3ζ with three immunoreceptor tyrosine-based activation motifs (ITAMs). The co-stimulatory domain improves the half-life *in vivo*, proliferation and cytotoxity of the CAAR T cell.

disease. It would also be possible to perform an antibody mapping first, e.g. using domain-specific ELISAs, and tailor the CAAR strategy to the individual epitope profile.

Although the CAR T cell therapy has revolutionized the treatment of malignancies, it has several potential flaws. First, the manufacturing of personalized CAR T cells is very complex, time-consuming and expensive. Second, CAR T cell therapy could involve serious adverse effects, such as the cytokine release syndrome (CRS), caused by the activation of CAR T cells and their production of proinflammatory cytokines (66, 67), or neurotoxicity, which often accompanies CRS, possibly due to cerebral endothelial dysfunction (68). Third, it is questionable whether the generation of memory T cells is always desirable in light of many patients achieving full remission of disease after some time.

The generation of CAR NK cells represents an alternative strategy to overcome some of these undesired effects. Due to their limited lifespan, CAR NK cells show a relatively low toxicity towards normal tissue of the recipient (69). They offer the possibility of "off the shelf" therapy as they can be obtained from peripheral blood mononuclear cells of healthy donors, umbilical cord blood, induced pluripotent stem cells, and even NK92 cell lines (70). Currently, most of the CAR NK constructs derive from CAR T cell approaches, but there are attempts to design also CARs tailored specifically for NK cells (71). To date, CAR NK cell therapy seems to be a safe additional approach for cancer therapy, with several clinical trials running (72, 73). In conclusion, CAAR T cell therapy and, alternatively, the safer and likely more cost-efficient and easier to manufacture CAAR NK cell therapy represent an elegant and promising therapeutic strategy for patients with MN.

Notably, is also possible to approach T cell therapy from the opposite site, by regaining immune tolerance. To achieve this, the function of one key player of immune tolerance has to be

modified: the so called regulatory T cells or Tregs. About 5-7% of all CD4+ T cells in the human body are Tregs. The identity and function of Tregs are characterized by the expression of various markers such as the cytotoxic T lymphocyte-associated protein-4 (CTLA-4), the interleukin 2 receptor subunit α (CD25), the transcription-maintaining factor STAT5, and the forkhead box P3 (FOXP3). FOXP3 has a key function for the maintenance of immune tolerance (74).

Tregs can be transduced with so called BARs (chimeric B cell-targeting antibody receptors) containing an extracellular domain consisting of the immunodominant parts of the antigen, which means they are basically comparable to CAARs on cytotoxic T cells. Instead of killing the B- cell upon binding, such BAR Tregs suppress antigen-specific B cells directly without affecting the T cell response (75).

CONCLUSION

MN is an antibody-mediated autoimmune disease and several autoantigens have been identified over the past years. The direct pathogenicity of the involved autoantibodies has either been shown or is strongly suspected (20, 22), and treatment strategies have shifted towards targeting of B cells (30, 31, 76). In light of these developments, the establishment of antigen-specific treatments represents the consequential continuation of MN therapy. We are aware that the therapeutic concepts discussed in this article have not been tested in MN, not even at an experimental level. However, future research should take into account such potential strategies, particularly as antigen-specific animal models become more and more available (22, 77). Clearly, the possible adverse effects of such treatments should not be overlooked, but there is a realistic chance that they would not be as severe as the ones of currently used therapeutics. Especially in case of CAR T cells, huge efforts are made to reduce side effects and make them more controllable, e.g. through transient transduction, suicide genes or elimination markers (78).

The actual charm of the two treatment approaches that we presented here is the option to combine and apply them as a therapeutic package tailored for the individual patient. As an example, one could imagine the following procedure: After confirming the diagnosis of PLA2R1-associated MN by kidney biopsy, the antibody profile is analyzed using a domain-specific ELISA, revealing reactivity with the cysteine-rich domain. Subsequently, sweeping antibodies containing the cysteine-rich domain are applied to eliminate the circulating autoantibodies. This acutely reduces binding of pathogenic antibodies at the glomerular filtration barrier and additionally paves the way for CAAR T or NK cells carrying a CAAR which contains the cysteine-rich domain. The CAAR T or NK cells thus eliminate autoreactive B cells, which prevents further production of pathogenic antibodies. Without previous clearance of antibodies, CAAR cells may in fact be neutralized by the circulating antibodies binding to the CAAR, potentially making them ineffective in eliminating autoreactive B cells. As an alternative or additive strategy, PLA2R1-specific immune tolerance could be restored by suppression of autoreactive B cells using Tregs expressing a BAR that contains the cysteine-rich domain.

In summary, the increasing knowledge about the targets of autoimmunity in MN offers a huge potential for the application of antigen-specific treatment strategies, which would ideally spare protective immunity.

AUTHOR CONTRIBUTIONS

SK and LS contributed equally to this review. NT conceptualized the work. All authors discussed the concept and revised the manuscript. All authors approved the final version of the manuscript.

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New and Old Anti-CD20 Monoclonal Antibodies for Nephrotic Syndrome. Where We Are?

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Nephrotic proteinuria is the hallmark of several glomerulonephritis determined by different pathogenetic mechanisms, including autoimmune, degenerative and inflammatory. Some conditions such as Minimal Change Nephropathy (MCN) and Focal Segmental Glomerulosclerosis (FSGS) are of uncertain pathogenesis. Chimeric anti-CD20 monoclonal antibodies have been used with success in a part of proteinuric conditions while some are resistant. New human and humanized monoclonal anti-CD 20 antibodies offer some advantages based on stronger effects on CD20 cell subtypes and have been already administered in hematology and oncology areas as substitutes of chimeric molecules. Here, we revised the literature on the use of human and humanized anti-CD 20 monoclonal antibodies in different proteinuric conditions, resulting effective in those conditions resistant to rituximab. Literature on the use of human anti-CD 20 monoclonal antibodies in different proteinuric diseases is mainly limited to ofatumumab, with several protocols and doses. Studies already performed with ofatumumab given in standard doses of 1,500 mg 1.73m^2 suggest no superiority compared to rituximab in children and young adults with steroid dependent nephrotic syndrome. Ofatumumab given in very high doses (300 mg/1.73m^2 followed by five infusion 2,000 mg/1.73 m^2) seems more effective in patients who are not responsive to common therapies. The question of dose remains unresolved and the literature is not concordant on positive effects of high dose ofatumumab in patients with FSGS prior and after renal transplantation. Obinutuzumab may offer some advantages. In the unique study performed in patients with multidrug dependent nephrotic syndrome reporting positive effects, obinutuzumab was associated with the anti-CD38 monoclonal antibody daratumumab proposing the unexplored frontier of combined therapies. Obinutuzumab represent an evolution also in the treatment of autoimmune glomerulonephritis, such as membranous nephrotahy and lupus nephritis. Results of randomized trials, now in progress, are awaited to add new possibilities in those

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cases that are resistant to other drugs. The aim of the present review is to open a discussion among nephrologists, with the hope to achieve shared approaches in terms of type of antibodies and doses in the different proteinuric renal conditions.

Keywords: nephrotic syndrome, anti-CD20 antibodies, rituximab, ofatumumab, obinutuzumab, daratumumab, glomerulonephritis, immune dysfunction

INTRODUCTION

Nephrotic Proteinuria is the hallmark of several renal diseases characterized by age dependent peculiarities and different pathogenesis. In adulthood, nephrotic proteinuria is generally due to autoimmune or degenerative diseases, such as Membranous Nephropathy (MGN) and Lupus nephritis (LN) or Myeloma. Pathologies of uncertain origin, such as Minimal Change Nephropathy (MCN) and Focal Segmental Glomerulosclerosis (FSGS), occur most frequently in children and young adults and account for above 90% of cases of nephrotic syndrome under 24 years.

Prednisone is the first line therapy in many cases, however, prevalently patients with MCN may develop steroid dependence (SDNS) requiring steroid sparing-agents to minimize drug-related adverse effects (1–3). Between 10 and 20% of patients have steroid-resistant nephrotic syndrome (SRNS), that requires alternative therapies such as calcineurin inhibitors or mycophenolate and 5% are resistant to all associations (3).

In last decade, clinical trials have shown that rituximab, a chimeric monoclonal antibody targeting the CD20 antigen expressed on B cells (4), may represent an effective treatment in all the spectrum of proteinuric glomerulonephritis in spite of their different origin (5–15). The existence of patients who are resistant to rituximab and others who developed anti-rituximab antibodies following multiple treatments with the drug (16–18) have stimulated the search of novel anti-CD20 molecules (19).

Several monoclonal human or humanized anti-CD20 antibodies have been developed, based on the technology for reshaping therapeutical human antibodies, as described by Riechmann et al. in 1988 (20) and many of them have been already administered in hematology and oncology areas.

In the present review, we will describe the impact and future perspectives of three new anti-CD20 antibodies already approved in different clinical settings (ofatumumab, obinutuzumab, ublituximab) and resulted promising in treatment of proteinuric disease. Depending on the structural aspects and on the number of binding sites, first and second generation anti-CD20 antibodies play different effects on CD20 cell subtypes by direct cytotoxicity, antibody-mediated cytotoxicity (ADCC), phagocytosis (ADCP) or complement-mediated cytotoxicity (CDC) (**Table 1**). Ofatumumab induces a potent stimulus for CDC, whereas obinutuzumab is a powerful activator of ADCC and ADCP and has also a strong direct cytotoxicity, but not a relevant CDC. Overall, fully human and humanized anti-CD20 antibodies demonstrated stronger *in vitro* activities than rituximab. Whether these cellular effects may translate into superior clinical benefits is unknown. The number of studies testing new anti-CD20 antibodies in glomerular diseases has grown in parallel with the expansion of studies in other clinical areas and results from randomized clinical trials are now appearing that may modify therapeutic strategies in a near future.

Whether human anti-CD20 should be preferred to humanized antibodies and the key question of dosages are main items to be discussed and shared.

RITUXIMAB: CELL TARGET AND ANTI-PROTEINURIC MECHANISM

Rituximab, the first class of anti-CD20 antibody used in renal diseases (21) is a chimeric monoclonal antibody composed of a murine immunoglobulin variable region mounted on a human immunoglobulin G1 heavy chain. It targets the CD20 antigen of B cells that appears early during maturation but not expressed by B cell precursors. Upon CD20 binding, B cells are killed trough different mechanisms, as previously reported. The lowering effects of rituximab on B cells is delayed over time with a median of 6 months, whereas the effect on memory B cells perdures for more than one year.

Reduction of antibodies production by B cells seems the obvious mechanism for the protective effect of rituximab in antibody-mediated renal diseases, such as MGN and LN. It is so far unknown how the drug works in SDNS, not an antibody mediated disease. Lack of clear evidence on mechanisms

TABLE 1 | Chimeric and humanized anti-CD20 determines different effects on their cell targets depending on their structure, number and extension of the binding sites.

Anti-CD20 Antibodies	Type	ADCC	Direct Cytotoxicity	CDC	ACDP
Rituximab	I	++	+	++	++
Ofatumumab	I	++	+	++++	+++
Obinutuzumab	II	++++	++++	+	++++
Ubituximab	I	++++	++	++	++++

ADCC, antibody-dependent cell-mediated cytotoxicity; CDC, complement-dependent cytotoxicity; ACDP, antibody-dependent cellular phagocitotsis.
+, really low; ++, low; +++, moderate; ++++, high.

responsible of SDNS complicates any further evolution on the drug activity (7). Early studies suggested a cross interaction of rituximab with podocyte spingomyelinase-like phosphodiesterase 3b precursor (SMPDL3B) which regulates acid sphingomyelinase (ASMse) in the raft of podocytes and partially co-localizes with synaptopodin, a regulator of thecytoskeleton. In many circumstances the effect of rituximab was associated with depletion of B cells (22), but there are cases of treatment failure despite B cells depletion (23). On the opposite, persistent remission induced by rituximab can be maintained in some patients also after CD19+ recovery (24). Memory B cells have been associated with SDNS disease activity, which may explain the effects of B cell depletion (25). Other immune cells may be involved in rituximab activity, including regulatory T cells (26).

NEW ANTI-CD20 ANTIBODIES

Structure and Binding Affinity

Ofatumumab is a type I humanized anti-CD20 monoclonal antibody that binds the CD20 target through the Fab domain at a distinct epitope respect to rituximab (**Figure 1**) and determines its immune effect through the Fc domain (27). The epitope is closer to the cell surface and the binding site is more extended if compared respect of other anti-CD20 antibodies. Ofatumumab possess a binding site for C1q that mediates an enhanced CDC activity (28).

Obinutuzumab is a type II humanized anti-CD20 monoclonal antibody that induces a direct cell death and antibody-dependent cell-mediated cytotoxicity. It has a glyco-engineered Fc region

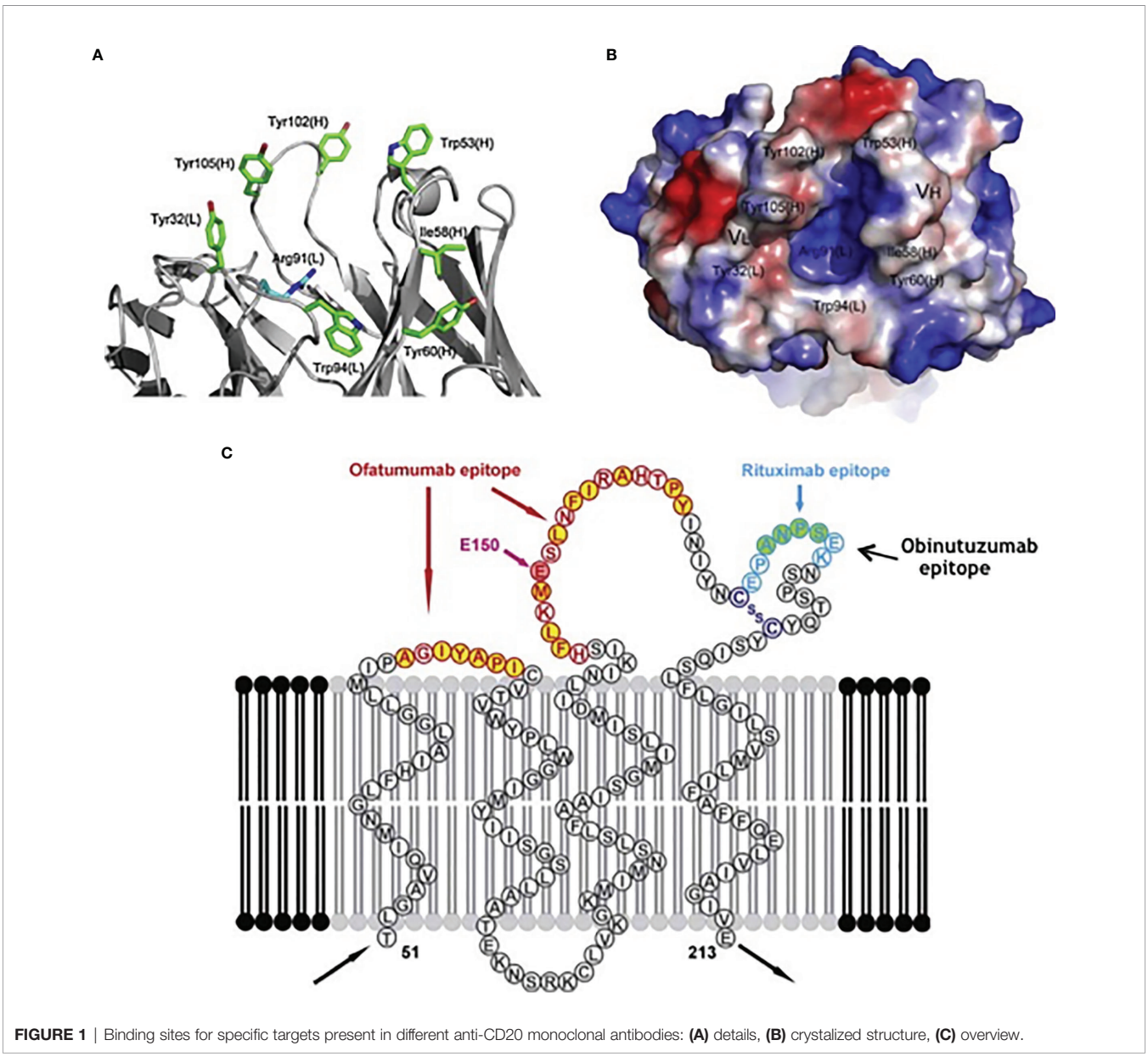

FIGURE 1 | Binding sites for specific targets present in different anti-CD20 monoclonal antibodies: **(A)** details, **(B)** crystalized structure, **(C)** overview.

which can enhance binding affinity to the Fc receptor (FcR) on immune effector cells (28, 29). Since the characteristic of type II antibodies is that they do not localize into the lipid rafts, CDC is reduced compared to rituximab and Ofatumumab.

Ublituximabs is a chimeric IgG1 monoclonal antibody that recognizes a unique epitope in CD20 and has enhanced affinity for the FcγRIIIa of effector cells and macrophages that mediates increased ADCC and ADCP (30, 31).

Extra-Renal Fields of Application

Hematolgy is the main area of application of the anti-CD20 antibodies. Ofatumumab has been approved by the FDA for the treatment of multidrug-resistant chronic lymphocytic leukemia (CLL) and has been shown to have efficacy against rituximab-resistant B-cell cancers (32) in combination with other chemotherapeutics (33). Ofatumumab has also been approved as maintenance therapy for the same condition (34). In 2013, Obinutuzumab was approved by FDA for the treatment of CLL after the open-label, three arms trial CLL11 comparing obinutuzumab with rituximab and chlorambucil (35). Similar results were reported in the phase 2 and 3 GALTON trials (36). Efficacy and safety of ofatumumab and obinutuzumab compared with rituximab in different clinical conditions are still controversial. The HOMER randomized study compared the effects of rituximab *vs.* ofatumumab in patients with non-Hodgkin lymphoma (NHL) (37): the trial was stopped early because of futility at the planned interim analysis, indicating non-superiority of ofatumumab. Analysis of results deriving from 11 RCTs comprising 5261 patients with CD20+ NHL showed that ofatumumab, compared to rituximab, had no significant differences in terms of progression-free survival, overall survival and complete response rate, but was inferior in consideration of the overall response rate. Compared with rituximab, obinutuzumab significantly prolonged the progression free survival but it had no improvement on overall response rate, and on complete response rate. Obinutuzumab also increased the incidence of serious adverse effects (OR 1.29, 95% CI 1.13–1.48, $P < 0.001$) (38). Studies *in vitro* strengthened the effectiveness of obinutuzumab in combination with other agents against rituximab resistant cells (39).

OFATUMUMAB FOR PROTEINURIC RENAL DISEASES

Immunoglobulin A Vasculitis With Nephritis

IgAVN is a systemic leukocytoclastic vasculitis characterized by purpuric skin manifestation, usually enteritis and arthritis and frequently glomerulonephritis with IgA deposition in the glomerular mesangium (40). Evidence-based optimal therapy recommendations in cases of IgAVN are not available due to the variability of clinical presentation (41). A recent report described 3 cases treated with rituximab and one with the association of rituximab plus 4 doses ofatumumab (8). Massive B-cell depletion was in this case associated with decrease of proteinuria and stabilization of renal function (**Table 2**).

Membranous Nephropathy

In the literature, only one patient affected by MGN was treated with ofatumumab. Podestà et al. (52) described the case of a young male with podocyte phospholipase A2 receptor positive MGN resistant to 4 cycles of rituximab and then treated with the fully human anti-CD20 monoclonal antibody ofatumumab, achieving remission of the NS, without significant side effects. Four doses of ofatumumab were administered over 4 years allowed long lasting maintenance of normal urinary parameters (**Table 2**).

Childhood Steroid Dependent Nephrotic Syndrome

According to recent case reports and small case series, ofatumumab may induce disease remission in children with SDNS (43, 44) and it was administered in place of rituximab in patients with circulating anti-rituximab antibodies (53).

Positive results stimulated a monocentric randomized clinical trial comparing rituximab and ofatumumab in children with calcineurin dependent SDNS: as main result, we reported that a single dose of ofatumumab was not superior to a single dose of rituximab in maintaining remission in children with steroid- and calcineurin inhibitor-dependent NS (54). After 12 months, the same percent of patients in the ofatumumab and rituximab groups were in remission (i.e. 46 and 47% respectively) then the curve diverged and after 24 months emerged a higher percent of patients in remission in the rituximab *vs.* the ofatumumab group (i.e. 34% and 24% respectively). An ancillary finding was that ofatumumab produced better results in children >16 years than below this age (**Table 2**).

Childhood Multidrug Resistant Nephrotic Syndrome

Treatment of children with multidrug resistant nephrotic syndrome (MRNS) is a major clinical concern due to a very high probability of evolution to end stage renal failure. The optimal dosing of ofatumumab for renal conditions has not been established yet, especially in children, and studies so far published are mainly limited to case reports and case series. Three papers reporting small case series suggested that the fully humanized anti-CD 20 antibody ofatumumab could be more effective than the chimeric compound in MRNS and encouraged clinical testing. Basu et al. (19) treated 5 children with nephrotic syndrome with well-defined resistance to rituximab, tacrolimus/ciclosporin and cyclophosphamide with high dose ofatumumab (300 mg/1.73m^2 followed by five infusion 2,000 mg/1.73 m^2) and observed normalization of proteinuria within 6 weeks. Similarly Wang et al. (44) showed promising results with high dose. Bonanni et al. treated 6 children with the same clinical characteristics with a 'low dose' approach (300 mg followed by 700 mg/1.73 m^2 in two weeks) and observed remission of proteinuria in 2 cases (45). Safety of ofatumumab given in high doses may represent a main problem that requires much attention.

Ravani et al. (46) designed a randomized placebo-controlled trial in children with MRNS comparing ofatumumab administered

TABLE 2 | Main studies reporting administration of humanized anti-CD20 in nephrotic disease.

Reference	Drug Type	N	F/U (mo)*	Studydesign	Dose	CR (%)
IgAVN						
Lundberg S, *Clin Kidney J*, 2017 10 (1):20-26 (8)	Rituximab + Ofatumumab	1	12	Case Report	RTX 375mg/m^2 + OFA 175mg/m^2	100
MGN						
Sethi S, *K Intern Rep*, 2020 5: 1515-1518 (42)	Obinutuzumab	10	18 (9–24)	Caseseries	N/A	40
MDNS						
Ravani P, *J Am Soc Nephrol*, 2021 32 (10):2652-2663 (14)	Ofatumumab	70	12	RCT	1.500 mg/m^2 (1 dose)	47
Vivarelli M, *Pediatr Nephron* 2017 32 (1):181-184 (43)	Ofatumumab	2	17 (15-19)	Case series	750 mg/m^2 (1 dose)	50
Fujinaga S, *Pediatr Nephrol*, 2018 33(3):527-528	Ofatumumab	1	5	Case Report	300mg/m^2	100
MRNS						
Basu B, *N Engl J Med* 2014, **370**:1268-1270 (19).	Ofatumumab	5	12	Case series	300mg/m^2 followed by 5 weekly infusions (2g/m^2)	80
Wang CS, *Pediatr Nephrol. 2017 32(5):835-841* (44)	Ofatumumab	4	9 (6-13)	Case series	300mg/m^2 followed by 5 weekly infusions (2g/m^2)	50
Vivarelli M, *Pediatr Nephrol*, 2017 Jan;32(1):181-184 (43)	Ofatumumab	2	17 (15-19)	Case series	750 mg/m^2 (1 dose)	50
Bonanni A, *BMJ Case Rep* 2015, 10.1136/bcr-2015-210208 (45)	Ofatumumab	6	12	Case series	375-700 mg/m^2 (1 dose)	33
Ravani P, *Pediatr Nephrol*, 2020 35(6):997-1003 (46)	Ofatumumab	7	12	RCT	1.500 mg/m^2 (1 dose)	0
Post Tx recurrence FSGS						
Wang CS, *Pediatr Nephrol.* 2017 *32(5):835-841* (44)	Ofatumumab	1	4	Case Report	300mg/m^2 followed by 5 weekly infusions (2g/m^2)	100
Colucci M, *Pediatr Nephrol*, 2020 35(2):341-345 (47)	Ofatumumab	2	12	Case series	750 mg/m^2 (1 dose)	50
Solomon S, *Pediatr Transplant, 2019 Jun;23(4):e13413* (48)	Rituximab + Ofatumumab	1	12	Case Report	RTX 375mg/m^2 (2 doses) + OFA 2g/m^2 (2 doses)	100
Reynolds BC, *Pediatr Nephrol*. 2021 Aug 12 (49)	Ofatumumab	7	24	Case series	300mg/m^2 followed by 5 weekly infusions (2g/m^2)	44
Kienzl-Wagner K, *Am J Transplant*, 2018 18(11):2818-2822 (50)	Ofatumumab	1	8	Case Report	1150 mg/m^2 + 1150 mg/m^2 at 6mo	100
Bernard J, *Pediatr Nephrol*, 2020 35(8):1499-1506 (51).	Ofatumumab	6	10 (8-12)	Case series	300mg/m^2 followed by 5 weekly infusions (2g/m^2)	0 (50 PR)

**Data are presented as common follow-up period for all the patients or as median (range).*

CR, complete remission; MDNS, multidrug dependent nephrotic syndrome; MGN, membranous glomerulonephropathy; N/A, not available; OFA, ofatumumab; PR, partial remission; RTX, rituximab; RCT, randomized controlled trial; MDNS, multidrug dependent nephrotic syndrome; SRNS, steroid resistant nephrotic syndrome; Tx, kidney transplantation.

at hematologic doses (1,500 mg/1.73 m^2) *vs.* placebo and did not support potential benefits of ofatumumab (**Table 2**).

Based on results of studies using high dose ofatumumab and the negative results with low cumulative dose, there is a need of more safety data. Confirmatory studies on the effect of high doses ofatumumab in patients with MRNS are also needed.

Post-Transplant Recurrence of Nephrotic Syndrome

The treatment of post-transplant recurrence of nephrotic syndrome is often challenging. Disease recurrence after renal transplantation occurs in around half of cases. Early recurrence is more common in pediatric patients who may present massive

proteinuria within hours or days after transplantation; efficacy of therapeutic strategies is often limited.

Ofatumumab has been proposed in treating post-transplant FSGS relapse based on single case reports and small series (44, 47–49). Kienzl-Wagner et al. (50) demonstrated decrease of proteinuria in a patient with second transplant after treating with daily plasma exchange and ofatumumab. At 8 months after kidney re-transplantation graft function was in normal range.

In the largest series involving six children with recurrence of nephrotic syndrome after renal transplantation with failure of previous treatments, ofatumumab demonstrated poor efficacy (51). Four children were treated with the high dose described by Basu (19) and were followed for 10.5 months. No patient achieved a complete remission, half of them had a partial remission and half had no response at all (**Table 2**).

Cell Monitoring

Human and chimeric anti-CD20 antibodies target the same polymorphonuclear sub-populations. A detailed comparison between ofatumumab and rituximab has been done in the recent randomized study comparing the two drugs in SDNS children (54): the whole B cells compartment was reduced to zero, with minimal differences in the re-population kinetics between ofatumumab and rituximab. Overall, B-cells started to recover after 3-4 months after infusion while Memory B cell, in particular IgM Memory B cells, remained very low during the first 12 months after infusion and then started to regenerate.

T cells were only minimally modified and remained stable during the follow up. A modest increment in the percent concentration of CD3Tcells, CD53 NK and Treg cells in the 6 months following infusion was observed.

OBINUTUZUMAB FOR RESISTANT GLOMERULONEPHRITIS

Childhood Multidrug Dependent Nephrotic Syndrome

Only one study used obinutuzumab (single dose 1,000 mg 1.73m^2) in nephrotic syndrome in combination with sequential administration of the anti-CD38 (plasma cell) monoclonal antibody daratumumab (1,000 mg 1.73m^2) after 14 days (55). Fourteen patients who relapsed after conventional treatments with prednisone, rituximab and CNI and developed dependency with combination of more drugs (MDNS) were treated with the above association of obinutuzumab and daratumumab and were included in a retrospective analysis: 5 presented recurrence of proteinuria after about 10 months, 9 were in stable remission after 20 months of follow up. The use of 2 monoclonal antibodies at low doses is of interest in consideration of safety. On the other hand, to discern the separate effects of obinutuzumab and daratumumab when given together is not possible; however, the positive results of combining therapy opens new ways in the treatment of nephrotic syndrome and supports the necessity of new studies in the future in those patients who require more than one drug to maintain remission.

Membranous Nephropathy

In a recent case series, Sethi et al. (42) treated with obinutuzumab 10 adults with MGN, with well-defined resistance to rituximab, tacrolimus and cyclophosphamide. Authors reported that 60% of patients achieved complete or partial remission at 6 months and almost 90% after 12 months of follow up.

Lupus Nephritis

In MRL/lpr mice, a murine model of Lupus, obinutuzumab resulted more effective in depleting B cells than rituximab (56). A Randomized Controlled Study comparing obinutuzumab and rituximab in subjects with Proliferative Lupus Nephritis (class III and IV) is now in progress: preliminary results are showing that obinutuzumab provides sustained clinical benefit through 2 years compared to rituximab. Results of the phase 2 NOBILITY trial (NCT02550652), comparing the efficacy and safety of obinutuzumab plus MMF with placebo plus MMF in participants with proliferative Lupus Nephritis, also showed that obinutuzumab was well-tolerated with no unexpected safety findings at two years of follow up.

SAFETY

Ofatumumab

Safety of new anti-CD20 monoclonal antibodies is an important issue considering that in many cases with resistance to other drugs, very high doses, particularly of ofatumumab, are required.

Previous observations in patients treated with standard dose of ofatumumab (1,500 mg 1.73 m^2) indicated an increased respiratory susceptibility when compared with rituximab. Several patients presented bronchospasm and required infusion of the drug in a protected condition (57). The association of nebulized salbutamol in the pretreatment drug schedule resolved almost completely this problem.

Bonanni et al. (57) compared safety of ofatumumab and rituximab in large cohorts of patients (268 *vs* 68 rispectively) showing higher incidence of respiratory symptoms with infusion of ofatumumab. Of note, the retrospective observatory study included infusions administered in the pre-salbutamol era. The results on safety of humanized and chimeric anti-CD20 presented in the randomized trial comparing their effects in SDNS confirmed low negative impact for both compounds (54).

Four of the 10 patients treated with ofatumumab due to recurrence of nephrotic syndrome after kidney transplantation (55) exhibited minor allergic reactions; one patient died of infection as a consequence of multiple factors.

Obinutuzumab

Three patients treated with obinituzumab presented mild infusion reactions, i.e. vomiting and urticaria and transient neutropenia was observed in other limited subjects (55).

One patient of the membranous series had respiratory symptoms during obinutuzamab infusion and 4 had leukopenia that lasted for more than 3 months in one of them (42).

CONCLUSION AND PERSPECTIVES

The introductions in clinical practice of human and humanized anti-CD20 monoclonal antibodies has represented a break-through for the treatment of proteinuric renal diseases and offer the opportunity to reconsider those clinical conditions resistant or partially responsive to rituximab. A main issue is multidrug resistant FSGS in the pre and post-transplant phases. Looking to a possible future clinical trial, the key questions remain which anti-CD20 antibody and at which dose should be administered. The extremely positive results obtained with the combination of obinutuzumab and daratumumab in patients with severe MDNS, offer the possibility to consider this association also for these patients. The opportunity to use both drugs in medium-low doses probably minimizes side effects and strengthens the necessity of formulation of new therapeutic approaches. Post-transplant recurrence of FSGS is another condition that should be considered for the association of obinutuzumab and daratumumab. Other combinations of daratumumab, e.g. with rituximab, could also be considered.

Obinutuzumab represent an evolution also in the treatment of autoimmune glomerulonephritis, such as MGN and lupus nephritis. Results of randomized trials now in progress are awaited to add new possibilities in those cases resistant to other drugs.

AUTHOR CONTRIBUTIONS

BB, AA, BI, and GG contributed to conception and writing of the work. BB and GG revising it critically. All the authors provide approval for publication of the content.

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REVIEW
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Therapies for Membranous Nephropathy: A Tale From the Old and New Millennia

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Primary Membranous Nephropathy (PMN) is the most frequent cause of nephrotic syndrome in adults. If untreated, PMN can lead to end-stage renal disease; moreover, affected patients are at increased risk of complications typical of nephrotic syndrome such as fluid overload, deep vein thrombosis and infection. The association of PMN with *HLA-DQA1* and the identification in around 70% of cases of circulating autoantibodies, mainly directed towards the phospholipase A2 receptor, supports the autoimmune nature of the disease. In patients not achieving spontaneous remission or in the ones with deteriorating kidney function and severe nephrotic syndrome, immunosuppression is required to increase the chances of achieving remission. The aim of this review is to discuss the evidence base for the different immunosuppressive regimens used for PMN in studies published so far; the manuscript also includes a section where the authors propose, based upon current evidence, their recommendations regarding immunosuppression in the disease, while highlighting the still significant knowledge gaps and uncertainties.

Keywords: membranous nephropathy, glomerulonephitis, nephrotic syndome, rituximab, cyclical therapy

INTRODUCTION

Membranous nephropathy (MN) is a disease induced by deposition of immune complexes in the subepithelial space of the glomerulus (1). In the primary form (PMN), immune complex formation is driven by autoantibodies, with the most frequent autoantigen being the M type phospholipase A2 receptor (PLA2R) (2), a protein normally expressed in podocytes. Of note, other antigens may be involved as well (3–6). The genetic association of PMN with *HLA-DQA1* (7) supports abnormal antigen presentation as a further key step contributing to disease. Yet, several aspects in the pathogenesis remain unclear, such as what are the mechanisms that induce loss of self-tolerance, how exactly the IgG4 autoantibodies cause podocyte injury and proteinuria, how the complement system is activated, and whether or not it plays a role in podocyte damage (8).

From a clinical point of view, the natural course of PMN is variable. Several retrospective studies with relatively short follow-up, including patients with and without nephrotic range proteinuria, reported that about 1/3 of patients do not present nephrotic syndrome or may enter a spontaneous remission, 1/3 maintain stable kidney function, with proteinuria fluctuating between nephrotic and sub-nephrotic range, while the remaining tend to have persistent nephrotic syndrome and progress to end-stage renal disease (ESRD) (9–14).

Studies with long-term follow-up (10 years or more) clearly showed that kidney survival is significantly affected by the length of patients' observation. In an Italian randomized controlled trial in patients with biopsy-proven PMN and nephrotic syndrome at presentation, after 10 years of follow-up only 5% of the cases assigned to symptomatic treatment were in complete remission, and another 28% were in partial remission (for more details on the definition of complete and partial remission, please see the **Appendix**). Of note, in the same group, 40% of patients developed ESRD or died within 10 years from randomization (15). In a systematic review including all studies published up to 1994, Hogan et al. (16) found that the kidney survival of patients with MN averaged around 50% at 14 years. DuBuf-Vereijken et al. (17) analysed the reports published during the previous 25 years, excluding patients with a follow-up shorter than 3 years, and identified a 100% renal survival rate in non-nephrotic patients; on the other hand, nearly 50% of patients with PMN and nephrotic syndrome experienced deterioration in kidney function. An increased risk of chronic kidney disease (CKD) progression is only one of the risks of persistent nephrotic syndrome, which is also associated with a number of severe extra-renal complications including vascular thrombosis, infection, and cardiovascular disease. Non-immunomodulatory and non-specific nephroprotective approaches, although necessary, are therefore not sufficient for patients with PMN and persistent or severe nephrotic syndrome. In this context, a role for immunomodulation has been proposed, in order to try to modify disease progression. The aim of this manuscript is to review evidence of historical and emerging immunomodulatory approaches for the treatment of PMN, with a particular focus on prospective randomised controlled studies (**Table 1**).

OLD THERAPIES

Corticosteroids or Alkylating Agents Versus Supportive Treatment

Glucocorticoids have been the first immunomodulatory drug employed for the management of PMN. Three randomized controlled trials failed to show significant benefits of prednisone in PMN and, when a benefit was shown, this was not sustained during follow-up (19–21). Moreover, the study demonstrating better response rates in the glucocorticoids arm included a higher proportion of patients with nonselective proteinuria in the placebo arm (21). This has to be interpreted in the context of retrospective, observational studies with controversial results, with some manuscripts reporting good remission rates (45, 46) and others showing that only a small subset of patients obtained transient and not sustained remission (47–49).

Other immunosuppressive drugs were explored in monotherapy for PMN at the early stages of clinical research in the field. A small trial randomized 22 participants with PMN to receive either cyclophosphamide or symptomatic therapy for one year. At the end of this period, no significant differences were detected between the two groups in terms of proteinuria and renal function (18). Alkylating agents were employed also in retrospective studies with contrasting results, with significant heterogeneity across different reports (45, 47–49). Of note, a safety signal emerged from a French cohort of heterogenous glomerular diseases treated with chlorambucil for at least one year: of 41 participants, 3 developed cancer (50). These controversial results supported the idea that a combination of different immunomodulatory drugs may be required for the management of PMN.

Corticosteroids and Alkylating Agents Versus Supportive Treatment

In 1984, a novel therapeutic approach was proposed. An Italian multicenter, prospective randomized trial assigned 67 patients with PMN and nephrotic syndrome to receive symptomatic treatment or 6 months of a cyclical therapy, with glucocorticoids and chlorambucil administered on alternate months. The month with glucocorticoids consisted of 1 g pulse of IV methylprednisolone repeated for three consecutive days, followed by oral prednisone 0.5 mg/kg for 27 days; then, glucocorticoids were stopped and oral chlorambucil (0.2 mg/kg/day) was given daily for one month. Patients with serum creatinine >1.7 mg/dL were excluded. After a mean follow-up of 30 months, 12/32 (37.5%) treated participants were in complete remission, and another 11/32 (34%) in partial remission, as compared with 9/30 (30%) complete or partial remission in the control group. Importantly, this was associated with a stabilisation of kidney function in the treated group. Among treated participants, one developed obesity and one had reversible increase in serum transaminases (22). The benefits of such an approach were also confirmed in the long term: a study with a median follow-up of 5 years demonstrated that the cyclical therapy led to more frequent and sustained remissions of nephrotic syndrome, in comparison with symptomatic treatment (23) The long-term follow-up of the 1984 study showed that the probability of being still alive and free from ESRD, as well as in complete or partial remission after 10 years, was higher in the cyclical therapy group compared to controls (92% vs 83% and 60% vs 30%, respectively) (15).

The efficacy of the cyclical therapy has been further confirmed in an Indian randomized controlled trial, reporting the 10 years outcomes of 93 patients assigned either to a 6-month regimen alternating glucocorticoids and cyclophosphamide or to symptomatic therapy. Survival without dialysis was 89% versus 65%, respectively. At the last follow-up, 62% of the treated participants had complete (32%) or partial remissions, compared with 35% of the controls. The treated arm experienced fewer

TABLE 1 | Randomized controlled studies in Primary Membranous Nephropathy.

Trial (year)	Interventions, number of patients	Follow-up (months)	CR+PR (as per study definition)	p	Relapse rate	p	Adverse events (number)	p
Corticosteroids or alkylating agents versus supportive treatment								
Donadio JV et al. (1974) (18)	Oral CYC (1.5 – 2.5 mg/kg/day) for 1 year (n= 11) Placebo (n=11)	12 from baseline	4/11[$] 2/11[$]	NA	NA	NA	Leukopenia (n=5); nausea (n=4); partial alopecia (n=3)	NA
Collaborative Study of the Adult Idiopathic Nephrotic Syndrome (1979) (19)	PDN 100-150 mg/every other day (according to body weight) tapered in case of response otherwise withdrawn (n=34) Supportive treatment (n=38)	Mean: 23 (range, 4-52)	22/34[%] 11/38[%]	0.002	8/22 NA	NA	Gastrointestinal bleeding (n=1) Psychiatric alterations (n=1)	ns
Cattran DC et al. (1989) (20)	PDN on alternate days (45 mg per square meter of BSA) for 6 months (n=81) Supportive treatment (n=77)	Mean: 48 ± 3.2	Similar proportion throughout the follow-up	ns	NA	NA	Cushingoid features (n=12); glucose intolerance (n=4); mood swings (n=5); excessive weight gain (n=3); gastrointestinal disturbances (n=2); acne (n=2); muscle weakness (n=2); headache (n=1); excessive hair loss (n=1); death (myocardial infarct, n=1) NA	NA
Cameron JS et al. (1990) (21)	PDLN 125 – 150 mg every other day (according to body weight) for 8 weeks (n=52) Placebo (n=51)	Mean: 52	10/52* 7/51*	ns	NA	NA	pulmonary embolism (n=2); stroke (n=2); death (n=1); duodenal ulcer (n=1); peripheral neuropathy (n=1); intracranial hypertension (n=1) neoplasia (n=2, 1 death); perforated ischemic bowel (n=1, 1 death) death for uncertain causes (n=1)	NA
Cyclical regimen versus supportive treatment								
Ponticelli C et al. (1984) (22)	IV MPDN for three days followed by oral MPDN (0.4 mg/kg/day) or PDN (0.5 mg/kg/day) for 27 days alternate to chlorambucil (0.2 mg/kg/day) for one month. Overall duration 6 months (n=33) Supportive treatment (n=30)	Mean: 31.4 ± 18.2 Mean: 31.4 ± 18.2	23/32* 9/30*	0.001	3/26[£] NA	NA	Peptic ulcer (n=1); gastric intolerance (n=1); glucose intolerance (n=1) 2 drop out: progressive renal failure (n=1); high titer of anti-dsDNA (n=1)	NA
Ponticelli C et al. (1989) (23)	IV MPDN (1g/day) for three days followed by oral MPDN (0.4 mg/kg/day) or PDN (0.5 mg/kg/day) for 27 days alternate to chlorambucil (0.2 mg/kg/day) for one month. Overall duration 6 months (n=42) Supportive treatment (n=39)	Median: 60 (range, 24–132)	28/42* 9/39*	NA	NA	NA	Gastrointestinal disturbances (n=3); peptic ulcer (n=2); tremors (n=2); leukopenia n=2); cramps (n=2); infections (n=1); anxiety (n=1); liver disfunction (n=1); diabetes mellitus (n=1); obesity (n=1)	NA
Ponticelli C et al. (1995) (15)	IV MPDN (1g/day) for three days followed by oral PDN (0.5 mg/kg/day) for 27 days alternate to chlorambucil (0.2 mg/kg/day) for one month. Overall duration 6 months. The cycle may be repeated after at least two years from the first treatment (n=42) Supportive treatment (n=39)	Up to 10 years	26/42* 13/39*	NA	4/35[%] NA	NA	Peptic ulcer (n=2); leukopenia (n=2); tremors (n=2); cramps (n=2); infections (n=1); gastric intolerance (n=1); anxiety (n=1); liver disfunction (n=1); obesity (n=1); diabetes mellitus (n=1); death (neoplasia, n=1) Death (cardiac arrest hepato-renal failure, cardiac infarct, n=3)	NA
Jha V et al. (2007) (24)	IV MPDN (1g/day) for three days followed by oral PDLN (0.5 mg/kg/day) for 27 days alternate to oral CYC (2 mg/kg/day) for one month. Overall duration 6 months. (n= 47) Supportive treatment (n=46)	Median: 132 (range, 126 – 144)	34/47[%] 16/46[%]	<0.0001	8/34[%] 4/16[%]	NA	Infections (n=10) leukopenia (n=3); thrombotic events (n=3); death cardiac event, n=1) Infections (n=14); thrombotic events (n=4); death (advanced uraemia n=2, and pneumonia n=1)	ns
Cyclical regimen versus active treatment								
Ponticelli C et al. (1992) (25)	IV MPDN (1g/day) for three days followed by oral MPDN (0.4 mg/kg/day) for 27 days alternate to chlorambucil (0.2 mg/kg/day) for one month. Overall duration 6 months (n=45) IV MPDN (1g/day) for 3 days at the beginning of month	Mean: 54 ± 16 Mean: 54 ± 17	28/45* 18/47*	<0.05 for the first 3 years of follow-up	NA	NA	Gastric discomfort (n=6); leukopenia (n=2); infections (n=2); amenorrhea (n=2) fever (n=1); liver disfunction (n=1); acne (n=1); mild myoclonus (n=1); diabetes mellitus (n=1); death (neoplasia, n=1) Gastric discomfort (n=5); acne (n=2); thrombotic event	NA

(Continued)

TABLE 1 | Continued

Trial (year)	Interventions, number of patients	Follow-up (months)	CR+PR (as per study definition)	p	Relapse rate	p	Adverse events (number)	p
	1, 3 and 5 followed by oral MPDN (0.4 mg/kg every other day). Overall duration of 6 months (n=47)						(n=1); cushingoid appearance (n=1); diabetes mellitus (n=1); death (neoplasia, n=1)	
Ponticelli C et al. (1998) (26)	IV MPDN (1g/day) for three days followed by oral MPDN (0.4 mg/kg/day) for 27 days alternate to chlorambucil (0.2 mg/kg/day) for one month. Overall duration 6 months. (n= 50) IV MPDN (1g/day) for three days followed by oral MPDN (0.4 mg/kg/day) for 27 days alternate to oral CYC (2.5 mg/kg/day) for one month. Overall duration 6 months. (n= 45)	Median: 36 (range, 12–78) Median: 42 (range 12 – 72)	36/44[%] 40/43[%]	0.116	11/36[%] 10/40[%]	NA	Infections (n=6); leukopenia (n=2); bone marrow hypoplasia (n=1); nausea (n=1); glucose intolerance (n=1); amenorrhea (n=1); neoplasia (n=1) Nausea (n=1); cerebral transient ischemic attack (n=1); glucose intolerance (n=1); neoplasia (n=1)	NA
Branten AJ et al. (1998) (27)	IV MPDN (1g/day) for three days followed by oral PDN (0.5 mg/kg/day) for 27 days alternate to chlorambucil (0.15 mg/kg/day) for one month. Overall duration 6 months. (n= 15) Corticosteroids at a comparable dose plus oral CYC (1.5–2 mg/kg/day) for 1 year (n= 17)	Median: 38 (range, 8 – 71) Median: 26 (range, 5 – 68)	2/15* 11/17*	<0.01	NA	NA	Infections (n=8); leukopenia (n=8); thrombocytopenia (n=3); anaemia (n=2); osteonecrosis (n=1); renal artery stenosis (n=1) Infections (n=5); leukopenia (n=3); anaemia (n=2); nausea (n=2); general malaise (n=1)	<0.01
Ponticelli C et al. (2006) (28)	IV MPDN (1g/day) for three days followed by oral MPDN (0.4 mg/kg/day) for 27 days alternate to oral cytotoxic agents (CYC 2.5 mg/kg/day or chlorambucil 0.2 mg/kg/day) for one month. Overall duration 6 months (n= 16) Synthetic ACTH (max 1mg twice per week) for 12 months (n= 16)	Mean: 21.8 ± 7.5 Mean: 21.8 ± 7.6	12/16* 14/16*	ns	7/15[%] 3/14[%]	ns	Leukopenia (n=2); glucose intolerance (n=2) Hyperpigmentation of the skin (n=6); glucose intolerance (n=2); dizziness (n=1); diarrhea (n=1); onycodystrophy (n=1); folliculitis (n=1)	NA
Howman A et al. (2013) (29)	IV MPDN (1g/day) for three days followed by oral PDLN (0.5 mg/kg/day) for 28 days alternate to chlorambucil (0.15 mg/kg/day) for one month. Overall duration 6 months. (n= 33) 12-month course of CyA (5 mg/kg, target through levels 100–200 μg/L.) (n=36) Supportive treatment (n=37)	Until the achievement of the primary endpoint (maximum of 3 years)	Lowest decline of renal function and highest decline of proteinuria in the cyclic regimen arm	0·003	NA	NA	20/33 pts[%]: haematological events (n=28); metabolic events (n=8); dermatological events (n=4); cardiovascular events (n=4); neurological events (n=3); infections (n=3); gastroenterological events (n=3); renal events (n=1) 18/37 pts[%]: infections (n=8); neurological events (n=6); haematological events (n=5); renal events (n=5); gastroenterological events (n=3); cardiovascular events (n=3); dermatological events (n=2); metabolic events (n=1) 16/38 pts[%]: metabolic events (n=5); neurological events (n=4); haematological events (n=3); cardiovascular events (n=3); renal events (n=2); gastroenterological events (n=2) infections (n=2)	NA
Ramachandran R et al. (2017) (30)	TAC (0.1 mg/kg/day, target trough levels 5–10 ng/ml in the first 6 months and 4–8 ng/mL in the next 6 months) for 12 months and oral PDLN (0.5 mg/kg/day) for 6 months, then tapered (n=35) IV MPDN (1 g/day) for 3 days followed by oral PDLN (0.5 mg/kg/day) for 27 days alternate to oral CYC (2 mg/kg/day) for one month. Overall duration: 6 months (n=35)	24 from baseline	21/35* 30/35*	0.03	10/23* 2/31*	0.0069	Infections (n=16); nephrotoxicity (n=8)[#]; diabetes mellitus (n=6); gastrointestinal disturbances (n=5); tremor (n=4); hypertension (n=3) Infections (n=13); amenorrhea (n=5) [#]; diabetes mellitus (n=5); leukopenia (n=4); gastrointestinal disturbances (n=3); hypertension (n=2)	[#]<0.05 Others: ns

(Continued)

TABLE 1 | Continued

Trial (year)	Interventions, number of patients	Follow-up (months)	CR+PR (as per study definition)	p	Relapse rate	p	Adverse events (number)	p
CNIs versus supportive treatment								
Cattran DC et al. (1995) (31)	CyA (3.5 mg/kg/day, target through levels 110–170 µg/L) (n=9) Placebo (n=8)	Mean: 49 (range, 17–75) Mean: 48 (range, 25–88)	6/8* 0/8*	NA	NA	NA	Hypertension (n=6); gastrointestinal disturbances (n=2); infections (n=1); tremor (n=1); hirsutism (n=1) Infections (n=3); hypertension (n=3) gastrointestinal disturbances (n=1); tremor (n=1); hirsutism (n=1)	ns
Praga M et al. (2007) (32)	TAC (0.05 mg/kg/day, target through level 3 – 5 ng/ml, to be increased to 5 – 8 ng/ml in case of lack of response at 2 months; full dose for 12 months, followed by tapering in 6 months) Overall duration: 18 months (n= 25) Supportive treatment (n= 23)	Up to 30 from baseline	19/25[$] 6/23[$]	0.003	9/19* NA	NA	Glucose intolerance (n=4); diarrhea (n=2); nausea (n=1); headache (n=1); tremor (n=1); gouty arthritis (n=1) Glucose intolerance (n=2); chest pain (n=2); infections (n=1)	ns
CNIs versus active treatments								
Cattran DC et al. (2001) (33)	Cya (3.5 mg/kg/day, target through level 125–225 µg/L) for 7 months and low-dose PDN (0.15 mg/kg/day, max 15 mg/day for 26 weeks, withdrawn at week 32) (n=28) Placebo and low-dose PDN (0.15 mg/kg/day, max 15 mg/day for 26 weeks, withdrawn at week 32) (n=23)	Up to 78	11/28* 3/23*	0.007	10/21[%] 3/5[%]	NA	Hypertension (n=10); nausea (n=4) Hypertension (n=5); nausea (n=1)	NA
Chen M et al. (2010) (34)	TAC (0.1 mg/kg/day, target through level 5–10 ng/ml for the first 6 months, then 2–5 ng/ml for 3 months) and PDN (1mg/kg/day for 1 month, withdrawn at month 8). (n=39) Oral CYC (100 mg/day for 4 months) and PDN (1mg/kg/day for 1 month, withdrawn at month 8). (n= 34)	12 from baseline	31/39* 23/34*	ns	6/33[%] 5/23[%]	ns	Glucose intolerance/diabetes (n=12) [#]; infections (n=8) [#], 3 SAE; liver disfunction (n=7, 1 SAE); hypertension (n=5) [#]; gastrointestinal symptoms (n=3, 2 SAE); tremor (n=3); transient worsening of renal function (n=1) Liver disfunction (n=9, 1 SAE); gastrointestinal symptoms (n=1 SAE); infections (n=1)	[#]0.00 Others: ns
Liang Q et al. (2017) (35)	TAC (0.05–0.1 mg/kg/day, target through level 5–10 ng/ml for the first 6 months, then 4–6 ng/ml for 3 months before starting the tapering). Overall duration: 12 months (n=30) IV CYC (0.5–0.75 g/m^2) monthly for the first 6 months, then every 2-3 months plus oral corticosteroids (1mg/kg/day). Overall duration: 12 months (n= 28)	Median: 10 (range, 0.2–18) after the end of the treatment Median: 10.5 (0.3–19) after the end of the treatment	24/30[$] 23/28[$]	ns	3/24[%] 0/23[%]	NA	Glucose intolerance/diabetes mellitus (n=7); leukopenia/anemia (n=2); liver disfunction (n=2); gastrointestinal disturbances (n=2); UTI (n=1)[#] Glucose intolerance/diabetes mellitus (n=19); UTI (n=8)[#]; leukopenia/anemia (n=8); transaminase elevation (n=6); gastrointestinal disturbances (n=5); pneumonia (n=1)	[#]0.01 Others: ns
Ramachandran R et al. (2017) (30)	TAC (0.1 mg/kg/day, target trough levels 5–10 ng/ml in the first 6 months and 4–8 ng/mL in the next 6 months) for 12 months and oral PDLN (0.5 mg/kg/day) for 6 months, then tapered (n=35) iv MPDN (1 g/day) for 3 days followed by oral PDLN (0.5 mg/kg/day) for 27 days alternate to oral CYC (2 mg/kg/day) for one month. Overall duration: 6 months (n=35)	24 from baseline	21/35* 30/35*	0.03	10/23* 2/31*	0.0069	Infections (n=16); nephrotoxicity (n=8)[#]; diabetes mellitus (n=6); gastrointestinal disturbances (n=5); tremor (n=4); hypertension (n=3) Infections (n=13); amenorrhea (n=5) [#]; diabetes mellitus (n=5); leukopenia (n=4); gastrointestinal disturbances (n=3); hypertension (n=2)	[#]<0.05 Others: ns

(Continued)

TABLE 1 | Continued

Trial (year)	Interventions, number of patients	Follow-up (months)	CR+PR (as per study definition)	p	Relapse rate	p	Adverse events (number)	p
Di J et al. (2018) (36)	PDN (0.5 mg/kg/day for 2 months, then tapered to 10 mg/day) and TAC (0.1 mg/kg/day, target through level 5–10 ng/ml for the first 6 months, then 2 – 4 ng/ml). Overall duration: 12 months (n=38) PDN (0.5 mg/kg/day for 2 months, then tapered to 10 mg/day) and TAC (0.1 mg/kg/day, target through level 5–10 ng/ml for the first 6 months, then 2 – 4 ng/ml). Overall duration: 24 months (n=38)	24 months	25/36* 30/36*	<0.05	8/25% 4/30%	<0.05	6 SAEs (infections n=5 pts, of whom 3 died, and interstitial lung disease n=1); glucose intolerance (n=1) Overall incidence: 13.1% vs 15.8%	ns
MMF versus supportive and active treatments								
Chan TM et al. (2007) (37)	MMF 1g bid and PDLN (0.8 mg/kg/day tapered until reaching 10 mg/day at around 4 months, then tapered by 2.5 mg/day every 2 weeks until withdrawal). Overall duration: 6 months (n=11) IV MPDN (1g/day) for three days followed by oral PDLN (0.4 mg/kg/day for 3 weeks, then 0.2 mg/kg till the end of the months) alternate to chlorambucil (0.2 mg/kg/day) for one month. Overall duration 6 months. (n=9)	15 from baseline	7/11% 6/9%	ns	2/7% 1/6%	ns	Dyslipidemia (n=6); infections (n=3); diabetes mellitus (n=1) Leukopenia (n=6)#; dyslipidemia (n=5); infections (n=2); nausea and vomiting (n=1); diabetes mellitus (n=1)	#0.002 Others: ns
Dussol B et al. (2008) (38)	MMF 2g/day for 12 months (n=19) Supportive treatment (n=17)	12 from baseline	7/19* 7/17*	ns	NA	NA	Myalgias (n=4); gastrointestinal disturbances (n=3); anemia (n=2); infections (n=1); cough (n=1); hypotension (n=1); bullous dermatosis (n=1); neoplasia (n=1) Myalgias (n=5); cough (n=2); gastrointestinal disturbances (n=1); anemia (n=1); infections (n=1); hypotension (n=1)	NA
Senthil Nayagam L et al. (2008) (39)	MMF 1g bid for 6 months and PDLN (0.5 mg/kg/day for 8–12 weeks) (n=11) IV MPDN (1g/day) for three days followed by oral PDLN for 27 days alternate to oral CYC (2mg/kg/day) for one month. Overall duration 6 months. (n= 10)	Mean: 18 (range, 15 – 21) Mean: 16 (range, 13 – 19)	7/11% 8/11%	ns	0/7% 1/8%	NA	Infections, liver disfunction, gastrointestinal symptoms, cytopenia (number of pts NA)	NA
Multitarget therapy versus active treatments								
Nikolopoulou A et al. (2019) (40)	TAC (2 mg bid, target through levels 5–12 ng/ml) combined with MMF (500 mg bid, target blood levels 1.5–3 mg/L); after 1 year of remission, MMF was withdrawn, and TAC was tapered over 6 months. Max duration of treatment: 24 months (n=20) TAC (2 mg bid, target through levels 5–12 ng/ml); withdrawn over 6 months after 1 year of remission. Max duration of treatment: 24 months (n=20)	Median: 71 (range, 9 – 106) Median: 72 (range, 6 – 106)	19/20% 16/20%	ns	8/19% 8/16%	ns	Gastrointestinal disturbances (n=2); bleeding/anemia (n=2); cholestatis (n=1); haemorrhoidectomy (n=1) Gastrointestinal disturbances (n=3); AKI (n=3); CNI toxicity (n=2); haematuria/UTI (n=2); blackout (n=1); headache (n=1); infections (n=1); gouty arthritis (n=1)	NA
Rituximab versus supportive and active treatments								
Dahan K et al. (2017) (41)	RTX 375 mg/m^2 at time 0 and at 1-week (n=37) Supportive treatment (n=38)	Median: 17 (IQR, 12.5–24.0) Median: 17 (IQR, 13.0–23.0)	24/37% 13/38%	<0.01	NA	NA	Cardiovascular disorders (n=4); infections (n=1); edema (n=1); pain/fever (n=1); diarrhea (n=1) AKI (n=2); cardiovascular disorders (n=2); pleural effusion (n=1); malignancy (n=1); edema (n=1); asthma (n=1)	ns
Fervenza FC et al. (2019) (42)	RTX 1g on days 1 and 15 (repeated at month 6 if proteinuria reduced more than 25% without experiencing a complete response) (n=65)	24 from baseline	39/65* 13/65*	<0.001	2/39* 18/34*	NA	179 AEs; the most common were: infections (n=22); infusion reactions (n=22) #; pruritus (n=8) # 218 AEs; the most common were: gastrointestinal	#p<0.05 Others: ns

(Continued)

TABLE 1 | Continued

Trial (year)	Interventions, number of patients	Follow-up (months)	CR+PR (as per study definition)	p	Relapse rate	p	Adverse events (number)	p
	CyA (3.5 mg/kg/day, target trough levels 125–175 ng/ml) for 12 months, then tapered and discontinued in 2 months (n=65)						disturbances (n=24)[#]; infections (n=20); worsening of renal function (n=17)[#]	
Fernandez-Juarez et al. (2021) (43)	IV MPDN (1 g/day) for 3 days followed by oral PDN (0.5 mg/kg/day) for 27 days alternate to oral CYC (2 mg/kg/day) for one month. Overall duration: 6 months (n=43) TAC (0.05 mg/kg/day, target trough levels 5–7 ng/ml) for 6 months, then tapered by month 9, plus RTX 1g at day 180 (n=43)	24 from baseline	36/43* 25/43*	0.002	1/36[%] 3/25[%]		NA	239 AEs; the most common were: leukopenia (n=22); infections (n=14); cushing syndrome (n=8) 170 AEs; the most common were: AKI (n=16); infections (n=14); diarrhea (n=13); hyperkalemia (n=6)
				0.04				
Scolari F et al. (2021) (44)	RTX 1g on days 1 and 15 (n=37) IV MPDN (1g/day) for three days followed by oral PDN (0.5 mg/kg/day) for 27 days, alternate to oral CYC (2 mg/kg/day) for one month. Overall duration 6 months. (n= 37)		17/20* 16/22*	ns	3/23[%] 6/27[%]	ns	Infusion reaction/drug intolerance (n=10)[#]; infections (n=6); malignancy (n=2); cardiovascular events (n=1); stroke (n=1); glucose intolerance (n=1) Infections (n=15); leukopenia (n=6); glucose intolerance (n=1); cardiovascular events (n=1); nausea/vomiting (n=1); infusion reaction/drug intolerance (n=1); malignancy (n=1)	[#]<0.01 Others: ns

**, at the end of the follow-up; [£], assessed; [$], after the first treatment; [%], cumulative; [#], different in a statistically significant way compared to the control group (for the p value, see within the text); AE, adverse event; AKI, acute kidney injury; bid, bis in die; BSA, body surface area; CR, complete remission; CyA, ciclosporine A; CNI, calcineurin inhibitors; CYC, cyclophosphamide; IV, intravenously; MMF, mycophenolate mofetil; MPDN, methylprednisolone; NA, not available; ns, not significant; PDN, prednisone; PDLN, prednisolone; PR, partial remission; RTX, rituximab; TAC, tacrolimus; SAE, severe adverse event; UTI, urinary tract infection*

infections as well as lower blood pressure and serum cholesterol levels and a higher quality of life. No case of malignancy was reported (24).

Corticosteroids and Alkylating Agents Versus Active Treatment

In another trial, the effects of the cyclical therapy were compared to those of glucocorticoids alone given with the same schedule and cumulative dosage. After a mean follow-up of 54 months, 28/45 (62%) participants in the cyclical therapy were in complete (30.5%) or partial remission, while, among the 47 participants assigned to glucocorticoids, only 18 (38%) were in complete (17%) or partial remission. Two patients died of cancer, one in the chlorambucil and one in the glucocorticoids group. Other reversible side effects occurred in 9 patients assigned to the cyclical therapy and in 8 assigned to glucocorticoids (25).

Due to concerns related to the potential side-effects of chlorambucil, a randomized controlled trial explored the non-inferiority of cyclophosphamide in the context of the cyclical therapy. In this open-label trial, 87 patients were assigned to receive a 6-month treatment, alternating every other month glucocorticoids with chlorambucil (0.2 mg/kg/24h) or cyclophosphamide (2 mg/kg/24h), respectively. After a median follow-up of 36 months, 12 participants of the 44 assigned to the chlorambucil arm (27%) achieved complete and 24 (55%) partial remission. Among the 43 participants randomized to cyclophosphamide, after a median follow-up of 42 months, 16 achieved complete (37%) and 24 (56%) partial remission. Among responders, 11 patients in the chlorambucil group (30%) and 10 in the cyclophosphamide group (25%) had a relapse, that responded to re-treatment. Six patients in the chlorambucil arm and two in the cyclophosphamide group withdrew treatment due to side-effects; four patients in the chlorambucil group but none in the cyclophosphamide one developed herpes zoster, one patient presented with laryngeal carcinoma four years after chlorambucil therapy, and one developed prostate carcinoma five years after cyclophosphamide therapy. Overall, both treatments showed similar efficacy, but cyclophosphamide appeared to be better tolerated (26). Another prospective randomised study in 32 patients compared two different cyclical regimen treatments: one based on glucocorticoids and chlorambucil, at a lower dose compared to previous studies, and one based on glucocorticoids and cyclophosphamide; the latter was associated with higher response rates and a lower risk of progression towards ESRD (27). Since then, cyclical therapy with cyclophosphamide became more widely employed (51–54).

The role of a cyclophosphamide-based immunosuppressive regimen has also been explored in patients with reduced kidney function. A multi-center randomized controlled trial undertaken in 37 renal units across the UK enrolled 108 patients with deteriorated kidney function, having a creatinine of less than 300 μmol/L (3.4 mg/dl) and at least a 20% decline in renal function measured in the 2 years before study entry. 33 patients received a cyclical therapy with prednisolone and chlorambucil, 37 cyclosporine, and 38 supportive therapy alone. Risk of further 20% decline in kidney function was significantly lower in the prednisolone and chlorambucil group than in the supportive care arm, while this was not significantly different between cyclosporine and supportive treatment; however, serious adverse events were more frequent in the prednisolone and chlorambucil group (29).

In a retrospective study, 9 patients with a baseline serum creatinine ranging from 135 μmol/L to 356 μmol/L (1.5-4 mg/dl) were treated with cyclophosphamide (1 to 2 mg/kg) and 6 of them received concurrent prednisone; they were compared with 17 controls (14 of whom received also prednisone). After a mean follow-up of 83 months, 4 of 9 treated patients achieved a complete remission and 5 a partial one. 1 out of 9 patients in the treatment group (11%) and 10 of the 17 controls (59%) reached ESRD (55). Another group prospectively treated 65 patients with PMN and renal failure (serum creatinine >135 micromol/l, 1.5 mg/dl) with oral cyclophosphamide (1.5-2.0 mg/kg/day for 12 months) and glucocorticoids (methylprednisolone pulses 3 x 1 g, i.v. at months 1, 3 and 5, and oral prednisone 0.5 mg/kg/48 h for 6 months). After a median follow-up of 51 months, 16 patients were in complete and 31 in partial remission, 8 had persistent nephrotic syndrome, one mild proteinuria; of note, 4 patients progressed to ESRD and 5 died. Overall kidney survival was 86% after 5 and 74% after 7 years, compared to 32% after 5 and 7 years in an historical control group. Treatment-related complications occurred in two-thirds of patients, mainly consisting of bone marrow depression and infections. One patient developed bladder cancer and one prostate cancer (56). An alternative regimen has been proposed by Brunkhost et al, who treated 17 PMN patients with a 6-month cyclical therapy scheme, where methylprednisolone was given at a dose of 0.5 g and chlorambucil at a reduced dose of 0.12 mg/kg. After one-year, serum creatinine decreased from 162 to 127μmol/L (1.8 to 1.4 mg/dl) and proteinuria from 16.9 to 5.5 g/d. Side effects were rare and mild (57).

THERAPIES IN THE NEW MILLENNIUM

In 2012 the KDIGO guidelines for PMN recommended that initial therapy should consist of a 6-month course of alternating monthly cycles of oral and intravenous glucocorticoids, and oral alkylating agents. In order to reduce the risk of toxicity, the doses of cyclophosphamide or chlorambucil should be adjusted according to patients' age and estimated glomerular filtration rate (eGFR) (58).

Despite that, and mainly due to the non-negligible risk of toxicity of a cyclical therapy approach, in the last 20 years several new treatment options have been proposed for PMN.

ACTH

Intramuscular administration of natural adreno-corticotropin hormone (ACTH) was one of the earliest treatments used for managing idiopathic nephrotic syndrome in children. In 2004, Berg and Arnadottir reported that synthetic ACTH, 0.75–1 mg twice weekly for nine months, allowed to achieve complete remission in 15 patients with MN and nephrotic syndrome, which was sustained for up to 18–30 months in 14 patients (59).

A small randomized controlled trial compared the six-month cyclical regimen, based on glucocorticoids alternated to an alkylating agent, with intramuscular synthetic ACTH given at a dose of 1 mg twice a week for one year. In the first group, 15 of 16 participants entered complete or partial remission as a first event, versus 14 of 16 in the second group. Median proteinuria decreased from 5.1 g/day to 2.1 g/day and from 6.0 g/day to 0.3 g/day, respectively, in the two arms. No significant side effects were seen in participants assigned to ACTH (28). However, it has to be noted that, although mitigated, ACTH side effects are potentially the same as glucocorticoids.

The role of ACTH in PMN has been further explored in other studies. In a retrospective series, 17 patients were treated with synthetic ACTH for nine months, four patients entered complete remission and seven partial remission. These results were inferior to those observed in historical controls treated with oral cyclophosphamide for one year (60). In the United States, the effects of a natural ACTH gel formulation were assessed in 11 patients with PMN. Two participants entered complete and seven a partial remission, while two failed to respond (61). In another study, 20 patients with MN and nephrotic syndrome received a subcutaneous dose of 40 or 80 IU ACTH twice weekly. At 12 months, proteinuria decreased from 9.1 g/day to 3.9 g/day, with improvement in serum albumin and cholesterol. No significant adverse effects were documented (62). Despite these encouraging results, evidence for a role of ACTH in PMN is still relatively weak and more data are needed. Also, the mechanism of action of ACTH in this context is unclear. It has been hypothesized that this may depend upon activation of melanocortin receptor-1 (MCR-1), which is co-localized with synaptopodin in podocytes. MCR-1 might interfere with catalase and RHO-1 protein activity, consequently regulating cytoskeletal stability and preventing podocyte apoptosis (63). However, it has been shown that, in MCR1-null mice, melanostimulating hormone can reduce proteinuria and protect podocytes from lipopolysaccharide injury *via* a MCR1-independent mechanism (64). Moreover, it is possible that the effects of ACTH are modulated by β-defensins, a new class of melanocortin ligands that can cross talk between MCRs and the immune system (65).

Calcineurin Inhibitors (CNIs)

In the early 1980s, the discovery of cyclosporine revolutionized the treatment of allotransplantation. A few years later, the role of this drug was also explored in PMN. Several observational studies reported a decline in proteinuria from nephrotic to non-nephrotic range and even complete remission in patients with PMN (66–69). A review of 73 patients with PMN who received cyclosporine reported complete remission in 20% of cases, partial remission in 25% and failure in 55% (70). However, disease flares were frequent when cyclosporine was withdrawn or reduced. Moreover, the potential nephrotoxicity of cyclosporine, which is dose- and time-dependent, can raise concerns regarding this treatment option. Two prospective randomised controlled studies investigated the role of cyclosporine in PMN. In a study published in 1995, 17 patients with PMN and worsening of kidney function, after a run-in phase of 1 year, were randomised to cyclosporine or placebo. Treatment with cyclosporine was associated with a significant reduction in the eGFR decline and a sustained improvement in proteinuria. Of note, only one patient per arm received renin-angiotensin-system (RAS) inhibitors (31). A second study compared a 26 week course of cyclosporine and low-dose prednisone (28 patients) with placebo and low-dose prednisone (23 patients). At 26 weeks (primary end-point), remission occurred in 75% versus 22% ($p<0.001$). However, during or after the tapering, 48% and 60% of the patients in remission in the cyclosporine and placebo arms, respectively, relapsed. Remarkably, only 19 patients received RAS-inhibitors during the study (1 cyclosporine and 8 placebo) (33).

Around 10 years after cyclosporine discovery, tacrolimus, another calcineurin inhibitor, was approved for prevention of rejection in organ transplantation. Like cyclosporine, the role of tacrolimus has also been examined in PMN. A few observational studies reported a good rate of partial remissions; however, as with cyclosporine, relapses were frequent after drug withdrawal (71).

Two big retrospective studies supported a role for tacrolimus in the management of PMN. A Spanish multicenter group reported the outcomes of tacrolimus monotherapy at a mean dose of 0.05 mg/kg/d in 122 PMN patients with nephrotic syndrome and stable kidney function. After a mean treatment duration of 17.6 ± 7.2 months, including a full-dose and a tapering period, 102 (84%) patients responded. Among responders, 42% achieved a complete and 58% partial remission (72). Another large Chinese study described outcomes in 408 consecutive patients with PMN and nephrotic syndrome treated with tacrolimus. The cumulative partial or complete remissions after therapy were 50% at 6 and 67% at 24 months. The cumulative complete remission rates were 4%, and 23%, respectively. A relapse occurred in 101 of the 271 (37.3%) patients (73).

The role of tacrolimus in PMN has been further established in randomized controlled trials, that compared it with either supportive or other active treatment. A Spanish controlled trial reported a high remission rate in patients assigned to tacrolimus, in comparison with untreated controls after 18 months of therapy (94% versus 35%), although 50% of the patients relapsed after tacrolimus withdrawal (32). In a Chinese multicenter trial, 73 patients with nephrotic PMN were randomized to tacrolimus plus prednisone for 9 months or cyclophosphamide plus prednisone for 4 months. Remission was reached earlier with tacrolimus, but at 12 months the remission rate was comparable in the two groups. Of note, patients receiving tacrolimus were more likely to develop diabetes, infection, and hypertension (34).

Another Chinese study explored the efficacy of a 12 month course of tacrolimus, compared with a 12 month course of cyclophosphamide and glucocorticoids. During the first year of follow-up, the probability of remission and the average time to remission were similar between the two groups. Relapses occurred only in the tacrolimus group in 3 patients after drug withdrawal, and 2 out of 3 were successfully retreated using the same scheme. The through levels of tacrolimus were significantly

lower in non-responders, compared with patients reaching complete or partial response (3.1 ± 1.1 ng/ml versus 5.8 ± 1.6 ng/ml and 4.8 ± 2.1 ng/ml; p <0.05). The safety profile was better in the tacrolimus group, especially in terms of infections (35).

A single-center randomized trial compared the effects of a 12 month course of tacrolimus plus prednisone versus a cyclical therapy with cyclophosphamide alternated with glucocorticoids in 70 patients with PMN and persistent nephrotic syndrome. At 12 months, remission rates were comparable (71% with tacrolimus vs 77% with cyclical therapy), while at 24 months, 43% of the patients assigned to tacrolimus and 80% of patients assigned to cyclical therapy were in remission. Patients on cyclophosphamide had a significantly higher risk of amenorrhea while those on tacrolimus experienced a greater risk of reversible nephrotoxicity (30).

A Chinese group compared a 12 and a 24 month course of tacrolimus plus glucocorticoids in 76 patients. At the 24 month assessment, the longer course was associated with higher remission rates and a lower incidence of relapses (p <0.05). Of note, six patients did not complete the treatment protocol because of pulmonary infections, that were fatal in 3 of them (36).

In summary, CNIs may be a useful therapeutic option for nephrotic patients with well-preserved kidney function. Most patients experience a remission with a significant reduction in the risk of deteriorating kidney function, however the relapse risk is high when this class of drugs is discontinued.

Mycophenolate Salts

A role for mycophenolate salts has also been proposed in MN. Observational studies involving small numbers of patients reported that mycophenolate mofetil (MMF), usually associated with prednisone, reduced proteinuria. In a retrospective study, MMF, 1 g twice daily, for 12 months was compared to cyclophosphamide, 1.5 mg/kg/d for 12 months. Both groups also received intermittent methylprednisolone pulses and alternate-day prednisone. Cumulative incidence of remission (66% vs 72%) and side effects (75% vs 69%) were similar, but the relapse risk was greater with MMF compared to cyclophosphamide (38% vs 13%) (74). A prospective, controlled, open-label study randomized 20 patients with MN to receive either the association of MMF and prednisolone for 6 months or a cyclical regimen. Remission (complete or partial) rates were 63.6% in the MMF group and 66.7% in the cyclical treatment group, and serum creatinine remained stable during a mean follow up of 15 months. Nephrotic proteinuria relapsed in two patients assigned to MMF and in one to cyclical therapy; chlorambucil resulted in higher risk of leukopenia compared to MMF (37). Similarly, another trial compared the efficacy of MMF with cyclical therapy in 21 nephrotic adults with MN. Of the 11 participants randomized to receive MMF (2 g/day for 6 months) and oral prednisolone (0.5 mg/kg/day for 2-3 months), 7 (64%) achieved complete or partial remission, compared to 8/10 (80%) treated with a 6-month regimen of glucocorticoids alternated with cyclophosphamide every other month (39).

In another controlled trial, 36 patients with primary MN were randomized to MMF (2 grams per day) for one year or symptomatic therapy. At 12 months, there was no difference between the two groups in terms of mean proteinuria reduction, as well as in terms of rate of complete and partial remissions. Serious adverse effects were observed in 4 of 19 (20%) patients receiving MMF (38). A more recent trial assigned 40 patients with PMN and nephrotic syndrome to receive either tacrolimus monotherapy or tacrolimus combined with MMF for 12 months. At the end of the follow-up, 16/20 (80%) patients in the tacrolimus group achieved remission compared to 19/20 (95%) in the tacrolimus/MMF group. Of note, no difference was detected in terms of relapse rate between groups (50% vs 42%, respectively) (40).

In summary, a role for MMF in improving proteinuria has been documented, at least in the short term and in the context of small studies. However, complete remissions are rare, relapses are frequent and long-term benefits are yet to be clarified.

Rituximab – Uncontrolled Experience

Rituximab is a chimeric human/murine monoclonal antibody that binds CD20, a membrane protein expressed on B cells, and induces killing of CD20+ B-cells. The efficacy of rituximab in PMN was initially tested in 9 patients treated with the dose of 375 mg/m^2 every week for 4 weeks: proteinuria decreased from a mean of 8.6± 1.4 g/day to 3.8± 0.8 g/day after 4 weeks (75). In a multi-center study, rituximab at a dose of 375 mg/m^2 every week for 4 weeks was administered to 20 patients with MN and proteinuria > 5 g/day, and treatment was repeated after 6 months (76). Two patients did not respond to the first course and 18 completed the treatment, with proteinuria decreasing from 11.9 to 2.0 grams per day. At the last visit, 4 patients were in complete remission (20%), 12 in partial (60%), 1 had limited response and 1 relapsed. Beck et al. administered rituximab to 25 patients with anti-PLA2R antibodies positive MN; in 17 patients the antibodies declined or disappeared within 12 months after rituximab, obtaining the so called "immunological response". Five of them (29%) achieved complete and 10 (59%) partial remission at 2 years. Among the 8 patients with persistently elevated levels of anti-PLA2R antibodies, none achieved remission after 1 year and 3 experienced partial remission at 2 years (77). A multi-center study collected data of 23 patients with PMN treated with rituximab. At 12 months, complete remission was achieved in 6 (26%) patients and partial in 13 (overall renal response, 82.6%). In 3 patients, nephrotic syndrome relapsed 27-50 months after treatment. Importantly, eGFR <45/ml/min/1.73 m^2 was an independent risk factor for rituximab failure (78). The largest uncontrolled series of PMN patients treated with rituximab was collected by the Bergamo group. Out of 132 patients with MN treated with rituximab and followed for a mean time of 30 months, 84 responded (63.6%), with 43 (32.6%) achieving complete and 41 (31.0%) partial remission. Among the responders, 25 (30%) had a relapse of nephrotic syndrome, with a higher risk of disease flares in patients who achieved partial remission, compared to the ones that achieved complete remission (50% versus 30%). The response rate was similar regardless of anti-PLA2R antibodies positivity; however, re-emergence of circulating antibodies predicted relapse of the

disease (79). No treatment-related serious adverse events were reported, although, in a previously published cohort of 100 PMN patients treated with rituximab, the same group described four deaths, three patients who developed cancer, four progressions to ESRD and 8 patients with serious cardiovascular events. However, it has to be noted that these adverse events were observed in the context of a significant burden of previous immunosuppression (80).

The use of rituximab in PMN has also been studied in combination with other immunomodulatory approaches. A cohort of 10 patients with MN and proteinuria > 10 grams per day was treated with rituximab, plasmapheresis and iv immunoglobulins, with achievement of partial remission in 90% of the cases (81). In another observational study, 15 patients with MN received a combination treatment with oral cyclophosphamide for 8 weeks, prednisone at 60 mg daily, slowly tapered at 6 months, and rituximab 1000 mg 2 weeks apart followed by 1000 mg every 4 months for two years. Among treated patients, 93% achieved complete remission at a median time of 13 months. Three patients experienced reversible serious side effects: severe neutropenia and viral infection in two cases and altered mental status in one patient (82). A meta-analysis of 8 studies including 542 patients with MN showed that, in comparison with controls who received different treatments, rituximab improved the total remission rate, achieving a higher rate of complete remission while reducing the anti-PLA2R antibody titre. Adverse events were mostly mild in nature, and serious adverse events rare (83). Of note, an increased risk of severe infections in rituximab treated patients has been found in those with CKD, diabetes, or hypogammaglobulinemia (84–87). Moreover, several studies reported late-onset neutropenia, occurring usually several months following the administration of rituximab (88–90).

The effect of the cumulative rituximab dose on response in PMN is unclear, with conflicting results across different cohorts (91, 92). In a study, different protocols of rituximab were used in patients with PMN. Among 55 participants, 28 received two infusions of rituximab 1g at 2-week intervals, whereas the other 27 participants received two infusions of 375 mg/m^2 at 1-week interval. Remission occurred in 24 patients (86%) in the first group versus 18 (67%) in the second group and the median time to remission was 3 and 9 months, respectively. This data suggested that higher rituximab dose may be more effective in PMN (93). Another study compared efficacy and safety of 3 different treatment regimens: low-dose rituximab (one dose of 375 mg/m^2), standard dose (four weekly doses of 375 mg/m^2) and controls treated with the cyclical regimen. At 24 months, a significant improvement in proteinuria was observed in all groups (from 7.5 g/d to 0.21 g/d in the low-dose rituximab, from 5.1 to 0.35 g/d in the standard-dose rituximab and from 8.27 to 2.2 g/d in the cyclical regimen group) (94). In a recent retrospective study, 60 patients with PMN were treated with rituximab, administered over a 2-year period, combined with an initial short course of low dose oral cyclophosphamide and a rapidly tapered course of prednisone. By 2 years, all patients reached partial remission, and 83% complete remission; response to treatment was durable with 90% of patients remaining relapse-free. In addition, all patients achieved immunological remission by 6 months after starting therapy. Adverse events were infrequent with the most common being late-onset neutropenia (95).

Rituximab Versus Supportive and Active Treatments

The randomized controlled trial GEMRITUX, including 75 patients with biopsy-proven MN, compared non-immunosuppressive antiproteinuric treatment alone with non-immunosuppressive antiproteinuric treatment plus rituximab 375 mg/m^2 on days 1 and 8. There was no difference in remission rates at 6 months. However, during the observational phase, complete or partial remission was achieved in 24 of 37 (64.9%) patients in rituximab group versus 13 of 38 (34.2%) controls, with a median time to remission of 7 months. Of note, the "immunological response" of anti-PLA2R antibodies predicted response to treatment. Eight serious adverse events occurred in each group (41). In the randomized controlled trial MENTOR, 130 patients with PMN, proteinuria ≥ 5 g/d and creatinine clearance ≥ 40 ml/min/1.73 m^2, were randomized to receive intravenous rituximab (two infusions, 1000 mg each, administered 14 days apart; repeated at 6 months in case of signs of partial response) or oral cyclosporine (starting at a dose of 3.5 mg/kg/d for 12 months), after a run-in phase with RAS inhibitors for at least 3 months. Cyclosporine was then tapered over two months (months 12-14) and patients were followed for 24 months. At 12 months, 39 of 65 patients (60%) in the rituximab group and 34 of 65 (52%) in the cyclosporine group had a complete or partial remission, while at 24 months, 39 patients (60%) in the rituximab group and 13 (20%) in the cyclosporine group had a complete or partial remission ($p<0.001$). Remarkably, the "immunological response" was quicker, more frequent, and more sustained in the rituximab group. Serious adverse events occurred in 11 patients (17%) in the rituximab group and in 20 (31%) in the cyclosporine group (42). The efficacy of rituximab and cyclosporine was therefore similar. However, when cyclosporine was withdrawn, the relapse rate was high, suggesting that the beneficial effect of rituximab is longer in the context of cyclosporine withdrawal (96). This finding, similar to what observed when comparing cyclical therapy and CNIs, may question the role of the latter as first-line agents, at least when the goal of treatment is sustained remission (97).

In another recently published randomized, open-label controlled trial (STARMEN), conducted in Spain and the Netherlands, 86 patients with PMN and persistent nephrotic syndrome after a 6-month observation period were assigned to receive a 6-month cyclical treatment with glucocorticoid and cyclophosphamide or sequential treatment with tacrolimus (full-dose for 6 months and tapering for another 3 months) and a single infusion of rituximab (1 gram at month 6). Primary outcome was complete or partial remission of nephrotic syndrome (composite endpoint) at 24 months (43). The primary outcome occurred in 36 patients (83.7%) in the glucocorticoid-cyclophosphamide group and in 25 patients (58.1%) in the tacrolimus-rituximab group (relative risk [RR] 1.44; 95% confidence interval [CI] 1.08 to 1.92). Complete

remission at 24 months occurred in 26 patients (60%) in the glucocorticoid-cyclophosphamide group and in 11 patients (26%) in the tacrolimus-rituximab group (RR 2.36; 95%CI 1.34 to 4.16). The "immunological response" was quicker in the cyclical regimen group and associated with remission at 24 months. Relapses occurred in one patient (2.7%) in the cyclical regimen group, and three (12%) in the tacrolimus-rituximab group. There were more adverse events in the cyclical regimen group, although the rate of serious adverse events was similar in both arms. This study provided evidence that cyclical treatment with glucocorticoid and cyclophosphamide is superior to a combination of tacrolimus and rituximab in inducing persistent remission in PMN. It should be noted that almost 60% of patients treated with tacrolimus-rituximab had a good clinical response, and few responders relapsed after discontinuing tacrolimus. This supports a potential role for rituximab in preventing the occurrence of relapses if administered at the time of calcineurin inhibitors withdrawal (98). The pilot trial RICYLO performed a head-to-head comparison of rituximab (1 g two weeks apart) and cyclical regimen in 74 patients, with the primary outcome of complete remission at 12 months. The study failed to show statistically significant differences in remission rates for the two groups (complete remission 32% vs 16% in the cyclical regimen and rituximab arms, respectively [OR 0.40, 95%CI 0.13-1.23]; combined complete and partial remission 62% and 73% [OR 0.61, 95%CI 0.23-1.63]). The time to complete or complete and partial remission up to month 24 in the two groups was similar (p=0.78 and p=0.47, respectively). No differences in adverse events were detected in the two arms. Taken together, the results of this pilot study suggest a similar effectiveness of the two regimens (44).

HOW TO TREAT PATIENTS WITH PMN?

Retrospective studies and the recently published randomized control trials showed that the efficacy of rituximab in PMN is similar to the cyclical regimen and CNIs, which have been historically considered as first-line approach in the management of the disease. Moreover, compared to CNIs, rituximab (similarly to the cyclical regimen) is more effective in inducing sustained remission, while the risk of relapse is high when CNIs are withdrawn. In this context, an approach of "consolidation" of remission with rituximab administration at the moment of CNIs tapering may be an attractive option to prevent relapses. Of note, the favourable safety profile of rituximab makes this drug a good first-line candidate for management of the disease. The recently published KDIGO 2021 Guideline for the management of glomerular diseases (99) suggests rituximab or CNIs as first-line approaches for patients at moderate risk of progressive loss of renal function (defined as normal and stable eGFR after diagnosis), while rituximab or cyclical regimen or CNIs with rituximab are recommended for patients at high risk (reduced eGFR, or proteinuria >8g/day, or normal eGFR associated with serum albumin <25 g/l, or PLA2R antibodies >50 RU/ml). On the other hand, the cyclical regimen is advised as first-line approach for patients at very high risk (life-threatening nephrotic syndrome or rapid deterioration of kidney function). The use of a risk stratification approach to guide therapy is indeed a new concept compared to the 2012 KDIGO Clinical Practice Guideline for Glomerulonephritis, where the initial recommended treatment was, for all patients eligible for immunosuppression, a 6 month course of cyclical therapy, with CNIs being the only alternative first-line approach.

Rituximab is therefore due to be considered the first therapeutic line for the majority of patients with PMN, even in the context of reduced, although stable, renal function (100). However, further issues remain still unresolved.

First, it has to be clarified if biomarkers may contribute to inform the treatment strategy. In fact, reports support a higher efficacy of cyclophosphamide-based regimens in inducing "immunological remission", compared to rituximab, in patients with high anti-PLA2R antibodies titres (101). The best approach in this context is yet to be confirmed; of note inadequate rituximab dose might have played a role.

It is also unclear what the optimal dosing strategy of rituximab is. Importantly, patients with higher proteinuria and lower serum albumin at time of rituximab infusion show lower residual serum rituximab levels at month-3 (102), and this was associated with subsequent lower response rates (103). These findings support the idea that rituximab dose can affect the

TABLE 2 | Main baseline characteristics, therapeutic schedule and response rates in the rituximab arms of randomized control studies exploring rituximab effectiveness in primary membranous nephropathy.

Study	Reference	Proteinuria	PLA2R-Ab	RTX dose	CR+PR 6 months	CR+PR 12 months
GEMRITUX	Dahan K et al. (2017) (41)	7.7 g/g (4.6-10.4)*	41 (0-276)$	375 mg/mq^2 day 1 and day 8	35.1%	NA£
MENTOR	Fervenza FC et al. (2019) (42)	8.4 g/day (6.8-12.3)$	409 (163-834)&	1 g day 1 and 15; repeated at month 6 if no CR and proteinuria reduction >25%°	35%	60%
RICYCLO	Scolari F et al. (2021) (44)	6 g/day (4-10)$	63 (52-87)	1 g day 1 and 15	51%	62%

**: protein to creatinine ratio; median (IQR).*

$: Median (IQR).

£: 65% after a median of 17 months (IQR 12.5-24).

&: different ELISA method compared to GEMRITUX and RICYCLO.

°: 37/65 patients retreated.

PLA2R-Ab, phospholipase A2 receptor antibodies; RTX, rituximab; CR, complete remission; PR, partial remission.

response, especially in patients with higher proteinuria. However, as already discussed, findings from retrospective studies are contradictory in this respect. The three prospective studies available so far employed different therapeutic strategies (**Table 2**); however, despite all three trials included patients with full-blown nephrotic syndrome, differences in terms of baseline characteristics need to be acknowledged, and prevent the possibility of performing a direct comparison across them. The issue of the optimal dosing regimen therefore remains unresolved. Dedicated prospective studies will be needed, ideally including homogenous cohorts of patients in terms of disease severity, proteinuria, kidney function, anti-PLA2R antibodies titre and histological characteristics.

Other uncertainties regarding the use of rituximab in PMN are how to identify patients that may experience relapses, and how this subgroup should be managed. For this purpose, the re-appearance of anti-PLA2R antibodies is well defined as predictor of flares (79), although the role of other biomarkers, such as the kinetics of CD20+ B-cells and their subsets, will need to be studied further.

With humanized anti-CD20 monoclonal antibodies becoming more easily available on the market, a rising issue is how to manage rituximab-resistant patients. For rituximab-treated patients with resistant or early relapsing disease, the presence of anti-rituximab antibodies should be considered. In this context, testing of such antibodies could identify patients that may benefit from further rituximab administrations (anti-rituximab antibodies undetectable) (104) or from humanized anti-CD20 monoclonal antibodies (105, 106).

Finally, it is also uncertain how to manage patients with refractory or multi-relapsing disease courses. In this setting, the role of pre-emptive rituximab administration, as well as of alternative immunomodulating approaches with less clear evidence, such as ACTH or MMF, needs to be further investigated.

CONCLUSIONS

PMN is a rare disease that requires immunosuppressive treatment in patients not achieving spontaneous remission. Achievement of remission (ideally complete) is advised in order to reduce the risk of ESRD and the complications of nephrotic syndrome *per se*. Rituximab, CNIs or the cyclical regimen are considered as first-line therapeutic approaches. In keeping also with the recently published KDIGO 2021 Guideline for the management of glomerular diseases (99), the cyclical regimen is to be reserved for patients at high risk of progressive loss of renal function or life-threatening nephrotic syndrome. For patients requiring CNIs, prolonged treatment at low doses needs to be considered, due to the high relapse rate following their withdrawal; the long-term risk of nephrotoxicity of such an approach has to be taken into account. Of note, in the case of CNI withdrawal, administration of rituximab at the time of CNI tapering may reduce the risk of relapse. Further studies are required to confirm the role of other immunomodulatory approaches and to identify patients more likely to benefit from the different first-line therapeutic options available, as well as to determine the ideal rituximab induction regimens and the timing of re-treatment.

AUTHOR CONTRIBUTIONS

FS, FA, FM, EDB, HT, MP and CP drafted and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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APPENDIX

Although the definitions of complete and partial remission have been established by international guidelines (58), the different randomized clinical trials in primary membranous nephropathy have applied slight modifications. For instance, the MENTOR trial (42) defined complete remission as proteinuria of no more than 0.3 g/24h and a serum albumin level of at least 3.5 g/dl, whilst the study by Ramachandran et al. (30, 107) used a proteinuria <500 mg/24h with normal serum albumin (≥3.5 g/dl) and serum creatinine. Likewise, the STARMEN (43) employed a proteinuria ≤0.3 g/24h but also included a stable kidney function defined as an estimated glomerular filtration rate (eGFR) ≥45 ml/min/1.73m2 whereas the RI-CYCLO (44) trial only applied the proteinuria parameter (≤0.3 g/24h). On the other hand, partial remission in the MENTOR (42) study was defined as a reduction in proteinuria of at least 50% from baseline plus final proteinuria between 0.3 g–3.5 g/24h regardless of creatinine clearance or serum albumin level, while Ramachandran et al. (30, 107) used a 24h urine protein ≥500 mg/24h, but <2 g/24h or <50% of baseline with normal serum albumin (≥3.5 g/dl) and serum creatinine. Additionally, the STARMEN (43) defined partial remission as a reduction of proteinuria >50% from baseline and a value <3.5 g/24h plus stable kidney function (eGFR ≥45 ml/min/1.73m2) and the RI-CYCLO (44) employed a proteinuria of at least 50% lower than the baseline and ≤3.5 g/24h without including albumin or creatinine levels.

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Concurrent IgA Nephropathy and Membranous Nephropathy, Is It an Overlap Syndrome?

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IgA nephropathy (IgAN) and membranous nephropathy (MN) are common glomerulonephritis, the presence of which in the same patient– concurrent of IgAN and MN (cIgAN/MN) has been described occasionally. This study aims to show clinical-pathological features of cIgAN/MN and attempts to suggest underlying pathogenesis using disease-specific biomarkers and a genomics approach. This retrospective cohort study described the clinical and pathological data from 137 patients with cIgAN/MN diagnosed in Peking University First Hospital from 2005 to 2019. One hundred primary IgAN and 100 MN cases were randomly selected as disease controls between the same time interval. Moreover, disease-specific biomarkers and polygenic risk score models were conducted to reveal the underlying pathogenesis. The median age of the cIgAN/MN cases was 45-year-old, and 46% were women. Compared to IgAN, patients with cIgAN/MN had a higher level of 24-hour proteinuria excretion but lower microscopic hematuria. They had a lower median level of galactose-deficient IgA1 (Gd-IgA1, 4.00 versus 5.45 μg/ml, P=0.002) as well as the standardized genetic risk scores of developing IgAN (GRSs: 0.05 versus 0.68, P<0.001). Compared to MN, patients with cIgAN/MN had a lower proportion of nephrotic syndrome and a lower level of albumin-to-creatinine ratio. However, the 24-hour proteinuria levels, serum lipid profiles, proportion of hypertension, and pathology classification were similar. Patients with cIgAN/MN had lower levels of plasma autoantibodies against the M-type transmembrane phospholipase A2 receptor (PLA2R) (11.23 versus 36.59 U/ml, P=0.005). Intriguingly, there were no statistical differences in standardized GRSs of developing MN between them (2.77 versus 3.02, P=0.326). Compared to IgAN, cIgAN/MN may lean towards MN more according to clinical-pathological features, disease-specific biomarker levels, and disease-specific genetic risk scores.

Keywords: IgA nephropathy, primary membranous nephropathy, galactose-deficient IgA1, anti-phospholipase A2 receptor, polygenic risk score

1 INTRODUCTION

Immunoglobulin A nephropathy (IgAN) and membranous nephropathy (MN) are two types of common glomerulonephritis (GN) worldwide. It was reported that IgAN is the most common GN in patients less than 59-year-old, and MN is the most frequently observed GN in patients at age ≥60 years (1). These two diseases differ in clinical features, pathology, treatment, and prognosis. IgAN is more unlikely to develop hypo-albuminemia but more likely to present episodes of gross hematuria. IgAN can only be diagnosed with a kidney biopsy (2). However, a kidney biopsy may not be required to confirm the diagnosis of MN in patients with a compatible clinical and serological presentation like antibodies against PLA2R according to the 2021 KDIGO guideline (3, 4). Proteinuria reduction to <1g/d is a surrogate marker of improved kidney outcome in IgAN, and immunosuppressive drugs should only be considered in patients with IgAN who remain proteinuria >1g/24h despite at least 90 days of optimized supportive care. Differently, immunosuppressive therapy may not be required in patients with MN when proteinuria <3.5 g/d and eGFR >60 ml/min/1.73 m^2. Thus, elucidating the underlying disease is pivotal in guiding management and treatment decisions, which seems to be difficult when two diseases coexist in the same patient.

It is suggested that concurrent IgAN and MN (cIgAN/MN) in the same patient is rare, but more and more cases have been reported since 1983 (5–11). From the literature, all the patients had hematuria and nephrotic range proteinuria. In our previous reports on clinical-pathological features of 26 patients with cIgAN/MN, we observed that these patients displayed similar clinical features with MN patients and milder pathological lesions than IgAN patients (12), and they had comparable serum levels of Gd-IgA1 with IgAN, but lower detectable serum levels of anti-PLA2R compared with MN. These patients showed characteristics of both diseases. We thus suggested that cIgAN/MN might result from superimposed MN on a background of preexisting mild IgAN. However, the limitations of the previous studies are the small sample size and lack of pathogenesis investigation.

Thus, the aim of this study is to show clinical-pathological features with a larger sample size in 137 patients of cIgAN/MN and attempt to suggest underlying disease pathogenesis using disease-specific biomarkers and a genomics approach. Any attempts to address the issue should be of clinical relevance as different treatment strategies may lead to different prognoses.

2 ARTICLE TYPES

Original Research.

3 MATERIALS AND METHODS

All the study protocols complied with the principles of the Declaration of Helsinki and were approved by the Ethics Committee of Peking University First Hospital (Institutional Review Board number: 2021[Y148]). Written informed consent was obtained from all the participants.

3.1 The Process to Select Patients With cIgAN/MN

A retrospective cohort study (2005-2019) was established in Peking University First Hospital to study the features of patients with concurrent cIgAN/MN. First, patients diagnosed with IgAN from November 2005 to June 2019 were included in the study cohort (n=10387). The diagnosis of IgAN was based on dominant staining for IgA in the glomerular mesangium on immunofluorescence microscopy and electron-dense deposits in the mesangium on electron microscopy. Patients who were secondary IgAN, such as Henoch-Schonlein purpura (IgA vasculitis), systemic lupus erythematosus, thin basement membrane disease, Alport syndrome, Fabry disease, or without biopsy samples in electron microscope were excluded from our study (n=912).

Secondly, we screened those patients with concurrent MN (n=180). The diagnosis of cIgAN/MN was additionally confirmed by kidney biopsy with membranous thickening of the glomerular capillary wall; dominant staining for IgG, C3 in glomerular capillary walls on immunofluorescence microscopy; the existence of subepithelial electron-dense on electron microscopy (**Figure 1**). Patients who were secondary MN, like autoimmune disease (lupus erythematosus), infection with hepatitis B, hepatitis C, or syphilis, certain medications (gold/mercury salts and nonsteroidal anti-inflammatory drugs) or solid cancerous tumors or blood cancers were excluded from our study (n=43). The flowchart of the recruitment process was displayed in **Figure 2**.

3.2 The Selection of the Disease-Control Groups

Meanwhile, we searched our database for disease-control groups from the same time period. One hundred patients with primary MN and 100 with primary IgAN were retrieved using the stratified random sampling method as disease controls based on the year of hospital admission.

3.3 General and Clinical Information of All the Enrolled Patients

Demographics and clinical features at the time of renal biopsy performance included age, gender, blood pressure, urinary sediment microscopy, 24-hour urine protein excretion, serum levels of IgA and IgG, uric acid, lipid profiles, and creatinine. Hyperlipidemia was defined as the total cholesterol above 6.2 mmol/L or triglycerides above 2.3 mmol/L. The estimated glomerular filtration rate (eGFR) was calculated by the Chronic Kidney Disease Epidemiology Collaboration equation (13).

3.4 Pathological Features

All the kidney sections were processed for immunofluorescence examination, light microscopy, and electron microscopy. Pathological data were also recorded for each patient,

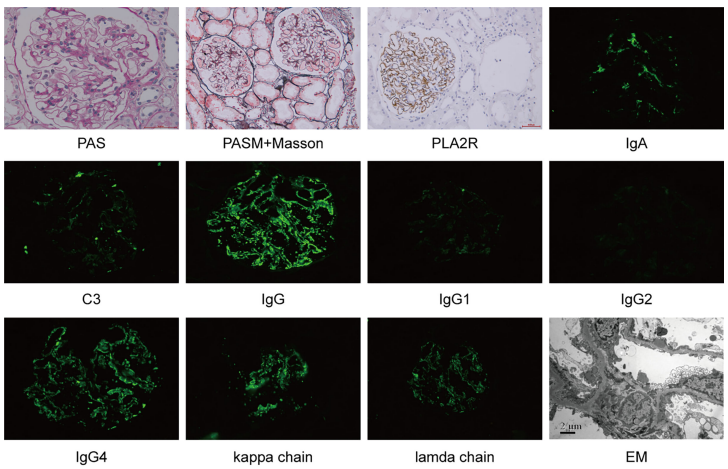

FIGURE 1 | Kidney biopsy from a case with concurrent IgAN and MN. PAS, Periodic Acid-Schiff; PASM, periodic acid-silver methenamine; EM, electron microscope.

including the degree of mesangial cell proliferation, the proportion of glomeruli with cellular/fibro cellular/fibrous crescents, the intensity of immunofluorescence staining (IgG/C3/IgA), and electron-dense deposits on the mesangial area or glomerular basement membrane (GBM). Because in the original development of Oxford classification, those with secondary causes of mesangial IgA deposits or those with comorbid conditions were excluded. The Oxford MEST scores may be not proper in evaluating pathology lesions in patients with cIgAN/MN, which were not reported here. The pathological stages of MN were based on the electron microscopic findings, referring to the immune deposits in the GBM, GBM reaction to the deposits, and resolution of glomerular injury with resorption of the deposits (14).

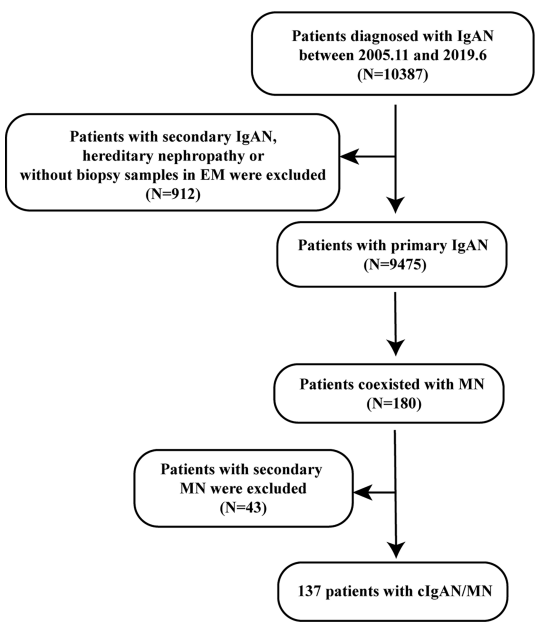

FIGURE 2 | Flowchart of the recruitment process.

3.5 Assay of Gd-IgA1 and IgA1

Levels of plasma Gd-IgA1 (1:200 dilution) were detected using the KM55 ELISA kit (27600, Immuno-Biological Laboratories, USA). All the experiments were done according to the manufacturer's instructions. Moreover, plasma IgA1 levels were determined using capture ELISA. High-absorption polystyrene plates (Thermo, USA) were coated with 2.5 mg/ml F(ab')2 fragment of goat anti-human IgA (Jackson ImmunoResearch, USA) overnight at 4°C. After washing and blocking with 1% bovine serum albumin in PBS with 0.1% Tween, the diluted plasma (1:80000) was added for incubation. The diluted plasma was then detected by the anti-human IgA1 horseradish peroxidase-conjugated antibody (25-783-72807, Gentaur, Belgium). The optical density at 450 nm was measured after the tetramethylbenzidine liquid substrate system was applied.

3.6 Assay of Anti-PLA2R Antibodies

Circulating anti-PLA2R antibodies were detected by using commercial ELISA kits (EUROIMMUN AG, Lübeck, Germany). The results were negative for <20 units (U)/mL and positive for ≥20 U/mL (15).

3.7 Single Nucleotide Polymorphisms (SNPs) Selection and Genotyping

Disease-specific genetic risk scores were calculated to deepen our understanding of the pathogenesis of cIgAN/MN. As previously reported, the IgAN risk score equation was based on the 15 SNPs associated with IgA nephropathy in the analysis of 20,612 individuals from 14 international case-control cohorts of European and Asian ancestry (16). The risk score was standardized using genotypes of 1,050 individuals from 52

worldwide populations included in the Human Genome Diversity Project (17). The MN risk score equation was derived from GWAS analysis of 12,820 individuals (3,782 primary MN and 9,038 controls), including 4,841 individuals of East Asian ancestry (1,632 cases and 3,209 controls) and 7,979 individuals of European ancestry (2,150 cases and 5,829 controls). We adopted the East Asians risk score, which is calculated separately for East Asians and standard-normalized using genotypes of healthy ancestry-matched controls (18).

Peripheral blood samples were collected from participants using anticoagulant EDTA. We obtained the genomic DNA from peripheral blood leukocytes using the salting-out method. Genotyping was performed using the Kompetitive Allele Specific PCR (KASP) assays (Compass Biotech, Tianjin, China) among 132 patients with cIgAN/MN, 96 with MN, and 98 with IgAN.

3.8 Genetic Risk Score Assessment

Individuals with 100% non-missing genotypes across all the scored loci were analyzed. The weighted genetic risk score (GRS) assessment was adopted to evaluate individual disease risk. The weighted-GRS utilized the allelic odds ratios to account for the strength of the genetic association within each allele because alleles might have different odds ratios. The weighted GRS was the weighted sum of risk allele counts, where the weight for each SNP was the natural log of the odds ratio. Details about the calculation formula were derived from http://www.columbiamedicine.org/divisions/kiryluk/resources.php.

3.9 Follow-Up Data

Follow-up was defined as the interval between renal biopsy and the last outpatient visit. Full details about the follow-up data, such as general clinical course, medication history, renal function, and urinalyses, were recorded. Time-averaged proteinuria (TA-proteinuria) was calculated as the weighted mean of all the 24-hour urine protein excretion measurements during follow-up, with the weight representing the time elapsed since the previous measurement (19). Patients were also classified according to the magnitude of TA-proteinuria (>1.0 or ≤1.0 g/d). Complete remission was defined as urinary protein excretion <0.5 g/24 h confirmed by two values at least one month apart, accompanied by a normal serum albumin concentration and normal serum creatinine level. Partial remission was defined as urinary protein excretion <3.5 g/24 h and reduced by at least 50% from peak values, accompanied by an improvement or normalization of the serum albumin concentration as well as stable serum creatinine.

3.10 Statistical Analysis

Continuous variables in this study were compared using an unpaired *t*-test or analysis of variance (ANOVA) between groups if the variables were normally distributed; otherwise, a Mann–Whitney U test or Kruskal-Wallis test was performed. Categorical variables were compared using the chi-square test or Fisher's exact test. Cumulative proteinuria remission rates were calculated according to the Kaplan-Meier method (log-rank test). The statistical analysis was performed with the STATA 15.0 (Texas, USA). A two-tailed *P*-value <0.05 was considered statistically significant.

4 RESULTS

4.1 Demographics

From November 2005 to June 2019 in Peking University First Hospital, a total of 9475 primary IgAN patients and 9335 MN patients were diagnosed, of which 137 patients met the diagnostic criteria of cIgAN/MN, accounting for 1.45% and 1.47% of the Chinese patients with IgAN and MN, individually.

Patient demographics were summarized in **Table 1**. Patients with cIgAN/MN had a median age of 45 years. Patients with cIgAN/MN were older than IgAN (35 years, $P<0.001$) but younger than MN (50.5 years, $P<0.001$), who were randomly selected at the same time interval. Among 137 cases with cIgAN/MN, there were 74 males (54.01%) and 63 females (45.99%). No gender distribution difference was observed among IgAN, cIgAN/MN, and MN.

4.2 Clinical Profiles of cIgAN/MN Compared to IgAN and MN

Compared to IgAN, patients with cIgAN/MN had higher 24-hour proteinuria excretion (3.96 versus 1.22 g/24h, $P<0.001$) but a similar urinary albumin-to-creatinine ratio (617.64 versus 458.04 mg/g, $P=0.247$). They showed less microscopic hematuria (25 versus 95 red blood cells/μl, $P<0.001$) and a lower incidence of gross hematuria (2.19% versus 27%, $P<0.001$). They showed better kidney function, with lower serum creatinine (63.89 versus 84.40 μmol/L, $P<0.001$) and higher eGFR level (105 versus 87 mL/min/1.73m^2, $P<0.001$) at the time of kidney biopsy. They had a higher frequency of hypertension (39.85% versus 22%, $P=0.004$).

Compared to MN, they showed comparatively lower 24-hour proteinuria excretion but with marginal significance (3.96 versus 4.63 g/d, $P=0.060$) and presented significantly less frequency of nephrotic syndrome (47.45% versus 72%, $P<0.001$). They had a lower urinary albumin-to-creatinine ratio (617.64 versus 1552.31 mg/g, $P=0.032$). Reversely, they had higher levels of serum albumin (28.10 versus 25.35 g/L, $P=0.025$). Serum total cholesterol concentration (7.20 versus 6.85 mmol/L, $P=0.977$), low-density lipoprotein cholesterol concentration (4.14 versus 4.12 mmol/L, $P=0.931$), and the proportion of hyperlipidemia (78.63% and 82.65%, $P=0.459$) did not show a significant difference between them. Patients with cIgAN/MN also showed better kidney function, with lower serum creatinine (63.89 versus 75.50 μmol/L, $P<0.001$) and higher eGFR level (105 versus 96 mL/min/1.73m^2, $P<0.001$) at the time of kidney biopsy compared to MN.

4.3 Pathological Features

Immunofluorescence showed that compared to IgAN, patients with cIgAN/MN had much weaker intensity of IgA deposition in

TABLE 1 | Demographics and clinical features of patients with MN, cIgAN/MN, and IgAN.

Characteristics	MN (n = 100)	cIgAN/MN (n = 137)	IgAN (n = 100)	*P* (cIgAN/MN vs. MN)	*P* (cIgAN/MN vs. IgAN)
Demographics					
age (y)	50.50 (41.00, 62.50)	45.00 (36.00, 55.00)	35.00 (29.00, 43.00)	<0.001	<0.001
female n(%)	42 (42.00%)	63 (45.99%)	47 (47.00%)	0.542	0.877
clinical features					
nephrotic syndrome n(%)	72 (72.00%)	65 (47.45%)	10 (10.00%)	<0.001	<0.001
proteinuria (g/24h)	4.63 (3.00, 7.89)	3.96 (2.06, 6.00)	1.22 (0.65, 2.75)	0.060	<0.001
albumin-to-creatinine ratio (mg/g)	1552.31 (391.77, 3070.74)	617.64 (254.30, 1764.42)	458.04 (237.33, 807.56)	0.032	0.247
serum albumin (g/L)	25.35 (21.80, 30.15)	28.10 (22.50, 33.80)	38.40 (35.05, 41.75)	0.025	<0.001
hyperlipidemia n(%)	81 (82.65%)	92 (78.63%)	30 (30.30%)	0.459	<0.001
total cholesterol (mmol/L)	6.85 (5.78, 8.50)	7.20 (5.70, 8.62)	4.61 (4.05, 5.25)	0.977	<0.001
triglycerides (mmol/L)	2.52 (1.60, 3.39)	2.03 (1.38, 2.82)	1.50 (1.15, 2.01)	0.046	<0.001
LDL-C (mmol/L)	4.12 (3.04, 5.42)	4.14 (3.00, 5.40)	2.71 (2.22, 3.30)	0.931	<0.001
serum IgG (g/L)	5.84 (4.39, 7.98)	7.05 (5.56, 8.98)	9.94 (8.29, 11.40)	0.016	<0.001
uric acid (μmol/L)	368.58 (101.03)	351.35 (98.03)	363.68 (90.42)	0.205	0.338
serum creatinine (μmol/L)	75.50 (62.50, 90.36)	63.89 (54.30, 78.00)	84.40 (65.50, 117.10)	<0.001	<0.001
eGFR (mL/min/1.73m^2)	96.00 (77.50, 106.00)	105.00 (94.00, 119.00)	87.00 (63.50, 112.00)	<0.001	<0.001
hypertension n(%)	38 (38.00%)	53 (39.85%)	22 (22.00%)	0.775	0.004
systolic blood pressure (mmHg)	130.00 (115.00, 140.00)	128.00 (115.00, 140.00)	120.50 (112.50, 130.00)	0.941	0.057
diastolic blood pressure (mmHg)	80.00 (75.00, 89.00)	80.00 (70.00, 90.00)	80.00 (70.00, 85.00)	0.746	0.450
gross hematuria n(%)	0 (0.00%)	3 (2.19%)	27 (27.00%)	0.265	<0.001
microscopic hematuria (red blood cells/ul)	37.70 (22.80, 75.20)	25.00 (9.44, 66.6)	95.00 (25.00, 253.80)	0.002	<0.001
plasma IgA (g/L)	2.13 (1.58, 2.69)	2.37 (1.94, 3.10)	3.00 (2.35, 4.19)	0.030	<0.001
plasma IgA1 (g/L)	2.01 (1.41, 2.83)	2.65 (1.70, 3.16)	2.72 (2.07, 3.53)	0.079	0.180

eGFR, estimated glomerular filtration rate; LDL-C, low-density lipoprotein cholesterol.

mesangial areas (grade of intensity: 1+, 2+, 3+, 4+: 3.65% versus 0%, 60.58% versus 17%, 34.31% versus 69%, and 1.46% versus 14%, *P*<0.001). Most of the cIgAN/MN patients showed 2+ of IgA intensity, whereas most of the IgAN patients showed 3+. Similarly, compare to MN, patients with cIgAN/MN displayed weaker IgG and C3 deposition along the GBM (grade of intensity of IgG: grade 0, 1+, 2+, 3+, 4+: 2.92% versus 1%, 5.84% versus 0%, 33.58% versus 17%, 48.91% versus 69%, and 8.76% versus 13%, *P*<0.001; grade of intensity of C3: grade 1+, 2+, 3+: 38.69% versus 21%, 35.77% versus 51%, and 6.57% versus 18%, *P*<0.001). The positive rates of tissue staining for PLA2R along capillary loops were of no difference between cIgAN/MN and MN (91.30% versus 92.30%, *P*=0.639).

The light microscope showed that compared to IgAN, patients with cIgAN/MN had fewer crescents with Oxford classification (C0: 91.24% versus 44%; C1: 8.76% versus 46%; C2: 0% versus 10%, *P*<0.001). Electron microscope showed that compared to MN, there was no significant difference in the four stages described by Ehrenreich and Churg's pathological stages in patients with cIgAN/MN (I: 50% versus 45.45%; II: 46.03% versus 49.49%; III: 3.97% versus 5.05%, *P*=0.769). Details about the pathological features are shown in **Table 2**.

4.4 Disease-Specific Biomarkers

4.4.1 Gd-IgA1

Compared to IgAN, the level of plasma IgA in patients with cIgAN/MN was lower (2.37 versus 3.00 g/L, *P*<0.001). Although the level of plasma IgA1 was similar, they had a lower median level of Gd-IgA1 (4.00 versus 5.45 μg/ml, *P*=0.002). In contrast, patients with cIgAN/MN had a comparable level of Gd-IgA1 compared to that of MN (4.00 versus 3.64 μg/ml, *P*=0.100).

4.4.2 Anti-PLA2R

Compared to MN, patients with cIgAN/MN had a lower frequency of plasma anti-PLA2R antibody positivity (40.43% versus 59.14%, *P*=0.036; 20U/ml as the cut-off value) and lower titers of antibody (11.23 versus 36.59 U/ml, *P*=0.005). Of certain, patients with IgAN did not have detectable anti-PLA2R antibodies in sera (**Table 3** and **Figure 3**).

4.5 Disease-Specific Genetic Risk Scores (GRSs)

To determine an individual's risk of developing IgAN and MN based on specific genetic markers, we calculated polygenic risk scores, which bridge the gap between initial discovery efforts and clinical applications for the estimation of disease risk using genetics. With the most updated genetic risk models suggested by Kiryluk et al., we genotyped 15 IgAN genetic variants and 5 MN genetic variants in 132 patients with cIgAN/MN (96.35%), 98 patients with IgAN (98%), and 96 patients with MN (96%), whose DNA were available. After quality controls, we had 111 patients with cIgAN/MN, 68 patients with IgAN, and 68 patients with MN with complete genotyping data for all the 20 SNPs (**Table 4** and **Figure 4**).

4.5.1 IgAN-GRSs

Compared to IgAN, patients with cIgAN/MN had lower standardized IgAN-GRSs (0.05 versus 0.68, *P*<0.001). By stratifying the IgAN-GRSs into three risk groups as reported (high >1; average -1~1; low<-1), patients with cIgAN/MN showed a significantly lower frequency of high-risk group

TABLE 2 | Pathological features of patients with MN, cIgAN/MN, and IgAN.

Pathological features	MN (n = 100)	cIgAN/MN (n = 137)	IgAN (n = 100)	*P* (cIgAN/MN vs. MN)	*P* (cIgAN/MN vs. IgAN)
immunofluorescence					
intensity of IgA in mesangial area					
0	100 (100.00%)	0 (0.00%)	0 (0.00%)	<0.001	<0.001
1	0 (0.00%)	5 (3.65%)	0 (0.00%)		
2	0 (0.00%)	83 (60.58%)	17 (17.00%)		
3	0 (0.00%)	47 (34.31%)	69 (69.00%)		
4	0 (0.00%)	2 (1.46%)	14 (14.00%)		
IgG in capillary loops					
0	1 (1.00%)	4 (2.92%)	96 (96.00%)	<0.001	<0.001
1	0 (0.00%)	8 (5.84%)	3 (3.00%)		
2	17 (17.00%)	46 (33.58%)	1 (1.00%)		
3	69 (69.00%)	67 (48.91%)	0 (0.00%)		
4	13 (13.00%)	12 (8.76%)	0 (0.00%)		
C3 in capillary loops					
0	10 (10.00%)	17 (12.41%)	96 (96.00%)	<0.001	<0.001
0.5	0 (0.00%)	9 (6.57%)	0 (0.00%)		
1	21 (21.00%)	53 (38.69%)	3 (3.00%)		
2	51 (51.00%)	49 (35.77%)	1 (1.00%)		
3	18 (18.00%)	9 (6.57%)	0 (0.00%)		
PLA2R in capillary loops (%)	92.30%	91.30%		0.639	
light microscope					
mesangial cell proliferation and matrix accumulation					
0	58 (58.00%)	4 (2.92%)	0 (0.00%)	<0.001	<0.001
1	42 (42.00%)	117 (85.40%)	30 (30.00%)		
2	0 (0.00%)	15 (10.95%)	62 (62.00%)		
3	0 (0.00%)	1 (0.73%)	8 (8.00%)		
Oxford classification of IgAN					
C0 n (%)		125 (91.24%)	44 (44.00%)		<0.001
C1 n (%)		12 (8.76%)	46 (46.00%)		
C2 n (%)		0 (0.00%)	10 (10.00%)		
electron microscope					
morphologic classification of MN					
I	45 (45.45%)	63 (50.00%)		0.769	
II	49 (49.49%)	58 (46.03%)			
III	5 (5.05%)	5 (3.97%)			
IV	0%	0%			
subepithelial electron-dense	100%	100%	0%		<0.001
mesangial electron-dense	0%	100%	100%	<0.001	

C, crescents.

(18.92% versus 41.18%, *P*=0.001). In contrast, this score was not significantly different from MN (0.05 versus -0.10, *P*=0.376).

4.5.2 MN-GRSs

In contrast, using MN-specific-GRS, patients with cIgAN/MN did not show a significant difference (2.77 versus 3.02, *P*=0.326) compared to MN. Patients with IgAN had the lowest MN-GRS, no matter when compared to MN (2.38 versus 3.02, *P*<0.001) or cIgAN/MN (2.38 versus 2.77, *P*<0.001).

4.6 Follow-Up Evaluation

Table 5 showed the medications and follow-up details for each group. At the time of renal biopsy check, the percentage of angiotensin-converting-enzyme inhibitors (ACEI) or angiotensin II receptor blockers (ARB) usage in patients with cIgAN/MN was 73.47%, which was lower than IgAN (88%, *P*=0.026), but showed no significant difference compared to MN (69.70%, *P*=0.634). 26.53% of cIgAN/MN patients had ever used glucocorticoids at the time of renal biopsy check. This percentage showed no significant difference compared with

TABLE 3 | Disease-specific biomarkers for patients with cIgAN/MN, MN, and IgAN.

Characteristics	MN (n = 93)	cIgAN/MN (n = 47)	IgAN (n = 88)	*P* (cIgAN/MN vs. MN)	*P* (cIgAN/MN vs. IgAN)
plasma anti-PLA2R (U/ml)	36.59 (2.97, 161.04)	11.23 (2.00, 47.00)	2.00 (2.00, 2.12)	0.005	<0.001
positive rates of anti-PLA2R n (%)	55 (59.14%)	19 (40.43%)	0 (0%)	0.036	<0.001
plasma Gd-IgA1 (μg/ml)	3.64 (2.81, 4.69)	4.00 (2.94, 5.58)	5.45 (4.20, 6.88)	0.100	0.002

Gd-IgA1, galactose-deficient IgA1; anti-PLA2R, anti-phospholipase A2 receptor.

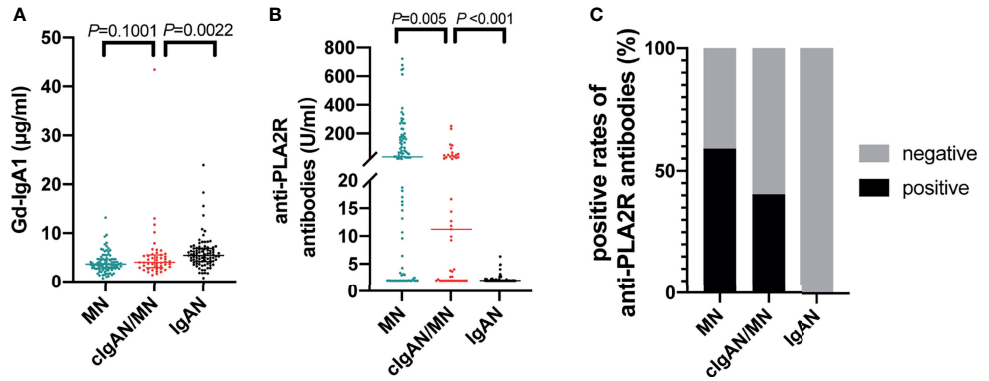

FIGURE 3 | Disease-specific biomarkers detection. The comparison of the level of Gd-IgA1 **(A)**, anti-PLA2R antibodies **(B)**, and the positive rates of anti-PLA2R antibodies **(C)** among patients with IgAN, cIgAN/MN and MN.

IgAN (20%, *P*=0.367) but was much lower than MN (54.55%, *P*=0.001).

Follow-up information was available from 30 cases with cIgAN/MN, 46 IgAN, and 38 MN. The percentage of ACEI or ARB usage in patients with cIgAN/MN was 85.19%, which was not significantly different compared to MN (75.68%, *P*=0.35) nor IgAN (89.13%, *P*=0.62). 18.52% of cIgAN/MN patients had ever used glucocorticoids, which also showed no significant difference compared with IgAN (17.39%, *P*=0.90) but was much lower than MN (48.65%, *P*=0.013). The level of TA-proteinuria in patients with cIgAN/MN was 0.79 g/d, which showed no significant difference between MN (1.26, *P*=0.08) and IgAN (0.79, *P*=0.93).

Comparisons across patients with or without complete follow-up data were performed, confirming no selection bias

TABLE 4 | GRSs of MN and IgAN in three disease groups.

Genetic risk	MN (n = 68)	MN/IgAN (n = 111)	IgAN (n = 68)	*P* (cIgAN/MN vs. MN)	*P* (cIgAN/MN vs. IgAN)
Standardized GRS of MN, median (IQR)	3.02 (2.63, 3.16)	2.77 (2.38, 3.16)	2.38 (1.54, 2.95)	0.326	<0.001
Standardized GRS of IgAN, mean (SD)	-0.10 (1.00)	0.05 (1.05)	0.68 (0.98)	0.376	<0.001
Genetic risk stratification of IgAN					
high risk	8 (11.76%)	21 (18.92%)	28 (41.18%)	0.452	0.001
average risk	48 (70.59%)	72 (64.86%)	37 (54.41%)		
low risk	12 (17.65%)	18 (16.22%)	3 (4.41%)		

GRS, genetic risk score.

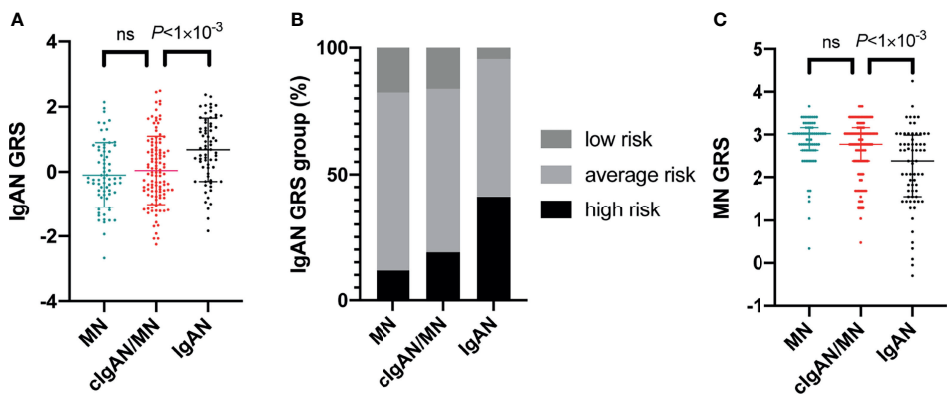

FIGURE 4 | Genetic risk scores of developing IgAN or MN. The genetic risk scores of developing IgAN **(A)**, genetic risk stratification of IgAN **(B)**, and genetic risk scores of developing MN **(C)**. ns, not significant.

TABLE 5 | Follow up data from patients with MN, cIgAN/MN, and IgAN.

	MN	cIgAN/MN	IgAN	*P* (cIgAN/MN vs. MN)	*P* (cIgAN/MN vs. IgAN)
therapies	N = 99	N = 49	N = 100		
ACEI or ARBs n (%)	69 (69.70%)	36 (73.47%)	88 (88.00%)	0.634	0.026
glucocorticoids n (%)	54 (54.55%)	13 (26.53%)	20 (20.00%)	0.001	0.367
any other immunosuppressive agents n (%)	56 (56.57%)	13 (26.53%)	8 (8.00%)	<0.001	0.002
follow-up details[a]	N = 38	N = 30	N = 46		
ACEI or ARBs n (%)	28 (75.68%)	23 (85.19%)	41 (89.13%)	0.35	0.62
glucocorticoids n (%)	18 (48.65%)	5 (18.52%)	8 (17.39%)	0.013	0.9
any other immunosuppressive agents n (%)	18 (48.65%)	6 (22.22%)	3 (6.52%)	0.031	0.068
follow-up period (months)	60.00 (29.00, 108.00)	41.50 (26.00, 71.00)	54.50 (34.00, 93.00)	0.16	0.13
time-average proteinuria (g/d)	1.26 (0.62, 4.18)	0.79 (0.28, 2.24)	0.79 (0.42, 1.45)	0.08	0.93
>1.0 (g/d)	25 (65.79%)	14 (46.67%)	20 (43.48%)	0.11	0.78
≤1.00 (g/d)	13 (34.21%)	16 (53.33%)	26 (56.52%)	0.11	0.78
Complete/partial remission[b/c] n (%)	26 (68.42%)	22 (73.33%)	44 (95.65%)	0.66	0.005
not remission n[c] (%)	12 (31.58%)	8 (26.67%)	2 (4.35%)	0.66	0.005

[a]*137 cases with cIgAN/MN were systematically found from kidney pathology database without selection. However, only 1/3 of cases were regularly followed-up with a median of 4.5 years, which may limit the generality and need external validations from other centers or ethnic populations.*

[b]*Complete remission is defined as urinary protein excretion <0.5 g/24 h confirmed by two values at least one month apart, accompanied by a normal serum albumin concentration and normal serum creatinine level. Partial remission was defined as urinary protein excretion <3.5 g/24 h and reduced by at least 50% from peak values, accompanied by an improvement or normalization of the serum albumin concentration as well as stable serum creatinine.*

[c]*Categorical variables were compared using the chi-square test or Fisher's exact test.*

ACEI, angiotensin-converting enzyme inhibitors; ARB, angiotensin II receptor blockers.

(**Supplementary Table 1**). We updated all the details among the patients who were regularly followed up until February 09th, 2022. The median follow-up period was 53 months. Twenty-two (73.33%) patients with cIgAN/MN achieved complete or partial remission, which was similar to MN (68.42%, *P*=0.66). 26.67% and 31.58% of patients with cIgAN/MN and MN showed no proteinuria remission. However, the percentages of glucocorticoids and other immunosuppressive agents in patients with cIgAN/MN were lower than those in MN. We assume milder pathology lesions and low anti-PLA2R titers in patients with cIgAN/MN might explain this difference.

Kaplan-Meier analysis (**Figure 5**) showed that the cumulative incidence of complete or partial remission was similar between patients with cIgAN/MN and MN or IgAN. Compared to IgAN, the cumulative incidence of persistent proteinuria after therapy in patients with cIgAN/MN was lower than in IgAN (*P*=0.001).

5 DISCUSSION

The present study mainly shows the clinical and pathological data about 137 patients with cIgAN/MN. Additionally, disease-specific biomarkers and genomics analysis were checked for a better understanding of the pathogenesis of cIgAN/MN.

From 1983, an increasing number of studies reported the concurrent of IgAN and MN in the same patient. Doi et al. firstly described their clinical and pathological characteristics, showing that cIgAN/MN had features from both IgAN and MN (5). Subsequently, more cases had been noticed in the clinic, and the reported patients had hematuria and nephrotic range proteinuria (6, 8–10). Magil et al. also showed an isolated case, suggesting that cIgAN/MN might be associated with hepatitis B surface antigenemia (7). Other related types of research showed that IgAN and MN occurred separately in one patient after some

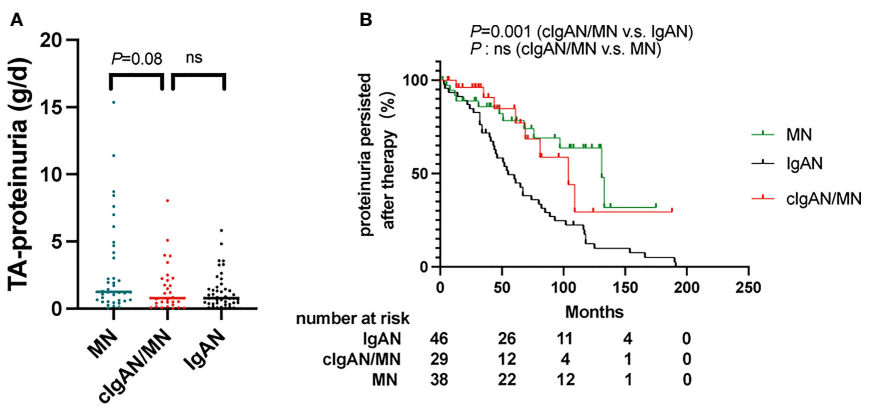

FIGURE 5 | **(A)** The comparison of the total-average proteinuria among patients with IgAN, cIgAN/MN and MN. **(B)** Proteinuria persisted means patients could not achieve complete or partial proteinuria remission during follow-up, which was were calculated according to the Kaplan-Meier method (log-rank test). ns, not significant.

years' interval (20, 21). Our previous case series summarized the clinical and pathological profiles of 26 patients with cIgAN/MN. The results showed that patients with cIgAN/MN displayed similar clinical features with MN patients and milder pathological lesions than IgAN patients (12). Besides, the prognosis of patients with cIgAN/MN was better than that in MN (11). However, due to a relatively small sample size and incomplete analysis, the pathogenesis of cIgAN/MN remained unclarified. In this study, we showed clinical-pathological features in patients of cIgAN/MN with a larger sample size.

The present cohort of patients with cIgAN/MN, to our knowledge, is the largest published to date. Our results showed that cIgAN/MN has common features of the two glomerular diseases. Firstly, the level of proteinuria and eGFR, the percentage of nephrotic syndrome, hypertension are much higher than IgAN. Whereas the rate of gross hematuria, the level of urinary microscopic hematuria, and plasma IgA in patients with cIgAN/MN are much lower than IgAN. Patients with cIgAN/MN showed fewer crescents than IgAN. Thus the clinical and pathological features of patients with cIgAN/MN are not in accordance with those of IgAN patients. Secondly, patients with cIgAN/MN and MN have comparable proteinuria levels, hyperlipidemia rates, serum total cholesterol levels, serum low-density lipoprotein cholesterol levels, hypertension rates, and gross hematuria rates. Pathological features also indicated no significant differences in the positive rates of PLA2R along capillary loops and pathological stages of MN between patients with cIgAN/MN and MN. These clinical and pathological features between patients with cIgAN/MN and MN patients were much similar. Thus, we suggest priority be given to MN when facing cIgAN/MN.

Moreover, we used disease-specific biomarkers and a genomics approach to shed some light on its pathogenesis. The plasma level of Gd-IgA1 in patients with cIgAN/MN is much lower than IgAN but had no significant difference with MN. Thus, Gd-IgA1 seems not to be a significant pathogenic factor in cIgAN/MN. On the other hand, nearly 40.43% of patients with cIgAN/MN had detectable plasma levels of anti-PLA2R antibodies. Although the plasma level of anti-PLA2R antibodies in cIgAN/MN is much lower than that in MN, anti-PLA2R antibodies may at least in part contribute to the pathogenesis of cIgAN/MN. To gain more insights into the pathogenesis of cIgAN/MN from a genetic perspective, we genotyped the majority of the subjects examined. The genetic risk score of developing IgAN in patients with cIgAN/MN is much lower than IgAN. Stratified analyses also showed that the high-risk group of developing IgAN in patients with cIgAN/MN is much lower than IgAN. Intriguingly, after calculating the genetic risk scores of developing MN, we did not observe a significant difference between patients with cIgAN/MN and MN. Thus, the genetic background of cIgAN/MN resembles that of MN. Therefore, the present study may demonstrate that cIgAN/MN seems to be just concurrent, not the overlap syndrome of IgAN and MN. cIgAN/MN may result from superimposed MN on a background of mild IgAN or IgA deposition (summarized in **Figure 6**).

Despite a relatively large-scale study on cIgAN/MN, we should note some limitations. First, the pathogenesis of cIgAN/MN is still not clear, but disease-specific markers in the circulatory system may be highlighted in disease differentiation. Second, patients with cIgAN/MN enrolled in the present study are diagnosed simultaneously by renal biopsy. We cannot rule out the possibility that IgAN or MN may superimpose on the basis of preexisting one of the other glomerular diseases. Third, our follow-up data are not complete. Only one-third of the patients enrolled from pathology dataset had been regularly followed-up. The composite endpoint, such as the decline rate in eGFR and the receipt of renal replacement therapy, should be further recorded in long-term follow-up. Fourth, as a growing number of cases will be discovered in the clinic, additional research with larger sample sizes is still warranted. More studies focusing on other biomarkers related to underlying mechanisms, such as T cells and activation of the complement system, will also be conducted (22–26). Fifth, we mainly recruited participants of Chinese Han ethnicity. IgA deposition was different among various ethnicities (27–30). Replication from different ethnicities or different populations was needed for similar studies.

In conclusion, although MN is concurrent with IgA deposition and mesangial proliferation in pathology, from the clinical, diagnostic, and prognostic point of view, cIgAN/MN is more likely to be MN accompanied by IgA deposition and may not be an independent disease.

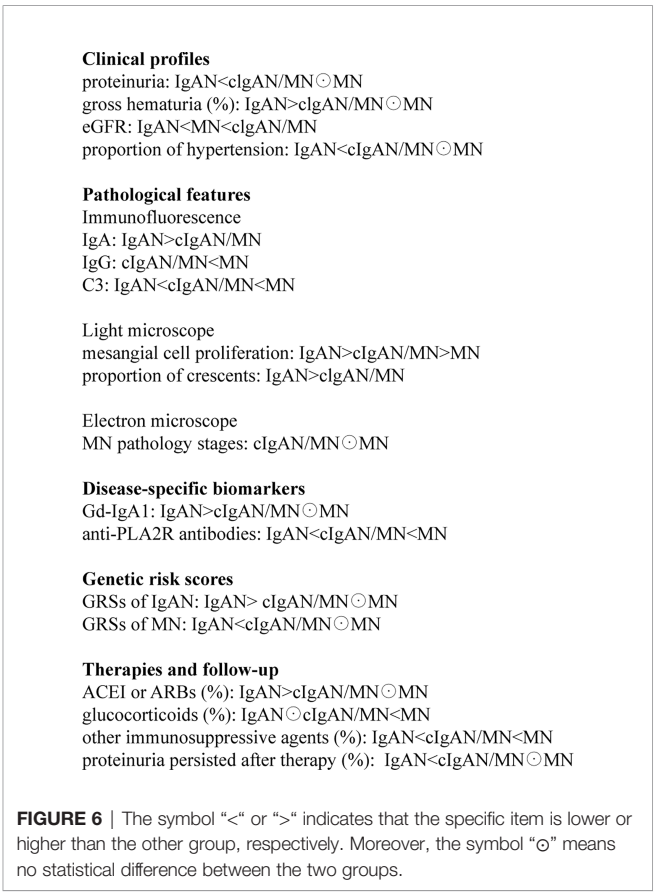

FIGURE 6 | The symbol "<" or ">" indicates that the specific item is lower or higher than the other group, respectively. Moreover, the symbol "⊙" means no statistical difference between the two groups.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

All the study protocols complied with the principles of the Declaration of Helsinki and were approved by the Ethics Committee of Peking University First Hospital (Institutional Review Board number: 2021[Y148]). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

HZ and X-JZ conceived and designed the study. X-JZ, S-FS, L-JL, J-CL, J-WH, D-FC, and LZ collaborated in patient recruitment, data acquisition, and organization. J-WH, PC, YL, XZ, and PH performed the laboratory analyses. J-WH, D-FC, Y-NW, and TG analyzed the data. X-JZ and J-WH made the figures. J-WH, D-FC, X-JZ, and HZ drafted and revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.846323/full#supplementary-material

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Serum IgE Levels Are Associated With the Prognosis of Minimal Change Disease

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Background: Previous reports showed that some patients with minimal change disease (MCD) had high serum immunoglobulin E (IgE) levels. This study aimed to explore the proportion of MCD patients with high serum IgE levels and evaluate the correlation between serum IgE levels and MCD remission and relapse.

Methods: This study enrolled 222 new-onset patients with renal biopsy-confirmed MCD from October 2012 to October 2019 at the First Affiliated Hospital of Zhejiang University in Hangzhou, China. Patients' demographics and clinical parameters were analyzed.

Results: The results indicated that 70.3% of 222 MCD patients had high serum IgE levels (IgE > 100.0 IU/mL). Moreover, 134 patients were treated with glucocorticoids alone and divided into the low- and high-IgE groups, according to the median serum IgE level (523.5 IU/mL). The mean time to complete remission of the low- and high-IgE groups was 29.0 ± 2.2 and 45.7 ± 4.2 days, respectively (log-rank test; $P = 0.002$). The mean time to total remission was 19.1 ± 1.4 and 31.6 ± 3.2 days of the low- and high-IgE groups, respectively (log-rank test; $P < 0.001$). The mean time to first relapse in the low- and high-IgE groups was 701.2 ± 65.0 and 425.0 ± 52.6 days, respectively (log-rank test; $P = 0.002$). Serum IgE ≥ 523.5 IU/mL was an independent correlation factor affecting the patients' remission and relapse.

Conclusion: Serum IgE level was an independent correlation factor for MCD remission and relapse. MCD patients with high serum IgE levels were prone to delayed remissions and early relapses.

Keywords: minimal change disease, serum IgE level, remission, relapse, risk factor

1 INTRODUCTION

Minimal change disease (MCD) is a common pathological type of idiopathic nephrotic syndrome (INS). MCD accounts for 70%–90%, 50%, and 10%–15% of patients with INS in children < 10 years old, children > 10 years old, and adults, respectively (1). Typical MCD clinical manifestations include hypoalbuminemia and massive proteinuria, possibly accompanied by edema and

hyperlipidemia (1). Glucocorticoids are usually the first choice for initial immunosuppressive therapy in patients with MCD (2). About 90% and 70% of children and adults with MCD, respectively, can achieve complete remission after receiving a course of glucocorticoid treatment but are prone to relapse (3). About 56%–76% of MCD patients will experience at least one relapse, and some patients may experience frequent relapses or steroid dependence (4). Changing the therapeutic regimen in time is crucial for these patients. Therefore, evaluating the clinical efficacy of glucocorticoids in MCD is important.

Reports in the 1970s showed that the serum immunoglobulin E (IgE) levels of several MCD patients were higher than normal (5). Serum IgE level was low in normal conditions, and elevated level was usually associated with allergic reactions (6). Several case reports reported the onset of nephrotic syndrome caused by food, allergen inhalation, insect bites, and vaccination (7–10). Numerous reports indicated that INS could be precipitated by allergic reactions, and INS patients could exhibit increased serum IgE levels (11). However, many MCD patients with high serum IgE levels had no history of allergy. Therefore, although some INS cases were associated with allergies, evidence that INS was a type of allergic disorder was weak (11). Shu et al. (12) reported that the serum IgE levels in MCD patients with frequent relapses were significantly higher than that in patients with non-relapse or infrequent-relapse, indicating that high serum IgE levels may be related to frequent MCD relapse. But the relevant studies on the correlation between serum IgE levels and prognosis of MCD were lacking. This study aims to explore the proportion of MCD patients with high serum IgE levels and evaluate the correlation between serum IgE levels and MCD remission and relapse.

2 METHODS

2.1 Study Design and Population

This retrospective observational study was performed at a single center, the Kidney Disease Center of the First Affiliated Hospital, College of Medicine, Zhejiang University (Hangzhou, China), from October 2012 to October 2019. The study complied with the Declaration of Helsinki. The Clinical Research Ethics Committee of the First Affiliated Hospital, Zhejiang University School of Medicine, provided ethical approval and waived informed consent.

The study included patients who met the following criteria: (1) 24-h urinary protein ≥ 3.5 g/day or urine protein to creatinine ratio (UP/Cr) ≥ 3.5 g/g for adults, and UP/Cr ≥ 2.0 g/g or ≥ 3+ on urine dipstick for children; (2) serum albumin < 30 g/L; (3) pathologically proven MCD by renal biopsy; and (4) new-onset of disease or discontinuation of immunosuppressive therapy for more than 1 year. Patients were excluded if they had any of the following conditions: (1) infectious diseases (e.g. hepatitis B, AIDS, syphilis, and tuberculosis); (2) malignant tumors; (3) connective tissue diseases; (4) diabetes mellitus; and (5) missing data on serum IgE levels at the onset.

2.2 Data Collection

As shown in **Table 1**, patiens' demographics and clinical parameters at the time of renal biopsy were collected, including gender, age, disease duration, body mass index, serum total IgE level, serum albumin (Alb), serum creatinine (SCr), estimated glomerular filtration rate (eGFR), serum uric acid (UA), serum triglyceride (TG), serum total cholesterol (TC), urine protein to creatinine ratio (UP/Cr), systolic blood pressure, diastolic blood pressure, fasting blood glucose, allergic history, comorbidities, treatment regimen, *etc.*

The eGFR was calculated using the four-variable Modification of Diet in Renal Disease Study equation (13). The serum total IgE levels of MCD patients were analyzed using the ImmunoCAP system (14). The normal value of serum total IgE was ≤ 100 IU/mL.

2.3 Immunosuppressive Treatment Regimens

For the new-onset biopsy-proven MCD patients, daily prednisolone (0.5-1.0 mg/kg/d, up to 80 mg/d) was generally used as the initial immunosuppressive treatment, and was maintained for 2-4 weeks if patients achieved complete remission or for a maximum of 16 weeks if patients didn't achieve complete remission. After remission, the dosage of glucocorticoids was tapered over 6 months. For relapse patients, the same initial dosage of glucocorticoids was used and was gradually tapered after remission was achieved. For patients with a contraindication to or intolerance of high-dose glucocorticoids, and patients with frequent relapses or steroid dependence, second-line agents such as cyclophosphamide, tacrolimus, cyclosporine, or rituximab were used. The choices of second-line agents were up to the individual nephrologists.

2.4 Follow-Up

Patients' clinical parameters at 1, 2, 3, 6, and 12 months were collected and analyzed. And the subsequent follow-ups were also recorded. The data were collected at specific time points if patients achieved remission or relapse at other times (within 1 month or beyond 12 months, for example). The last follow-up point was the latest clinical visit available in the follow-up system. The tolerated time-frame for follow-up time points were 30 days ± 1 week in this study. Clinical parameters included serum IgE levels, Alb, SCr, eGFR, UA, TG, TC, 24-h urine protein, and UP/Cr.

2.5 Outcomes Definition

The primary endpoint of this study was remission and relapse. Remission included partial remission (PR), complete remission (CR), and total remission (TR). The secondary endpoint included time to remission and time to first relapse. These indexes were defined as follows (15):

For adults, PR was the decrease in 24-h urine protein to < 3.5 g/day but > 0.3 g/day or in UP/Cr to < 3.5 g/g but > 0.3 g/g with a 50% reduction from its peak value. CR was the serum albumin ≥ 30 g/L with a decrease in 24-h urine protein to ≤ 0.3 g/day or in UP/Cr to ≤ 0.3 g/g. For children, PR was the decrease in UP/Cr

TABLE 1 | Baseline characteristics of patients with minimal change disease.

	Mean ± SD/median (Q1, Q3)/ *n* (%)			*P**
	Overall, *n* = 222	**Low-IgE, *n* = 111**	**High-IgE, *n* = 111**	
IgE level, IU/mL	389.5 (79.5, 1087.2)	79.4 (40.0, 157.0)	1090.0 (685.5, 1807.5)	**<0.001**
Female	88 (39.6%)	64 (57.7%)	24 (21.6%)	**<0.001**
Age, years old	25.5 (19.0, 43.8)	33.0 (20.0, 50.5)	22.0 (18.0, 28.5)	**<0.001**
Adult	182 (82.0%)	96 (86.5%)	86 (77.5%)	0.081
Disease duration, days	10.0 (7.0, 20.0)	10.0 (7.0, 20.0)	10.0 (7.0, 25.5)	0.900
Treatment regimens				**0.013**
GC	134 (60.4%)	58 (52.3%)	76 (68.5%)	
GC + TAC	58 (26.1%)	30 (27.0%)	28 (25.2%)	
TAC	13 (5.9%)	12 (10.8%)	1 (0.9%)	
GC + CsA	7 (3.2%)	3 (2.7%)	4 (3.6%)	
GC + CTX	2 (0.9%)	2 (1.8%)	0 (0.0%)	
GC + RTX	1 (0.5%)	1 (0.9%)	0 (0.0%)	
Non-immunosuppressive treatment	7 (3.2%)	5 (4.5%)	2 (1.8%)	
BMI, kg/m^2	22.7 (20.8, 25.8)	22.4 (20.8, 24.7)	23.1 (20.8, 26.4)	0.350
Allergy	32 (14.4%)	18 (16.2%)	14 (12.6%)	0.445
FBG, mmol/L	4.4 (4.1, 4.8)	4.4 (4.1, 4.9)	4.3 (4.0, 4.8)	0.252
SBP, mmHg	121.6 ± 13.4	120.4 ± 13.5	122.8 ± 13.3	0.180
DBP, mmHg	75.2 ± 9.9	75.6 ± 9.3	74.8 ± 10.5	0.565
Hypertension	30 (13.5%)	18 (16.2%)	12 (10.8%)	0.239
Alb, g/L	17.6 (15.2, 21.0)	18.3 (15.4, 21.9)	17.2 (14.7, 19.8)	**0.013**
SCr, μmol/L	79.5 (62.0, 102.0)	73.0 (60.5, 94.5)	84.0 (66.5, 117.5)	**0.006**
eGFR, mL/(min × 1.73 m^2)	96.9 ± 38.0	96.2 ± 37.3	97.6 ± 38.7	0.788
eGFR ≥ 90 mL/(min × 1.73 m^2)	130 (58.6%)	66 (59.5%)	64 (57.7%)	0.785
eGFR < 90 mL/(min × 1.73 m^2)	92 (41.4%)	45 (40.5%)	47 (42.3%)	
UA, μmol/L	402.3 ± 124.8	374.9 ± 121.7	429.7 ± 122.4	**<0.001**
Abnormal UA level	89 (40.1%)	35 (31.5%)	54 (48.6%)	**0.009**
AKI	39 (17.6%)	14 (12.6%)	25 (22.5%)	0.052
TG, mmol/L	2.0 (1.5, 3.0)	2.0 (1.5, 2.8)	2.2 (1.5, 3.2)	0.623
TG ≤ 1.7 mmol/L	77 (34.7%)	37 (33.3%)	40 (36.0%)	0.672
TG > 1.7mmol/L	145 (65.3%)	74 (66.7%)	71 (64.0%)	
TC, mmol/L	9.7 (7.7, 11.9)	8.7 (7.1, 11.3)	10.1 (8.3, 12.3)	**0.010**
TC ≤ 5.86 mmol/L	16 (7.2%)	11 (9.9%)	5 (4.5%)	0.119
TC > 5.86 mmol/L	206 (92.8%)	100 (90.1%)	106 (95.5%)	
UP/Cr, g/g	4.9 (3.7, 7.3)	5.1 (3.8, 7.4)	4.7 (3.6, 7.0)	0.323

*AKI, acute kidney injury; Alb, albumin; BMI, body mass index; CsA, cyclosporine; CTX, cyclophosphamide; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; FBG, fasting blood glucose; GC, glucocorticoids; Q1, lower quartile; Q3, upper quartile; RTX, rituximab; SBP, systolic blood pressure; SCr, serum creatinine; TAC, tacrolimus; TC, total cholesterol; TG, triglyceride; UA, uric acid; UP/Cr, urine protein to creatinine ratio. *P High-IgE vs. Low-IgE. P < 0.05 was shown in bold.*

to < 2.0 g/g but > 0.2 g/g with a 50% reduction from its peak value. CR was the serum albumin ≥ 30 g/L with a decrease in UP/Cr to ≤ 0.2 g/g or trace or negative results on repeat urine albumin dipstick.

TR was the achievement of PR or CR.

Time to remission was the time interval from treatment initiation to the first day of remission.

For adults, relapse was the increase in 24-h urine protein to ≥ 3.5 g/day or in UP/Cr to ≥ 3.5 g/g in patients who underwent TR with a 50% increase from its valley value. For children, relapse was the increase in urine dipstick to ≥ 3+ or in UP/Cr to ≥ 2.0 g/g in patients who underwent TR with a 50% increase from its valley value.

Time to the first relapse was the time interval from TR initiation to the day when the first relapse occurred (16).

Steroid resistance was defined as not achieving remission despite at least 16 weeks of prednisone (1 mg/kg/d) treatment.

Frequent relapse was defined as 2 or more relapses per 6 months (or 4 or more relapses per 12 months).

Steroid dependence was defined as relapse during corticosteroid therapy or within 2 weeks of discontinuing corticosteroid therapy.

2.6 Statistical Analysis

The Kolmogorov–Smirnov test determined whether the continuous variables conform to the normal distribution. Normally distributed continuous variables were represented by mean ± SD, and the comparison between the two groups was performed using the unpaired *t*-test. Non-normally distributed continuous variables were represented by median (lower quartile, upper quartile), and the Mann–Whitney *U* test was used to compare the two groups. Categorical variables were expressed as frequency (percentage), and comparison between groups was performed by chi-square test, continuity-corrected chi-square test, or Fisher's exact test. A two-sided test was performed on all data, and $P < 0.05$ was regarded as statistically significant.

The remission rate and the probability of the first relapse between the two groups were estimated using the Kaplan–Meier

method, and survival curves were compared with the log-rank test. Cox proportional hazards analysis model was used to explore the effects of different variables on MCD remission and relapse. Variables with $P < 0.1$ in the univariate analysis were included in the multivariate analysis with covariates. The independent correlation factors for the endpoint event were obtained using forward stepwise regression ($\alpha_{included} = 0.05$ and $\alpha_{excluded} = 0.10$).

The SPSS 24.0, GraphPad Prism 9.0, R 4.0.3, and EmpowerStats software were used for data analysis in this study.

3 RESULTS

3.1 Demographic and Clinical Features

As shown in **Figure 1**, a total of 222 MCD patients were enrolled in this study. The baseline characteristics of these patients were listed in **Table 1**. The median (Q1, Q3) of the age was 25.5 (19.0, 43.8) years old, and the range of the age was 14.0-81.0 years old. A total of 182 patients (82.0%) were adults. The median serum IgE level of these patients was 389.5 (79.5, 1087.2) IU/mL. And 156 (70.3%) patients had high serum IgE levels (IgE > 100.0 IU/mL) at the disease onset. Of the 222 patients, 134 patients received glucocorticoids alone as their initial immunosuppressive treatment, 58 patients received glucocorticoids plus tacrolimus, 13 patients received tacrolimus alone, 7 patients received glucocorticoids plus cyclosporin, 2 patients received glucocorticoids plus cyclophosphamide, 1 patient received glucocorticoids plus rituximab, and 7 patients didn't receive any immunosuppressive treatment.

And the 134 patients who received glucocorticoids alone were included for further analyses to explore the correlation between serum IgE levels and the efficacy of glucocorticoids for MCD. As shown in **Table 2**, of the included patients, 46 were females which accounted for 34.3% of the total population. 27 were children which accounted for 20.1% of the total population. And the median age of the patients was 24.0 (19.0, 43.8) years old. The median follow-up period of the patients was 15.2 (12.2, 46.6) months. A total of 22 patients that accounted for 16.4% of the total population had allergic history, including bronchial asthma, allergic rhinitis, atopic dermatitis, urticaria, and other allergic conditions. 33 patients that accounted for 24.6% of the total population had infections at the disease onset, including upper respiratory tract infections, suppurative tonsillitis, pneumonia, and gastroenteritis. The median serum IgE level was 523.5 (91.1, 1230.8) IU/mL and was set as the cutoff point, and 134 MCD patients were equally divided into two groups: low-IgE (IgE < 523.5 IU/mL) and high-IgE (IgE ≥ 523.5 IU/mL) groups.

The median serum IgE level was 90.6 (42.0, 256.5) and 1238.0 (808.5, 2239.5) IU/mL in the low- and high-IgE groups, respectively. In the high-IgE group, the ages of the patients were significantly lower than that in the low-IgE group [21.0 (18.0, 28.0) *vs.* 32.0 (19.0, 50.0); $P = 0.024$]; the proportion of female patients was significantly lower than that in the low-IgE group (19.4% *vs.* 49.3%, $P < 0.001$); and the proportion of patients with eosinophilia was significantly higher than that of the low-IgE group (16.4% *vs.* 1.5%; $P = 0.006$). Moreover, the total dosages of glucocorticoids used in patients in the high-IgE group were significantly higher than that in the low-IgE group [1.4 (0.9, 2.9) *vs.* 1.0 (0.7, 1.8); $P = 0.008$]. There were no significant differences in the allergic history or other baseline parameters between the two groups ($P > 0.05$).

3.2 Outcomes

3.2.1 Remission

The Kaplan–Meier curves were used for analysis to compare the cumulative remission rate of MCD patients in the low- and the

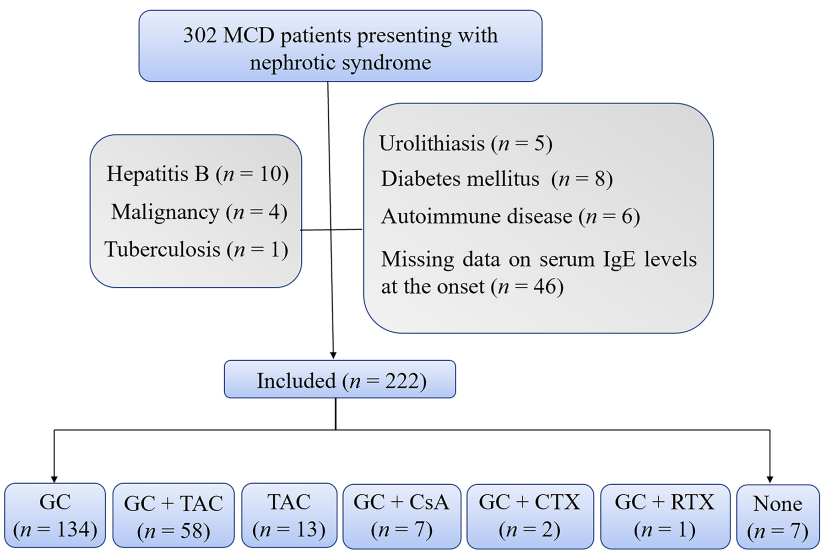

FIGURE 1 | Flowchart. The flowchart shows 222 enrolled patients with 134 patients treated with glucocorticoids alone and 88 patients treated with other treatment regimens. CsA, cyclosporine; CTX, cyclophosphamide; GC, glucocorticoids; TAC, tacrolimus; RTX, rutuximab.

TABLE 2 | Baseline characteristics of patients with minimal change disease treated with glucocorticoids.

	Mean ± SD/median (Q1, Q3)/ *n* (%)			*P**
	Overall, *n* = 134	**Low-IgE, *n* = 67**	**High-IgE, *n* = 67**	
IgE level, IU/mL	523.5 (91.1, 1230.8)	90.6 (42.0, 256.5)	1238.0 (808.5, 2239.5)	**<0.001**
Female	46 (34.3%)	33 (49.3%)	13 (19.4%)	**<0.001**
Age, years old	24.0 (19.0, 43.8)	32.0 (19.0, 50.0)	21.0 (18.0, 28.0)	**0.024**
Adult	107 (79.9%)	55 (82.1%)	52 (77.6%)	0.518
Disease duration, days	10.0 (7.0, 20.8)	10.0 (7.0, 20.0)	10.0 (7.0, 25.5)	0.881
Follow-up period, months	15.2 (12.2, 46.6)	15.7 (12.2, 49.5)	14.7 (12.2, 42.2)	0.805
BMI, kg/m^2	23.3 (20.9, 25.8)	23.6 (21.4, 25.9)	22.5 (20.8, 25.5)	0.259
Alcohol	19 (14.2%)	9 (13.4%)	10 (14.9%)	0.804
Smoking	28 (20.9%)	10 (14.9%)	18 (26.9%)	0.089
Infections	33 (24.6%)	14 (20.9%)	19 (28.4%)	0.316
Upper respiratory tract infections	24 (17.9%)	10 (14.9%)	14 (20.9%)	0.367
Suppurative tonsillitis	2 (1.5%)	1 (1.5%)	1 (1.5%)	1.000
Pneumonia	4 (3.0%)	2 (3.0%)	2 (3.0%)	1.000
Gastroenteritis	3 (2.2%)	1 (1.5%)	2 (3.0%)	1.000
Allergy	22 (16.4%)	10 (14.9%)	12 (17.9%)	0.641
Bronchial asthma	2 (1.5%)	2 (3.0%)	0 (0.0%)	0.496
Allergic rhinitis	4 (3.0%)	1 (1.5%)	3 (4.5%)	0.619
Atopic dermatitis	6 (4.5%)	2 (3.0%)	4 (6.0%)	0.680
Urticaria	3 (2.2%)	2 (3.0%)	1 (1.5%)	1.000
Other allergic conditions	9 (6.7%)	4 (6.0%)	5 (7.5%)	1.000
FBG, mmol/L	4.4 (4.1, 4.8)	4.5 (4.2, 4.8)	4.3 (4.0, 4.7)	0.059
SBP, mmHg	122.2 ± 12.2	122.3 ± 13.0	122.1 ± 11.5	0.922
DBP, mmHg	75.0 ± 9.6	76.2 ± 9.9	73.7 ± 9.2	0.133
Hypertension	20 (14.9%)	14 (20.9%)	6 (9.0%)	0.052
Alb, g/L	17.6 (15.3, 20.8)	18.3 (15.7, 21.4)	17.0 (14.6, 19.8)	0.055
SCr, μmol/L	82.5 (62.0, 107.2)	75.0 (61.0, 102.5)	84.0 (65.0, 115.0)	0.295
eGFR, mL/(min × 1.73 m^2)	97.1 ± 41.6	94.0 ± 41.5	100.1 ± 41.8	0.402
eGFR ≥ 90 mL/(min × 1.73 m^2)	77 (57.5%)	38 (56.7%)	39 (58.2%)	0.861
eGFR < 90 mL/(min × 1.73 m^2)	57 (42.5%)	29 (43.3%)	28 (41.8%)	
Eosinophilia	12 (9.0%)	1 (1.5%)	11 (16.4%)	**0.006**
UA, μmol/L	417.4 ± 129.5	401.6 ± 131.5	433.2 ± 126.6	0.159
Abnormal UA level	67 (50.0%)	32 (47.8%)	35 (52.2%)	0.604
AKI	36 (26.9%)	16 (23.9%)	20 (29.9%)	0.436
TG, mmol/L	2.0 (1.5, 3.0)	2.2 (1.6, 2.9)	1.9 (1.4, 3.2)	0.245
TG ≤ 1.7 mmol/L	51 (38.1%)	20 (29.9%)	31 (46.3%)	0.050
TG > 1.7 mmol/L	83 (61.9%)	47 (70.1%)	36 (53.7%)	
TC, mmol/L	9.4 (7.5, 12.0)	8.8 (7.1, 11.7)	9.7 (8.2, 12.2)	0.186
TC ≤ 5.86 mmol/L	10 (7.5%)	6 (9.0%)	4 (6.0%)	0.742
TC > 5.86 mmol/L	124 (92.5%)	61 (91.0%)	63 (94.0%)	
UP/Cr, g/g	5.0 (3.7, 7.1)	4.8 (3.7, 7.2)	5.0 (4.2, 6.9)	0.772
Total steroid dosages, g	1.2 (0.8, 2.2)	1.0 (0.7, 1.8)	1.4 (0.9, 2.9)	**0.008**
Steroid-resistance	2 (1.5%)	0 (0.0%)	2 (3.0%)	0.496
Steroid-dependence	30 (22.4%)	10 (14.9%)	20 (29.9%)	0.038
Frequent relapse	9 (6.7%)	1 (1.5%)	8 (11.9%)	0.033

*AKI, acute kidney injury; Alb, albumin; BMI, body mass index; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; EOS, Eosinophil; FBG, fasting blood glucose; GC, glucocorticoids; Q1, lower quartile; Q3, upper quartile; SBP, systolic blood pressure; SCr, serum creatinine; TC, total cholesterol; TG, triglyceride; UA, uric acid; and UP/Cr, urine protein to creatinine ratio. *P High-IgE vs. Low-IgE. P < 0.05 was shown in bold.*

high-IgE groups. And the Cox regression model was used to further explore the correlation between serum IgE levels and the remission rate of patients treated with glucocorticoids.

Figure 2A shows that the average time to CR was 29.0 ± 2.2 and 45.7 ± 4.2 days in the low- and high-IgE groups (log-rank test; P = 0.002), respectively. **Figures 2B, C** shows the independent correlation factors for CR of MCD. Serum IgE ≥ 523.5 IU/mL (hazard ratio [HR] = 0.615, 95% confidence interval [CI] = 0.400–0.946; P =0.027), acute kidney injury (AKI; HR = 0.437, 95% CI = 0.257–0.742; P = 0.002), dosages of glucocorticoids (GC-dose; HR = 0.053, 95% CI = 0.030–0.094; P < 0.001), and female (HR = 0.533, 95% CI = 0.334–0.852; P = 0.009) were independent CR correlation factors. **Figure 2D** shows the cumulative CR rates of MCD patients in the low- and the high-IgE groups after adjusting for AKI, UA levels, age, eGFR, GC-dose, and gender in the multivariate Cox regression model. The cumulative CR rate of MCD patients in the high-IgE group was significantly lower than that in the low-IgE group (P = 0.027).

Figure 2E shows that the average time to TR was 19.1 ± 1.4 and 31.6 ± 3.2 days in the low- and high-IgE groups (log-rank test; P < 0.001), respectively. **Figures 2F, G** shows the

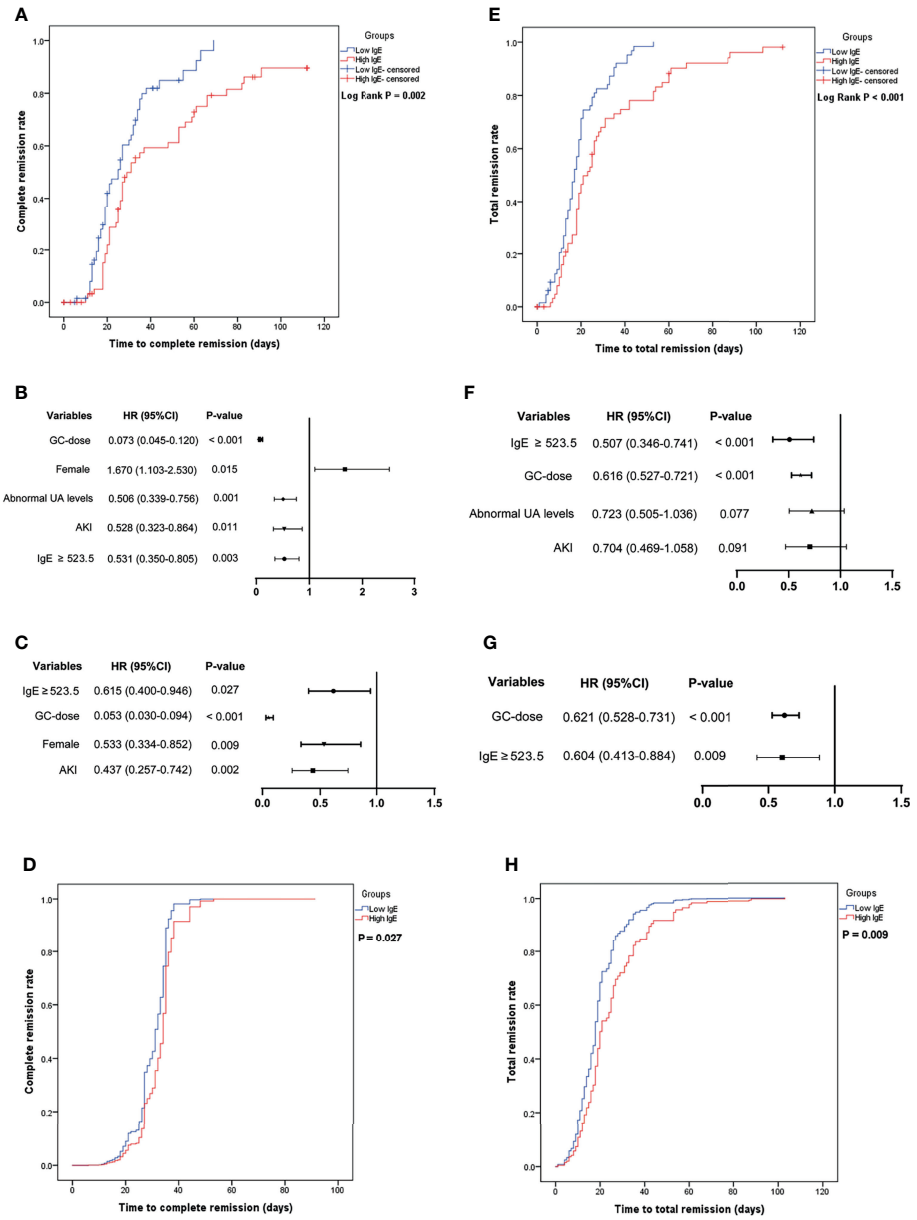

FIGURE 2 | The cumulative remission rate of minimal change disease (MCD) in the low- and high-IgE groups and the identification of independent correlation factors for remission. **(A)** Cumulative complete remission rate. Independent correlation factors for complete remission by univariate **(B)** and multivariate **(C)** cox regression analysis. **(D)** Serum IgE levels were independent correlation factors for complete remission of MCD. **(E)** Cumulative total remission rate. Independent correlation factors for total remission by univariate **(F)** and multivariate **(G)** cox regression analysis. **(H)** Serum IgE levels were independent correlation factors for total remission of MCD. *AKI*, acute kidney injury; *GC-dose*, the dosages of glucocorticoids; *MCD*, minimal change disease, and *UA*, uric acid.

independent correlation factors for TR of MCD. Serum IgE ≥ 523.5 IU/mL (HR = 0.604, 95% CI = 0.413–0.884; P =0.009), and GC-dose (HR = 0.621, 95% CI = 0.528–0.731; $P < 0.001$) were independent TR correlation factors. As shown in **Figure 2H**, after adjusting for age, AKI, GC-dose, and UA levels in the multivariate Cox regression model, the cumulative TR rate of MCD patients in the high-IgE group was significantly lower than that in the low-IgE group ($P = 0.009$).

Moreover, 2 patients exhibited steroid-resistance in the high-IgE group (**Table 2**). One of them (IgE = 2874 IU/mL) was re-diagnosed with focal segmental glomerular sclerosis (FSGS) after repeat renal biopsy 5 months later. The other one (IgE = 904 IU/mL) achieved complete remission after a combined treatment of tacrolimus plus glucocorticoids for 5 months, but experienced frequent relapses later during the treatment period, and was suspected as FSGS clinically. In addition, 10 (14.9%) and 20

(29.9%) patients were steroid-dependent in the low- and high-IgE groups, respectively (P =0.038). And 8 (11.9%) patients in the high-IgE group and 1 (1.5%) patient (IgE = 420 IU/mL) in the low-IgE group experienced frequent relapses (P =0.033).

3.2.2 Relapse

Figure 3A shows that the mean time to the first relapse in the low- and high-IgE groups was 701.2 ± 65.0 and 425.0 ± 52.6 days, respectively (log-rank test; P = 0.002). **Figures 3B, C** shows that serum IgE ≥ 523.5 IU/mL (HR = 2.087, 95% CI =1.224–3.558; P = 0.007), and abnormal UA level (HR = 2.237, 95% CI = 1.304–3.839; P = 0.003) were independent risk factors for the first relapse in MCD patients. In the multivariate Cox regression model, the probability of the first relapse of MCD patients (**Figure 3D**) in the high-IgE group was significantly higher than that in the low-IgE group (P = 0.007) after adjusting for age and UA levels.

3.2.3 Laboratory Data

Serum albumin, eGFR, UP/Cr, and UA levels were compared between the two groups during the follow-up period. **Figure 4** shows that the UA levels in the high-IgE group were significantly higher than that in the low-IgE group at month 1, 2, 3, and 6 of follow-up (P < 0.05), and there were no significant differences between serum albumin, eGFR, and UP/Cr of the MCD patients in the two groups at month 0, 1, 2, 3, 6, and 12 of follow-up (P > 0.05). Although 26.9% of the patients experienced AKI at the onset (as shown in **Table 2**), their renal functions gradually recovered as the proteinuria disappeared.

4 DISCUSSIONS

70.3% of the 222 MCD patients had high serum IgE levels at the onset in this study, including 75.0% of children and 69.2% of adults (P = 0.470). This result was consistent with previous reports. A previous study including 46 Chinese adult MCD patients found that 83.7% of the patients had high serum IgE levels, although only one patient had allergic history (17). Another study included 32 children with MCD and reported that 62.5% of the patients had high serum IgE levels (18). Elevated IgE levels usually indicated the occurrence of allergy (19). Though there were no significant differences in the history of allergy between the low- and high-IgE groups in this study, a higher percentage of eosinophilia was observed in the high-IgE group, indicating that the allergic condition may exist. Ni et al. (20) and Cheung et al. (21) reported that serum IgE levels of MCD patients were higher in the atopic subgroup than that in the non-atopic subgroup at the time of remission. We assessed the IgE levels when patients were in remission. However, only 61 of the 134 patients had data on serum IgE levels at the time of disease remission, including 12 patients in the atopic subgroup and 49 in the non-atopic subgroup. When we compared IgE levels between non-atopic *vs.* atopic subgroups at the time of remission, there was no significant difference between the two groups [145.0 (41.3–435.0) *vs.* 166.0 (76.3–501.5); P = 0.599]. Because there were many missing data on IgE levels at the time of remission, which was a limitation of retrospective studies, we did not include these data in the results. Further analysis of IgE levels at the time of MCD remission will be required in the prospective studies.

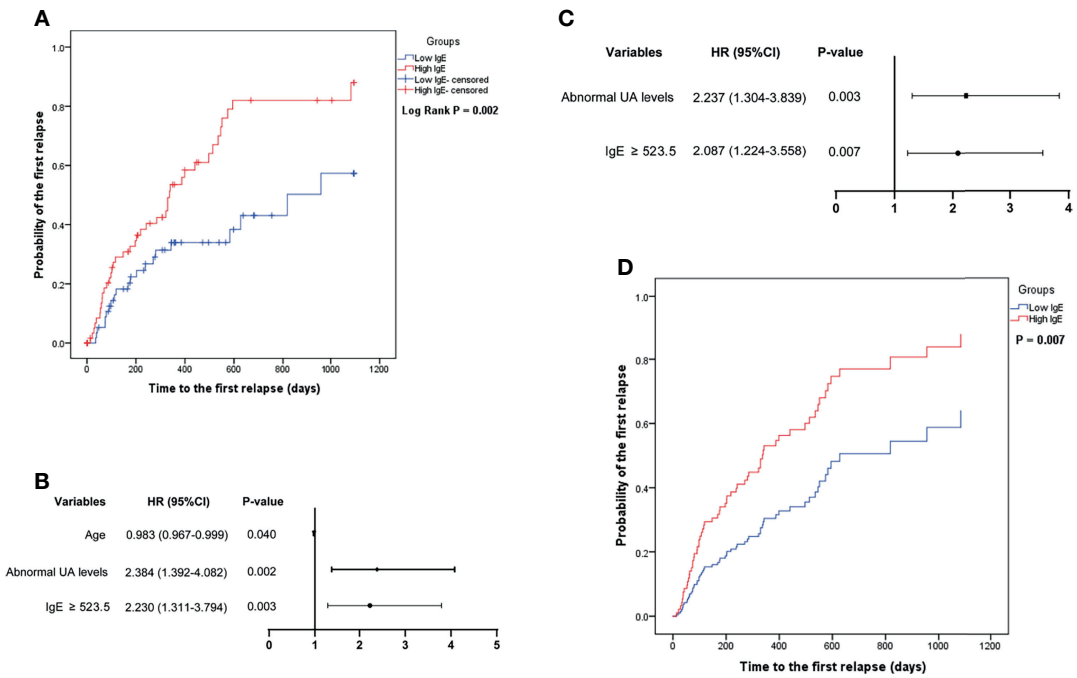

FIGURE 3 | Comparison of the probability of the first relapse of minimal change disease in the low- and high-IgE groups **(A)**, and the identification of independent correlation factors for relapse by univariate **(B)** and multivariate **(C)** cox regression analysis. **(D)** Serum IgE levels were independent correlation factors for relapse of minimal change disease. *UA*, uric acid.

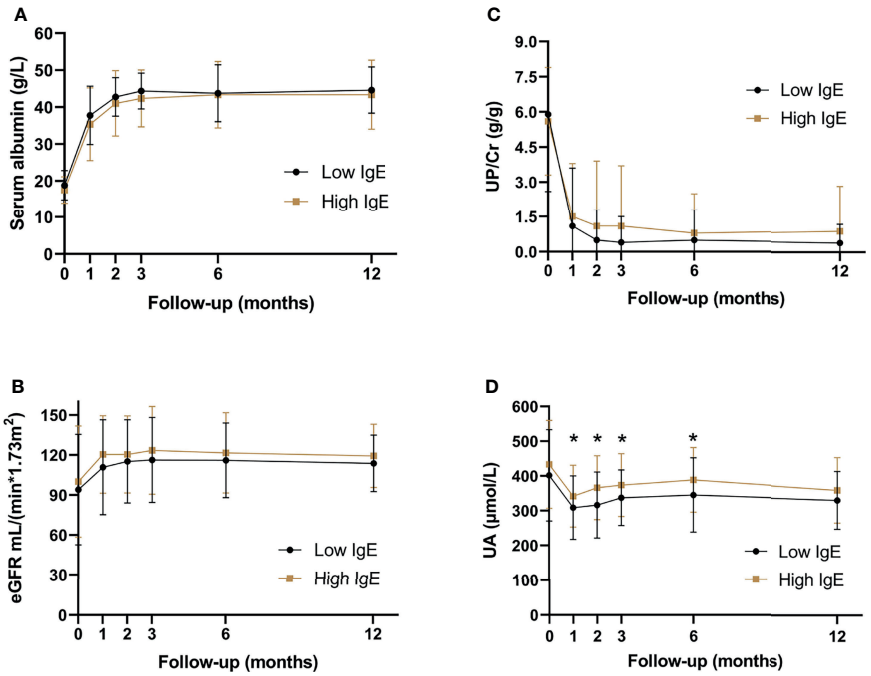

FIGURE 4 | Serum biochemical indexes of patients with minimal change disease during follow-up period. **(A)** serum albumin levels, **(B)** estimated glomerular filtration rate, **(C)** urine protein-creatinine ratio, and **(D)** serum uric acid levels. *$P < 0.05$, significant difference between the high- and low-IgE groups.

It's known that children and adults differed in the prognosis of MCD. In this cohort, there were 40 children patients with the ages ranging from 14.0-17.0 years old, which accounted for 18.0% of the 222 patients. And the age was recognized as a confounding factor and was included in our analysis. There was no difference in the proportion of children between the low- and high-IgE groups (17.9% *vs.* 22.4%, $P = 0.518$). Results showed that age was not an independent correlation factor for remission or relapse. Serum IgE levels were independent correlation factors for remission and relapse after adjusting for age and other covariates in the multivariate cox regression model.

In this cohort, 134 patients received glucocorticoids alone, and the remaining 88 patients received other regimens, of which 58 patients received glucocorticoids plus tacrolimus. However, the time of adding tacrolimus to these 58 patients was different due to the shortcomings of retrospective studies, resulting in inconsistent treatment regimens and difficulty in further comparative analysis. And the number of patients in other treatment groups were insufficient for comparative analysis. Therefore, these patients were not included for further analysis. And the 134 patients treated with glucocorticoids alone were included in the further analysis.

Previous studies indicated that the serum IgE levels might serve as a prognostic indicator for steroid responsiveness in MCD patients (12, 22). A 2015 study including 30 children with steroid-sensitive nephrotic syndrome reported that patients with normal IgE levels mostly responded in week 1 after steroid therapy, and patients with high serum IgE levels mostly responded to glucocorticoids in weeks 2 or 3 after therapy (23). Another study compared the clinical characteristics of INS patients with normal IgE and high IgE levels, and reported that the high-IgE group required a significantly longer time to remission, and was more susceptible to frequent relapse (18). In this study, a delayed remission and an early relapse for MCD patients in the high-IgE group was observed, indicating that the serum IgE levels were closely related to glucocorticoid responsiveness in MCD patients, which was consistent with previous studies.

Up to 85% of MCD patients will relapse within 5 years, although glucocorticoids are effective in treating most patients with first-onset MCD (24). In this study, the time to first relapse of MCD patients found in the high-IgE group was significantly shorter than that in the low-IgE group, consistent with other studies' results (25–27).And a higher percentage of MCD patients were steroid-dependent in the high-IgE group than that in the low-IgE group in this study, and more patients experienced frequent relapses in the high-IgE group, as well. A previous study reported that the mean serum IgE levels of pediatric MCD patients at the time of relapse in frequent-relapse group was more than 3 times higher than that in infrequent-relapse group (25). And the serum IgE level decreased to normal at the time of remission in infrequent-relapse group, but it was still high in frequent-relapse group, indicating a persistent immune disorder in the patients with high IgE levels (25).

The IgE synthesis requires two signals: the first signal is transmitted by the cytokine interleukin (IL)-4 or IL-13

produced by type 2 helper T cells (Th2), and the second signal is transmitted by CD40 and CD40L activation (28). This indicates that the increase in serum IgE levels might be related to Th2 activation (29, 30). Some evidence suggests that the Th2 cytokine, IL-13, may play a potential regulatory role in MCD pathogenesis and high serum IgE levels: multiple reports have shown that serum IL-13 levels in MCD patients are elevated; IL-13 can regulate IgE production; and IL-13 can induce glomerular podocyte damage in animal models and caused MCD-like pathological changes (11, 20, 21). Therefore, IL-13 may drive the onset of the nephrotic syndrome and the increase of serum IgE levels. Thus, IgE is expected to play a role in MCD diagnosis and prognosis evaluation.

5 CONCLUSIONS

This study investigated the correlation between serum IgE levels and the clinical efficacy of glucocorticoids in MCD. It demonstrated that serum IgE level was an independent correlation factor for MCD remission and relapse. MCD patients with high serum IgE levels were prone to delayed remissions and early relapses. These findings could lay the foundation for further studies on MCD pathogenesis and theranostics.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Clinical Research Ethics Committee of the First Affiliated Hospital, Zhejiang University School of Medicine. The ethics committee waived the requirement of written informed consent for participation

AUTHOR CONTRIBUTIONS

HL and LW contributed to the study design, data acquisition, statistical analysis, and manuscript writing. XL, WC, and YZ contributed to data analysis. JC contributed to commentary and revision of the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Advances in the Management of Primary Membranous Nephropathy and Rituximab-Refractory Membranous Nephropathy

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Primary membranous nephropathy (pMN) is an auto-immune disease characterized by auto-antibodies targeting podocyte antigens resulting in activation of complement and damage to the glomerular basement membrane. pMN is the most common cause of nephrotic syndrome in adults without diabetes. Despite a very heterogeneous course of the disease, the treatment of pMN has for many years been based on uniform management of all patients regardless of the severity of the disease. The identification of prognostic markers has radically changed the vision of pMN and allowed KDIGO guidelines to evolve in 2021 towards a more personalized management based on the assessment of the risk of progressive loss of kidney function. The recognition of pMN as an antibody-mediated autoimmune disease has rationalized the use immunosuppressive drugs such as rituximab. Rituximab is now a first line immunosuppressive therapy for patients with pMN with proven safety and efficacy achieving remission in 60-80% of patients. For the remaining 20-40% of patients, several mechanisms may explain rituximab resistance: (i) decreased rituximab bioavailability; (ii) immunization against rituximab; and (iii) chronic glomerular damage. The treatment of patients with rituximab-refractory pMN remains controversial and challenging. In this review, we provide an overview of recent advances in the management of pMN (according to the KDIGO 2021 guidelines), in the understanding of the pathophysiology of rituximab resistance, and in the management of rituximab-refractory pMN. We propose a treatment decision aid based on immunomonitoring to identify failures related to underdosing or immunization against rituximab to overcome treatment resistance.

Keywords: membranous nephropathy, rituximab, autoimmunity, immunomonitoring, PLA2R1 autoantibodies, KDIGO (Kidney Disease: Improving Global Outcomes), immunosuppressive therapy, nephrotic syndrome

1 INTRODUCTION

Primary membranous nephropathy (pMN) is an autoimmune disease affecting kidney glomerulus and the most common cause of nephrotic syndrome in non-diabetic Caucasian adults. The formation of subepithelial immune deposits and complement activation are responsible for the functional impairment of the podocyte, leading to the onset of the nephrotic syndrome (1, 2). The course of the disease is highly variable, ranging from spontaneous remission to persistent proteinuria or end-stage renal disease (3, 4). The treatment of pMN has long been based on a uniform management of patients with a 6-month supportive therapy period (5), which can lead to the persistence of a nephrotic syndrome that can be complicated in few cases by long-term renal failure (3, 4). The recent identification of prognostic markers drastically changed the vision of pMN and allowed KDIGO Clinical Practice Guideline for the Management of Glomerular Diseases to evolve in 2021 towards a more personalized management based on the assessment of the risk of progressive loss of kidney function (6).

The recognition of pMN as an autoantibody-driven disease and the identification of podocyte target antigens have been major advances in the diagnosis, prognosis and follow-up of patients with pMN. In addition to the M-type phospholipase A2 receptor type 1 (PLA2R1) and thrombospondin type-1 domain-containing 7A (THSD7A), other new antigens have recently been identified (7). These findings have rationalized the use of B-cell depleting agents such as rituximab, a chimeric monoclonal antibody targeting CD20. Over the last decade, rituximab became a first line therapy for pMN with proven safety and efficacy (8–10). However, 20 to 40% of patients do not respond to the first course of rituximab (8–10) and 5 to 28 % of patients relapse after a remission period (9–12). A more personalized approach is needed for these patients to understand the causes of treatment failure and to propose alternative treatment options. Recently described markers can predict the response to rituximab and new promising treatment options may help to overcome rituximab resistance. This review covers recent advances in the management of pMN and rituximab-refractory pMN, and proposes a therapeutic algorithm for an optimal and personalized management of pMN.

2 MANAGEMENT OF PRIMARY MEMBRANOUS NEPHROPATHY

2.1 New Advances in Treatment

The KDIGO 2012 Clinical Practice Guideline for the Management of Glomerular Diseases recommended a supportive therapy with inhibitors of the renin angiotensin aldosterone system for at least six months before initiating immunosuppressive therapy in nephrotic pMN without serious complications (5). This uniform management strategy has been a source of controversy given the highly variable evolution of the disease. The KDIGO 2021 Clinical Practice Guideline for the Management of Glomerular Diseases have been modified taking into account the numerous advances in the field of pMN since the previous issue in 2012 (6). Important progress has been made most notably in the identification of prognostic markers. Thus, the new guidelines propose to categorize patients with pMN and nephrotic syndrome according to their risk of kidney disease progression in order to provide personalized management. However, several additional markers can help to stratify disease severity as well as response to treatment.

2.2 Biomarkers of Disease Severity

The risk of persistent nephrotic syndrome or kidney disease progression should be considered as a combination of factors (**Figure 1**). A management algorithm based on these risk factors has been proposed in the KDIGO 2021 guidelines and in a recent review (6, 15).

2.2.1 Proteinuria Level

The severity of the nephrotic syndrome and more specifically the level of proteinuria is associated with the risk of deterioration of renal function (4, 16). Patients with proteinuria >8 g/d for more than six months are at greater risk of impaired kidney function in the long term (16). Therefore, immunosuppressive therapy should be considered in patients with persistent high proteinuria despite a well conducted supportive therapy (6).

2.2.2 Glomerular Filtration Rate

Reduced renal function defined as a glomerular filtration rate (GFR) < 60 ml/min/1.73m^2 at diagnosis is associated with a higher risk of progression to renal failure (4, 16, 17). Patients with deteriorating kidney function may therefore immediately be treated with immunosuppressive therapy. In this case, rituximab and cyclophosphamide are recommended as first-line therapies in the KDIGO 2021 guidelines (6).

2.2.3 Urine Low-Molecular-Weight Proteins and Urinary IgG Excretion

Urinary excretion of beta-2 microglobulin (uβ2m) and alpha-1 microglobulin (uα1m) – i.e. low-molecular-weight proteins – has been shown to correlate with disease progression and the evolution of renal function in patients with pMN (18–20). Urinary IgG excretion has been shown to predict the evolution of renal function in pMN patients (20, 21). However, these markers are rarely used in daily practice.

2.2.4 Phospholipase A2 Receptor Type 1 Antibody Titer

In 2009, PLA2R1 was identified as the major podocyte antigen in 70-80% of pMN patients (22). Anti-PLA2R1 antibodies are highly specific for the diagnosis of pMN (23). Thus, according to the KDIGO 2021 guidelines, a kidney biopsy is not required to confirm the diagnosis of pMN in patients with nephrotic syndrome and a positive anti-PLA2R1 antibody test (6). PLA2R1 antibody titers correlate with disease activity, as antibodies usually disappear during spontaneous or treatment-induced remission and reappear in relapse (24).

Anti-PLA2R1 antibody titers also correlate with disease prognosis: a high titer at diagnosis was associated with a lower rate of spontaneous (25–29) or treatment-induced clinical remission (30). Patients with high anti-PLA2R1 titer achieved clinical remission significantly later than patients with low titer

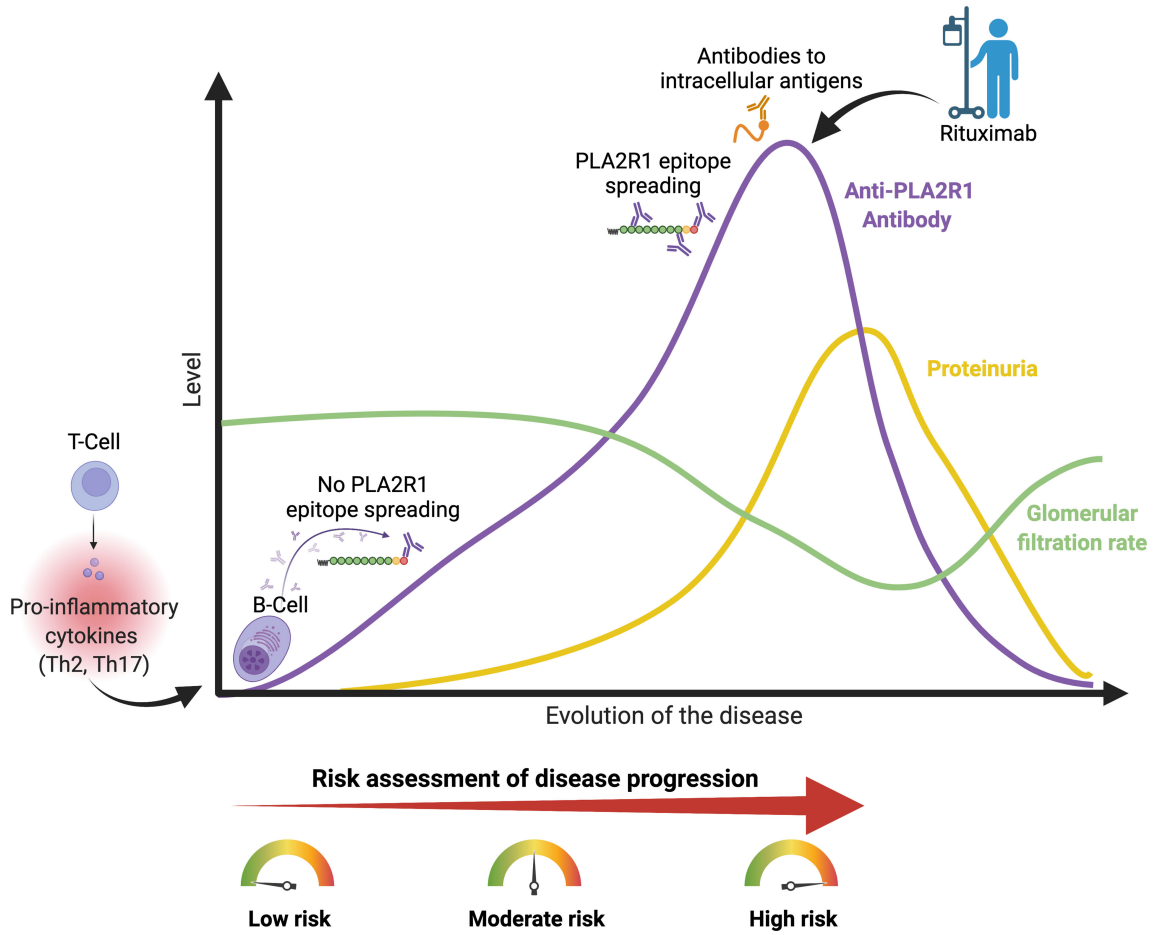

FIGURE 1 | Risk of disease progression in membranous nephropathy. PLA2R1, phospholipase A2 receptor type 1. Th2 and Th17 pro-inflammatory cytokines are increased in patients with pMN (13, 14). High anti-PLA2R1 antibody titer, positive epitope spreading, antibodies to intracellular antigens, high proteinuria despite optimal supportive care and deterioration of kidney function are associated with the risk of disease progression. Urine low-molecular-weight proteins and urinary IgG excretion have been shown to correlate with disease progression in patients with pMN, but these markers are rarely used in daily practice. Figure created with BioRender.com.

(26). In addition, a high anti-PLA2R1 titer at diagnosis was associated with a higher risk of long-term renal impairment (31, 32) and a higher risk of developing nephrotic syndrome in previously non-nephrotic patients (33). However, the thresholds used to define a high titer varied in these studies, it therefore remains unclear what specific antibody level should be used to predict the risk of disease progression. An anti-PLA2R1 antibody titer greater than 50 or 150 RU/mL appears to be reasonable to define a high antibody titer (6, 15). Between 50 and 150 RU/mL, the PLA2R1 epitope spreading analysis may be an important additional tool to assess patient prognosis. On the contrary, a low titer or the absence of anti-PLA2R1 antibody in patient with PLA2R1-associated pMN is correlated with a higher likelihood of spontaneous clinical remission (25–29, 33, 34). The decrease or disappearance of autoantibodies during follow-up usually precedes clinical remission (24, 30). Therefore, low baseline and decreasing anti-PLA2R1 antibody titers during follow-up strongly predict spontaneous remission, thus favoring supportive therapy alone. High baseline or increasing anti-PLA2R1 antibody titers, on the other hand, are associated with persistent nephrotic syndrome and progressive loss of kidney function, which should lead to the prompt initiation of immunosuppressive therapy (6).

Since re-emergence or increase in antibody titers precedes a clinical relapse, anti-PLA2R1 antibody titers should also be followed after immunosuppressive therapy (24, 30). The immunological response to immunosuppressive therapy may guide the adaptation of the treatment regimen. The KDIGO 2021 guidelines recommend monitoring of anti-PLA2R1 antibody titers with a first assessment three months after the start of therapy. In the case of stability or increased antibody titer, additional doses of treatment might be proposed (6).

2.2.5 Phospholipase A2 Receptor Type 1 Antibody Epitope Spreading

Epitope spreading refers to the development of an immune response directed against epitopes distinct from the dominant epitope,

without any cross-reactivity. This process is common in the fight against infectious agents. The epitopes may be located on the same antigen (called intramolecular epitope spreading) or on a different antigen (called intermolecular epitope spreading) (35).

This phenomenon was described in Heymann nephritis, a rat model of membranous nephropathy (36). In this model, rats immunized solely with the dominant megalin epitope gradually developed immunization against other epitopes of megalin, which was associated with a worsening of the disease. The same process may happen in humans with PLA2R1. PLA2R1 consists of several domains: (i) a cysteine-rich domain (CysR), (ii) a fibronectin type II domain (FnII) and (iii) eight C-type lectin domains (CTLD1 to CTLD8) (37). We and others have identified several different epitopes that can be recognized by anti-PLA2R1 antibodies (37–40): CysR (the dominant epitope), CTLD1, CTLD7 and CTLD8. In one of the studies, 67% of patients with pMN associated with anti-PLA2R1 antibodies had antibodies to CTLD1 and/or CTLD7 in addition to the dominant epitope CysR, defining intramolecular epitope spreading. Patients with epitope spreading had higher proteinuria level at diagnosis, poorer renal survival and a lower rate of spontaneous remission rate compared to patients without epitope spreading (37). In the GEMRITUX cohort, non-spreader patients had a spontaneous remission rate of 45% at six months while spreaders had a rate of 5% (41). Epitope spreading was an independent risk factor for poor renal prognosis (defined as persistence of proteinuria > 4 g/g and/or a GFR estimated by the CKD-EPI formula < 45 ml/min/1.73m^2 at the end of follow-up) and treatment failure (37, 41).

However, a recent study using a different detection technique demonstrated no additional predictive value of epitope spreading (40). Probably due to this lack of consensus, the epitope spreading did not enter in the risk-based treatment algorithm of pMN proposed by the KDIGO 2021 guidelines. More studies are needed to clearly establish the predictive value of epitope spreading for patients with PLA2R1-associated pMN (42).

2.2.6 Anti-Thrombospondin Type-1 Domain-Containing 7A Antibody Titer

Anti-THSD7A antibodies are present in approximately 3% of pMN patients (43). Similarly to anti-PLA2R1 antibodies, anti-THSD7A antibody titers correlate with disease activity (44, 45). However, due to the rarity of THSD7A-associated pMN, data on the prognostic value of anti-THSD7A antibody titers are limited (44). Anti-THSD7A antibody monitoring is not included in the management algorithm proposed by the KDIGO 2021 guidelines, but antibody titer-based management should probably also be applied to patients with THSD7A-associated pMN.

The mechanism of epitope spreading has also been described in THSD7A-related pMN but due to lack of statistical power, the impact of spreading on prognosis could not be evaluated (46).

2.2.7 Cytokine Profile and Environment

Environmental factors may play a role in the pathophysiology of pMN. In China, the incidence of pMN was correlated to the level of exposure to fine particles in the air (47, 48). Based on a functional approach, we have shown that: (i) pMN patients had higher levels of pro-inflammatory Th2 and Th17 cytokines than healthy subjects, and (ii) that patients with high levels of Th17 cytokines lived in urbanized areas highly exposed to fine particles in the air (13). Increased levels of Th17 cytokines were associated with more venous thromboembolic events and a 10.5-fold higher risk of relapse (13). However, the cytokine profile is not measured in daily clinical practice and this criterion is not included in the management algorithm proposed by the KDIGO 2021 guidelines (6).

2.2.8 Antibodies to Intracellular Antigens

Antibodies targeting different intracellular podocyte antigens such as aldose reductase, superoxide dismutase 2, and α-enolase have been identified in a significant number of patients with pMN (49, 50). In PLA2R1-associated pMN, the immunization against superoxide dismutase 2 and α-enolase was associated with a lower rate of clinical remission and a greater impairment of renal function (51). Patients with PLA2R1 epitope spreading were at higher risk of immunization against these intracellular antigens (51). Immunization against intracellular antigens could be the result of a multi-hit pathogenic mechanism. First, antibodies recognize a dominant epitope on a primary autoantigen (e.g. CysR on PLA2R1). Subsequently (i) intramolecular epitope spreading within the primary autoantigen may occur, and (ii) intermolecular epitope spreading with the formation of antibodies could target intracellular autoantigens (e.g. aldose reductase, anti-superoxide dismutase 2 and anti-α-enolase). These intra- and intermolecular epitope spreading would lead to an amplification of the immune response against the podocyte and, potentially, a more severe disease progression (51). However, testing for the antibodies against intracellular antigens is not performed in daily clinical practice and this criterion is not included in the management algorithm proposed by the KDIGO 2021 guidelines (6).

2.3 Rituximab in Primary Membranous Nephropathy

To date, the alkylating agents (e.g. cyclophosphamide) are the only treatment with proven efficacy to prevent end stage kidney disease (ESKD) and death (52–54). The basis for recommendations on these treatments comes from studies conducted three decades ago in which alkylating agents improved nephrotic syndrome and renal disease progression over non-immunosuppressive antiproteinuric therapy (52, 53). However, alkylating agents combined with corticosteroids are associated with a risk of serious infections, late malignancy, infertility and other severe adverse events (55). Rituximab appears to be safer, and allows a high rate of clinical remission, which has been associated with preservation of renal function on the long term.

Rituximab is an anti-CD20 chimeric monoclonal antibody, which can trigger B-cell death by apoptosis, complement-mediated cytotoxicity, and antibody-dependent cellular cytotoxicity (10).

Since the first report of patients with pMN treated with rituximab in 2002 (56), rituximab has progressively emerged as the first choice treatment for pMN due to its high safety and efficacy. In several non-randomized studies, the remission rate for pMN patients treated with rituximab ranged from 57% to 89% (11, 12, 56, 57).

In a large retrospective observational cohort study, van den Brand et al. analyzed outcomes of 100 patients treated with rituximab compared with 103 patients treated with glucocorticoid plus oral cyclophosphamide. Over a median follow-up of 40 months, the rituximab group had significantly fewer adverse

events than the cyclophosphamide/glucocorticoid group. Although the cumulative incidence of partial remission was lower in the rituximab group, rates of complete remission and a composite end point of doubling of serum creatinine, end-stage kidney disease, or death did not differ significantly between groups (55).

In the multicentric randomized controlled trial GEMRITUX, rituximab (2 infusions of 375 mg/m^2 on day 1 and 8) combined with a non-immunosuppressive antiproteinuric treatment (NIAT) was compared with NIAT alone (8). At six months, there was no significant difference between the groups in the rate of clinical remission. However, in the extended follow-up (after a median follow-up of 17 months), a significant difference was reported, with clinical remission occurring in 65% of the NIAT-rituximab group but only in 34% of the NIAT alone group (p<0.01). In addition, anti-PLA2R1 antibodies depletion rates were greater in the NIAT-rituximab group than in the NIAT alone group at month-3 (56% and 4%, respectively, p<0.001) and at month-6 (50% and 12%, respectively, p=0.004). The delayed efficacy of rituximab in the GEMRITUX study could therefore be explained by the fact that immunological remission precedes clinical remission by several months (30).

In the multicentric randomized controlled trial MENTOR, rituximab (2 infusions of 1 g on day 1 and 15) was compared to cyclosporine. Rituximab was as effective as cyclosporine in inducing clinical remission at 12 months, but discontinuation of cyclosporine after 12 months resulted in an increased rate of relapse, resulting in a higher clinical remission rate at 24 months in the rituximab group than in the cyclosporine group (60% in the rituximab group *vs* 20% in the cyclosporine group, p<0.001). The decrease in autoantibody titer was faster, greater, and more durable in the rituximab group than in the cyclosporine group. Serious adverse events occurred in 11 patients (17%) in the rituximab group and in 20 (31%) in the cyclosporine group (9).

More recently, a randomized controlled trial RI-CYCLO compared the efficacy of cyclic cyclophosphamide/glucocorticoid regimen to rituximab (two infusions of 1 g on day 1 and 15) in inducing clinical remission (58). At 12 months, 23/37 (62%) patients in the rituximab group and 27/37 (73%) in cyclophosphamide/glucocorticoid group had a complete or partial remission. At 24 months, probabilities of complete and partial remission were comparable between the two groups. The frequency of serious adverse events was comparable between the groups (19% of patients in the rituximab group and in 14% of patients in cyclophosphamide/glucocorticoid group). The authors concluded that trial found no evidence of more benefit or less harm associated with rituximab compared with a cyclophosphamide/glucocorticoid regimen in the treatment of pMN.

All these studies show remarkable efficacy of rituximab with limited side effects compared to cyclophosphamide or cyclosporine. This supports the use of rituximab as a first-line treatment for pMN.

2.4 Calcineurin Inhibitors in Primary Membranous Nephropathy

Calcineurin inhibitors (CNIs) (e.g. cyclosporine and tacrolimus) are immunosuppressive therapies that inhibit T-cell activation. CNIs have also been shown to have an immunomodulatory effect by inhibiting the Th17 immune response, which is implicated in the development of autoimmune diseases such as pMN (59–61). CNIs have been used for many years in the management of pMN.

In the MENTOR trial, cyclosporine was less effective than rituximab in achieving long-term persistent clinical remission (9). Furthermore, cyclosporine was associated with a high incidence of relapse after discontinuation of treatment (53% in the cyclosporine group *vs* 5% in the rituximab group) and frequent side effects.

Ramachandran et al. compared the long-term efficacy of a combination of cyclophosphamide and glucocorticoid for six months to a combination of tacrolimus for one year and prednisone for six months. At the end-point of six years after the beginning of treatment, 62% of participants in the cyclophosphamide/glucocorticoid group and 28% in the tacrolimus group maintained remission without relapse, and 88% of patients in the cyclophosphamide/glucocorticoid group *vs* 53% of patients in the tacrolimus group were in remission. This study confirmed the long-term superiority of six months of cyclophosphamide/glucocorticoid therapy *vs* one year of tacrolimus (62).

In summary, these studies show that CNI monotherapy regimens are less likely to achieve long-term clinical remission than rituximab or cyclophosphamide/glucocorticoid-based protocols. For this reason, the KDIGO 2021 guidelines suggest that CNI monotherapy is justifiable only in patients with a normal glomerular filtration rate and a moderate risk of disease progression – as these patients have less severe disease with a greater likelihood of achieving remission – in order to shorten the period of proteinuria (6).

3 RITUXIMAB-REFRACTORY MEMBRANOUS NEPHROPATHY

Rituximab-refractory pMN may be defined by the absence of clinical and/or immunological remission (i.e. antibody titer below the detection threshold by ELISA or a negative indirect immunofluorescence assay) after a rituximab course. Understanding the mechanisms behind treatment failure allows the identification of additional therapeutic strategies for rituximab-refractory pMN. The main mechanisms behind treatment failure are summarized in **Figure 2**.

3.1 Causes of Rituximab Resistance

3.1.1 Rituximab Underdosing

Rituximab underdosing is not uncommon in pMN (63, 64). Indeed, rituximab bioavailability is significantly decreased in pMN compared to other non-nephrotic autoimmune diseases due to the binding of rituximab to albumin and its wasting in the urine (63, 65, 66). We have shown that rituximab was undetectable three months (month-3) after rituximab infusion (two 1 g infusions two weeks apart) in 56% of nephrotic pMN,

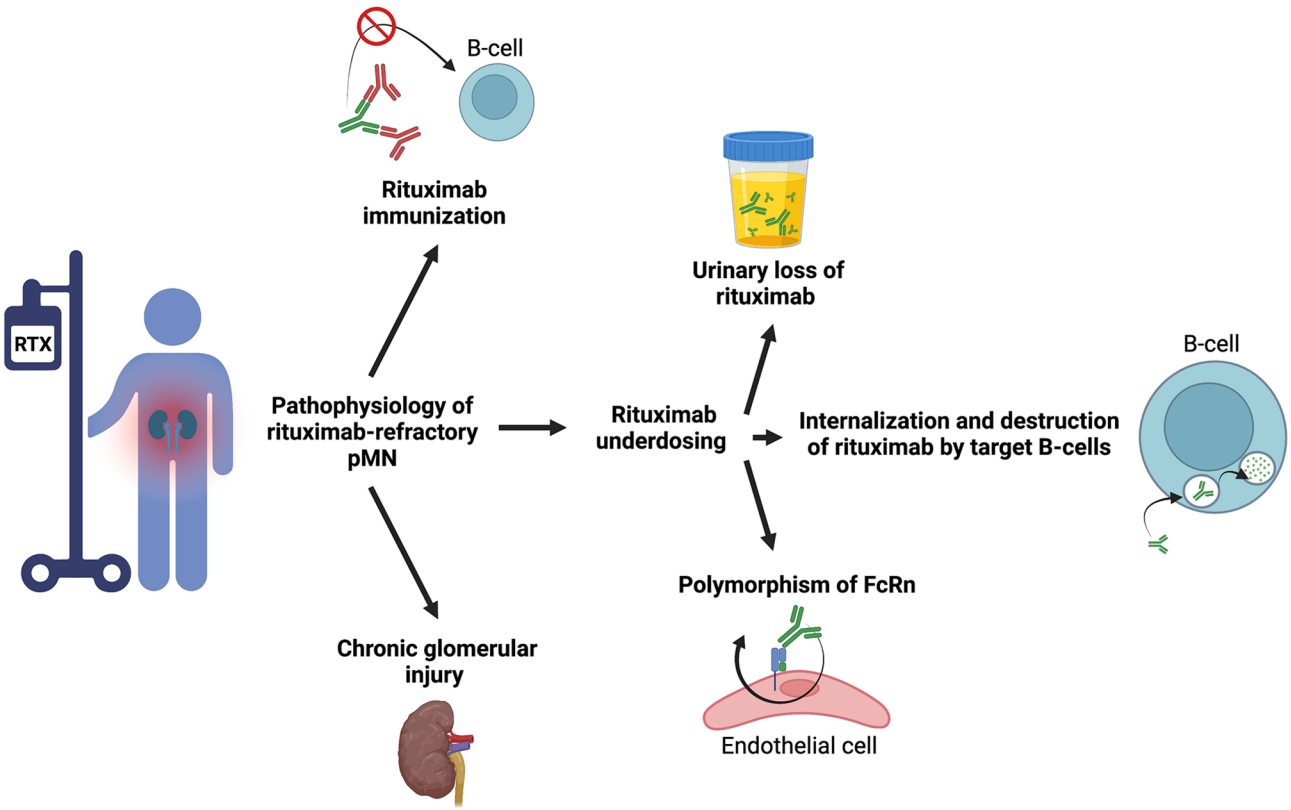

FIGURE 2 | Main causes of rituximab resistance in membranous nephropathy. ADAs, anti-drug antibodies; FcRN, neonatal Fc receptor for IgG; PLA2R1, phospholipase A2 receptor type 1; RTX, rituximab. Figure created with BioRender.com.

these patients were less likely to achieve clinical and immunological remission (64). Patients with the most severe nephrotic syndrome – with a baseline albumin level of less than 22.5 g/L – were more likely to have an undetectable serum rituximab level at month-3 (64). Serum rituximab level can be measured by ELISA (which costs about 40€ in France). However, this technique is not routinely available in all centers. If required, it is recommended to refer to an expert center. In addition to urinary excretion of rituximab, internalization and destruction of rituximab by target B-cells and polymorphism of the neonatal Fc receptor for IgG (FcRn) – which protects antibodies from degradation by lysosomes and thus reduces their clearance by allowing their recycling in the cellular environment – may also decrease rituximab bioavailability (67–70).

3.1.2 Rituximab Immunization

Rituximab is a chimeric monoclonal antibody including human IgG1 constant regions and a murine anti-human CD20 variable region. The use chimeric monoclonal antibodies may be complicated by the development of anti-drug antibodies (ADAs) such as anti-rituximab antibodies. Twenty-three to 43% of patients treated with rituximab develop anti-rituximab antibodies during follow-up (57, 71). These ADAs neutralized rituximab activity (complement-dependent cytotoxicity and antibody-dependent cell-mediated cytotoxicity) in 8 of 10 patients (80%). Anti-rituximab antibodies resulted in faster B-cell reconstitution and a higher rate of relapse (71). Therefore, in patients previously treated with rituximab, anti-rituximab antibodies should be systematically tested before starting a new course of rituximab. Anti-rituximab antibodies can be measured by ELISA (which costs about 40€ in France). However, this technique is not routinely available in all centers. If required, it is recommended to refer to an expert center.

3.1.3 Chronic and Irreversible Damage to the Glomerular Filtration Barrier

The presence of fibrous glomerular damage may be responsible for significant proteinuria. In patients who are refractory to multiple immunosuppressive therapies, it may be difficult to distinguish between primary immunosuppressive resistance and resistance secondary to chronic and irreversible glomerular damage. In these patients, a repeat kidney biopsy and monitoring of immunological activity (anti-PLA2R1 or anti-THSD7A titers) may be useful to distinguish between patients with an immunologically active disease who may benefit from additional immunosuppressive therapy (24, 44, 45), and patients with extensive chronic histologic lesions in whom additional immunosuppressive therapy is futile.

3.2 Management of Rituximab-Refractory Membranous Nephropathy

The KDIGO 2021 guidelines suggest that patients who are refractory to a first course of rituximab should receive a second course of rituximab and calcineurin inhibitors (CNIs) or cyclophosphamide and glucocorticoids (6). However, this does not take into account the cause of rituximab resistance. An approach based on immunomonitoring of rituximab and anti-rituximab antibodies would allow a more targeted management.

3.2.1 Optimized Supportive Therapy

In order to reduce proteinuria and urinary loss of rituximab, the supportive therapy should be optimized. It should include a renin-angiotensin-aldosterone system inhibitor (angiotensin-converting enzyme inhibitor or angiotensin II receptor blocker), a diuretic as well as a low-sodium diet (< 2 g/d) (6, 72, 73). The control of extracellular overload and arterial blood pressure are major components of the management of the nephrotic syndrome; systolic blood pressure should be < 120 mmHg (6). In the absence of renal failure, protein intake should be 0.8 to 1 g/kg/d (if proteinuria > 5 g/d: add 1g per 1g of protein loss) (6). High- or low-protein diets are not recommended. A low-fat diet is recommended for patients with high cholesterol to prevent cardiovascular complications (6).

3.2.2 Rituximab Dosing

In nephrotic patients, the bioavailability of rituximab is decreased due to the elimination of rituximab in the urine (63, 64). Uncertainty remains regarding the rituximab dosing protocol to be used in nephrotic patients.

In the GEMRITUX study, B-cells were not fully depleted six months after rituximab treatment, suggesting that the dose used (two 375 mg/m^2 injections one week apart) was suboptimal (8). This lack of B-cell depletion may explain the lack of significant difference in the 6-month clinical remission rate between the rituximab-NIAT group and the NIAT alone group (8).

More recently, we have shown that a high-dose rituximab regimen (two 1000 mg injections two weeks apart) was more effective on B-cell depletion and was associated with a higher clinical remission rate than the GEMRITUX regimen (10). The median residual rituximab level at month-3 was higher with the high-dose rituximab regimen compared to the GEMRITUX regimen and was correlated with higher rates of clinical remission.

Two recent randomized controlled trials also used a high-dose rituximab regimen (i.e. two 1000 mg injections). Remission rates at 6-month were comparable with high-dose rituximab in MENTOR and low-dose regimen in GEMRITUX despite higher baseline anti-PLA2R1 titers in MENTOR (8, 9). Remission rates at 6-month were higher with high-dose rituximab in RI-CYCLO compared to low-dose regimen in GEMRITUX despite comparable baseline anti-PLA2R1 titers (8, 58). These data would also favor high-dose rituximab.

Since patients with severe nephrotic syndrome are at higher risk of rituximab underdosing and treatment failure (64), higher initial doses of rituximab in these patients may limit the risk of treatment resistance and increase the likelihood of remission by fully depleting B-cells. High-dose rituximab regimen also appears to be required in patients with epitope spreading, while a low-dose of rituximab regimen may be sufficient for patients with anti-PLA2R1 activity limited to CysR (10). Indeed, all patients with antibodies restricted to the CysR domain (non-spreaders) entered into remission at last observation carried forward regardless of the rituximab protocol administered, while patients with epitope spreading had a higher likelihood of remission with a high-dose rituximab regimen (10). Similarly, patients with high anti-PLA2R1 antibody titers at baseline achieved clinical remission more frequently with the high-dose rituximab regimen (10). A clinical trial is underway in patients with PLA2R1-associated pMN to compare the efficacy of the GEMRITUX regimen (low-dose rituximab after six months of anti-proteinuric therapy) to a personalized management (stratifying the patients according to their epitope spreading status at month-0 and month-6 and treating them accordingly with either low- or high-dose rituximab regimen) (42).

3.2.3 Repeated Rituximab Injections

Reinfusion of rituximab can induce immunological and clinical remission in patients considered refractory to rituximab (74, 75). Dahan et al. described ten patients who were refractory to an initial course of rituximab and who were retreated with rituximab resulting in remission in eight of them (74). We have further expanded this study by showing that patients who failed to respond to a first course of rituximab and who did not develop anti-rituximab antibodies responded to repeated courses of rituximab while those who developed antibodies only responded to treatment with human or humanized anti-CD20 monoclonal antibodies (75).

For patients with PLA2R1-associated pMN, immunomonitoring of anti-PLA2R1 antibodies may be beneficial for guiding rituximab therapy. In the absence of immunological remission, repeated rituximab injections at three and/or six months after the start of treatment increased clinical remission rate at 12 months to 91% (76). KDIGO 2021 guidelines recommend anti-PLA2R1 antibody monitoring at month-3 and -6 and administering an additional rituximab dose to patients with persistent or increasing anti-PLA2R1 titers (6).

Since serum rituximab levels are lower during follow-up in patients with nephrotic pMN and since undetectable rituximab levels at month-3 are associated with a risk of treatment failure, rituximab immunomonitoring may be useful in treatment decision-making (63, 64). Patients with undetectable serum rituximab levels (< 2 μg/mL) at month-3 did not achieve clinical remission at month-6 if proteinuria was greater than 5.5 g/d at month-3 (64). In these patients, additional early doses of rituximab must be considered. In order to limit the urinary loss of rituximab in patients with active nephrotic syndrome, it is also important to ensure that supportive therapy is provided at the maximum tolerated dose (no extracellular overload without serum creatinine increase >30% and orthostatic hypotension).

3.2.4 Human and Humanized Anti-CD20 Antibodies

New generations of humanized or fully human anti-CD20 antibodies have been developed. Obinutuzumab and

ofatumumab are directed to a different epitope on CD20 and have higher affinity for CD20 than rituximab (77, 78). Obinutuzumab is a humanized and glycoengineered type II anti-CD20 monoclonal antibody. The glycan tree of the Fc fragment of obinutuzumab was modified to enhance antibody-dependent cellular cytotoxicity (78). Ofatumumab is a human type I anti-CD20 antibody that activates complement-dependent cytotoxicity more effectively than rituximab (77). Obinutuzumab and ofatumumab have superior *in vitro* and *in vivo* B-cell cytotoxicity and lower risk of immunization compared to rituximab (69, 77). These new monoclonal antibodies have shown efficacy in some autoimmune or inflammatory diseases (such as rheumatoid arthritis, systemic autoimmune diseases and chronic inflammatory demyelinating polyneuropathy) after the development of resistance to chimeric monoclonal antibodies following the appearance of ADA (79, 80). Anti-rituximab antibodies cross-reacted with obinutuzumab or ofatumumab in only 20% of patients with ADA (71). In a series of four cases with rituximab-refractory pMN, obinutuzumab and ofatumumab were effective in achieving clinical and immunological remission (75, 81–83). However, randomized controlled trials comparing ofatumumab or obinutuzumab to rituximab in patients with non-Hodgkin lymphoma have not found superiority of these new monoclonal antibodies over rituximab (84, 85). Randomized and well-powered clinical trials are needed to evaluate the efficacy and the optimal dose of humanized or human anti-CD20 in patients with pMN who develop anti-rituximab antibodies.

3.2.5 Calcineurin Inhibitors in Combination With Rituximab

The STARMEN trial, compared the effectiveness of a 6-month induction course with tacrolimus (followed by tapering over another three months) in combination with a single dose of rituximab (1 g) at six months with a 6-month cyclical therapy of methylprednisolone and cyclophosphamide (86). The tacrolimus/rituximab protocol was less effective than the cyclophosphamide/glucocorticoid protocol in achieving clinical remission at 24 months (58% *vs* 84%, respectively), while serious adverse events were similar in both groups. The immunological response (depletion of anti-PLA2R1 antibodies) was also significantly higher at three and six months in the cyclophosphamide/glucocorticoid group (77% and 92%, respectively) than in the tacrolimus/rituximab group (45% and 70%, respectively). It is important to note that the addition of rituximab was delayed by six months in this study, which may explain the limited effectiveness of this combination. Moreover, this study has some limitations, including a higher proportion of male patients and higher anti-PLA2R1 antibody titers in the tacrolimus/rituximab group than in the cyclophosphamide/glucocorticoid group.

A pilot trial combining rituximab (two infusions of 1 g on day 1 and 15, repeated at six months) and cyclosporine showed substantially higher rates of complete clinical remission and immunological response at 24 months than those observed with rituximab or cyclosporine alone in the MENTOR trial (87, 88).

Therefore, the combination of CNI/rituximab in patients refractory to rituximab because of underdosing may be of interest. In addition to their immunosuppressive and immunomodulating effects, CNIs may decrease the urinary loss of rituximab by causing glomerular arteriolar vasoconstriction. It is important to note that this combination is likely to result in significant immunosuppression with an increased risk of infection.

3.2.6 Other Treatments

Plasma exchange, immunoabsorption, mycophenolate mofetil (MMF), adrenocorticotropic hormone (ACTH) and bortezomib have been proposed for the management of refractory pMN.

Plasma exchange and immunoabsorption have been proposed in the management of pMN to rapidly decrease the titers of autoantibodies. However, only small series have been reported in patients with severe or refractory pMN (83, 89, 90). Plasma exchange and immunoabsorption do not directly modulate B-cell proliferation and activity, so the patients additionally received immunosuppressive treatment. Plasma exchange and immunoabsorption combined with immunosuppressive therapy seem to allow a faster remission than immunosuppressive therapy alone in patients with severe disease or refractory to conventional treatment.

MMF has also been proposed for the management of refractory pMN. In a study of 16 patients with pMN, including 15 steroid-resistant, six alkylating agent-resistant, and five cyclosporine-resistant patients, treatment with MMF for a mean duration of eight months resulted in a halving of proteinuria in six patients and partial clinical remission in two patients (91).

ACTH was administered to five pMN patients resistant to initial treatment. Three patients had previously received CNIs, two cyclophosphamide, two steroids, four MMF, and one patient previously received rituximab. Two of the five patients achieved partial clinical remission on ACTH therapy and three patients achieved immunological remission (92).

Finally, several case studies have reported the efficacy of bortezomib – an anti-plasma cell agent – in combination with glucocorticoids in the treatment of pMN refractory to first-line immunosuppressants (93–95).

High quality data demonstrating the effectiveness of these treatments are lacking. Well-conducted randomized clinical trials are needed to assess the effectiveness of these treatments in the management of refractory pMN. Therefore, these treatments should not be used as first line therapy and their use should be systematically discussed with an expert center.

3.2.7 Personalized Management

We propose a personalized management of pMN that takes into account (**Figures 3**–**5**): (i) the risk assessment of disease progression (see 2.2 Biomarkers of Disease Severity); (ii) a better understanding of the pathophysiology of rituximab-refractory pMN (see 3.1 Causes of Rituximab Resistance); and (iii) rituximab and ADAs immunomonitoring (see 3.2 Management of Rituximab-Refractory Membranous Nephropathy).

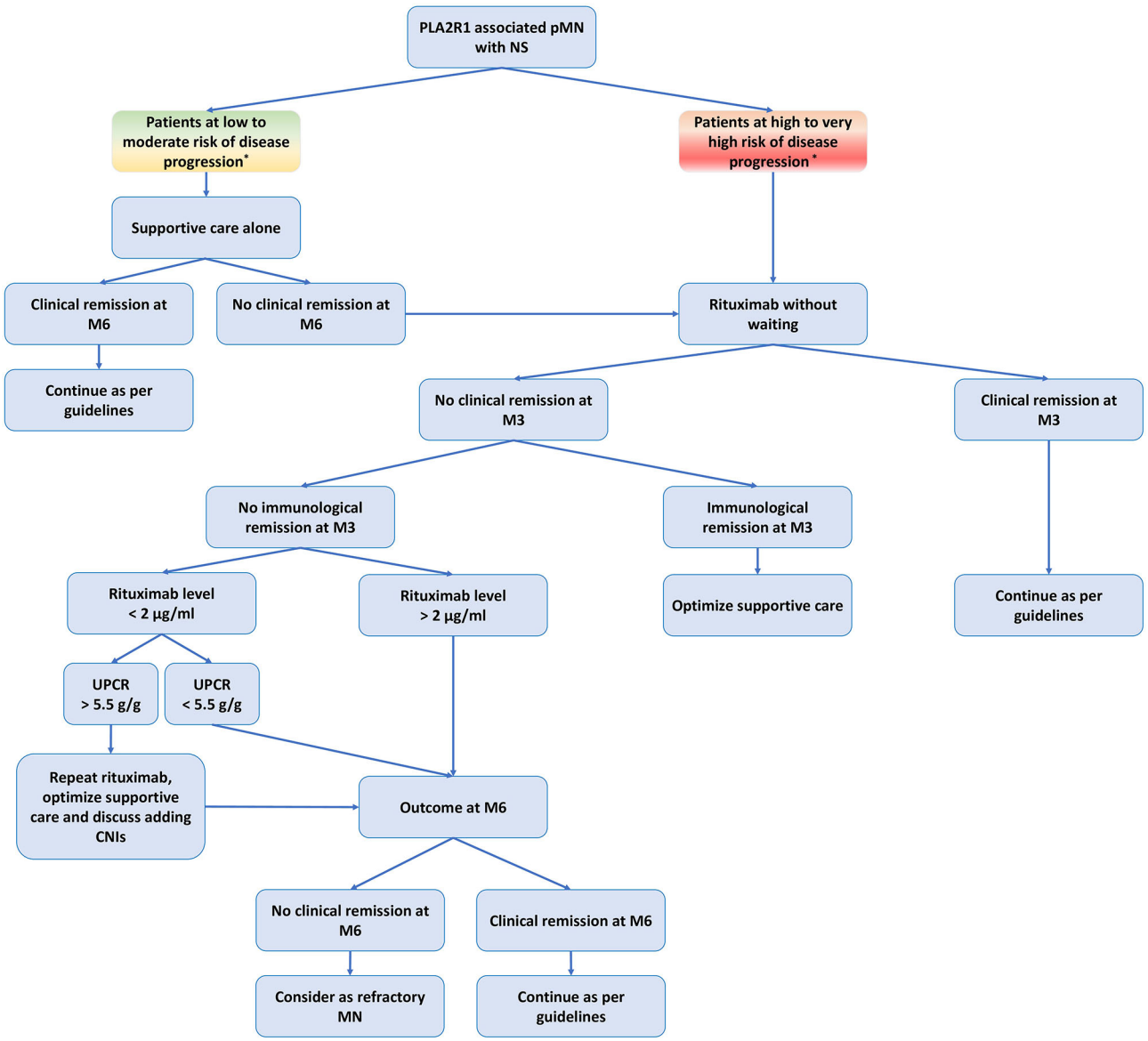

FIGURE 3 | Treatment of primary membranous nephropathy based on risk assessment of disease progression and rituximab immunomonitoring. CNIs, calcineurin inhibitors; M3, month-3 (3 months after rituximab infusion); M6, month-6 (six months after rituximab infusion); NS, nephrotic syndrome; pMN, primary membranous nephropathy; PLA2R1, phospholipase A2 receptor type 1; RTX, rituximab; UPCR, urine protein/creatinine ratio. *Low to moderate risk of disease progression: negative epitope spreading and anti-PLA2R1 titer < 150 RU/ml, and no severe complication related to NS, and reduction of proteinuria under optimal supportive care and no deterioration of kidney function. High to very high risk of disease progression: positive epitope spreading, and/or anti-PLA2R1 titer > 150 RU/ml, and/or severe complication related to NS, and/or high proteinuria (> 8 g/d) despite optimal supportive care and/or deterioration of kidney function. Urine low-molecular-weight proteins and urinary IgG excretion have been shown to correlate with disease progression in patients with pMN, but these markers are rarely used in daily practice. Serum rituximab level analyzed by ELISA (LISA-TRACKER Duo Rituximab, Theradiag © Croissy Beaubourg, France) three months after the last injection. The limit of detection defined by the manufacturer is 2 µg/ml.

In patients with high or very high risk of disease progression, immunosuppressive therapy should be initiated promptly, whereas it may be delayed by 3 to 6 months in patients with low or moderate risk.

Immunomonitoring allows the personalization of the initial therapeutic management as well as the management of refractory or relapsing pMN with a fairly low cost (**Figures 3**–**5**).

We propose to assess the residual serum rituximab level three months after rituximab infusion to identify underdosed patients. In these patients, additional doses of rituximab should be considered as early as month-3 if proteinuria is greater than 5.5 g/d (64). Optimized supportive therapy is required to prevent urinary loss of rituximab. The addition of CNI may also be discussed.

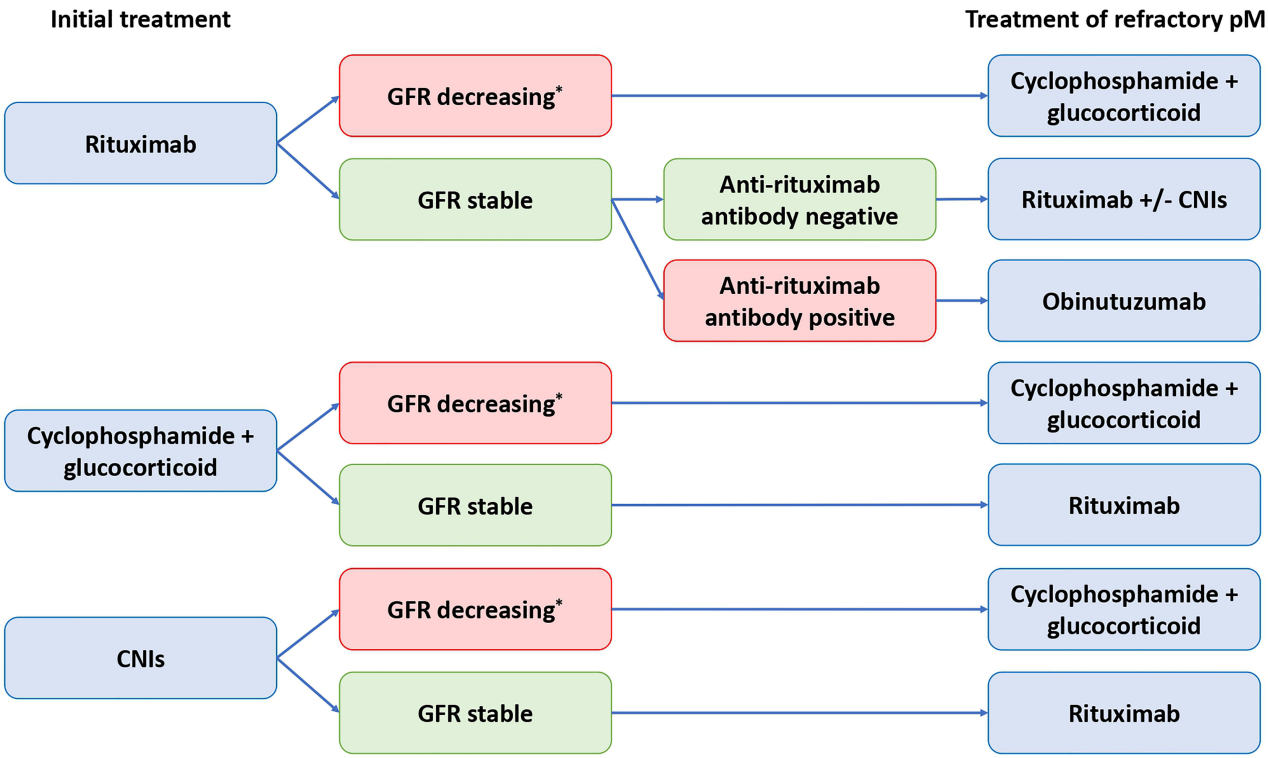

FIGURE 4 | Algorithm for management of patients with treatment-resistant primary membranous nephropathy. CNIs, calcineurin inhibitors; GFR, glomerular filtration rate; pMN, primary membranous nephropathy. *directly related to membranous nephropathy. To date, the alkylating agents (e.g. cyclophosphamide) are the only treatment with proven efficacy to prevent end stage kidney disease (ESKD) and death.

We propose to measure anti-rituximab antibodies in rituximab-refractory pMN or relapsing pMN after rituximab in order to identify patients who should receive a treatment with human or humanized anti-CD20 monoclonal antibodies (**Figures 4**, **5**).

4 PERSPECTIVES IN THE MANAGEMENT OF MEMBRANOUS NEPHROPATHY

4.1 Complement Inhibitors

The pathogenicity of complement in the development of pMN is well demonstrated (1, 2, 96). The complement is involved in the physiopathology of pMN, notably *via* the formation of the membrane attack complex C5b-9 following the activation of the classical pathway, the lectin pathway or the alternative pathway. C5b-9 forms a transmembrane pore that can cause osmotic lysis of the podocyte. C5b-9 can also cause damage to the glomerular filtration membrane by various other mechanisms, such as: (i) stimulation of the production of reactive oxygen species, proteases and prostanoids; (ii) modifications of the actin cytoskeleton and alterations of podocyte slit diaphragm; (iii) stimulation of the production of TGF-β and extracellular matrix components and (iv) limitation of podocyte cell proliferation (97). Blocking complement activation and C5b-9 formation is therefore an attractive therapeutic option. As IgG4 can activate the lectin pathway (98), the use of treatments that inhibit the common final complement pathway should be preferred to treatments targeting a single upstream complement pathway. While targeting the complement pathway seems to be an attractive option in pMN patients, a preliminary trial of eculizumab – a monoclonal antibody that binds to C5 and prevents its cleavage – was unsuccessful (99). Other anti-complement therapies are being evaluated in pMN (100).

While anti-complement therapies have not yet been proven to be effective when administered alone, they could represent a complementary strategy when used simultaneously with other immunosuppressive drugs or prior to other immunosuppressive drugs. They may allow rapid containment of glomerular damage by blocking complement activation until the immunosuppressive treatment results in a sufficient decrease in circulating antibody levels. However, since part of the efficacy of monoclonal antibodies (e.g. rituximab) is based on complement-mediated cytotoxicity, anti-complement therapies could limit the effect of monoclonal antibodies if used simultaneously.

4.2 Elimination of Autoreactive B-Cells and Induction of Immune Tolerance

Chimeric antigen receptor (CAR) T cells is a technology based on T cells genetically engineered to express an artificial T cell receptor specific to an antigen of interest. CAR T cells are currently used in cancer immunotherapy (101) and are a

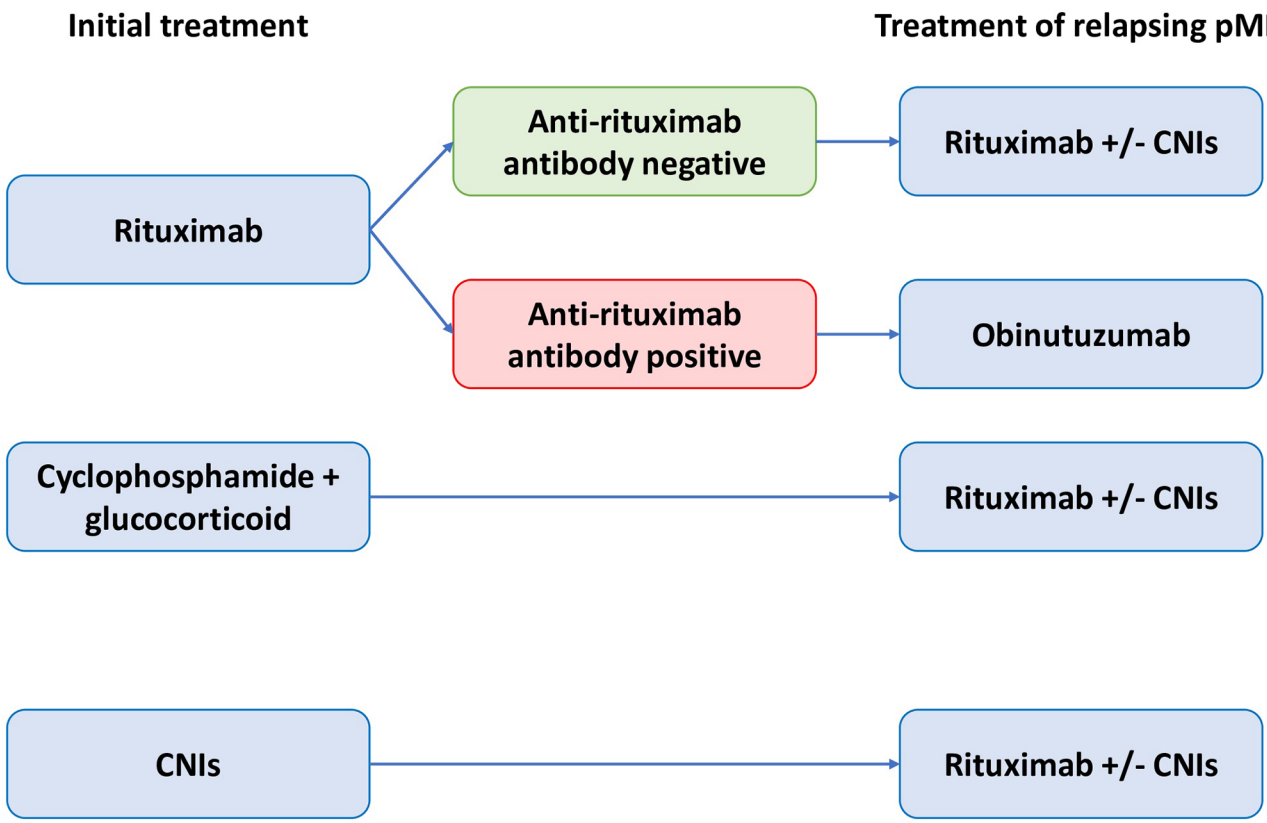

FIGURE 5 | Algorithm for management of patients with relapsing primary membranous nephropathy. CNIs, calcineurin inhibitors; pMN, primary membranous nephropathy.

promising therapeutic strategy to eliminate pathogenic B-cells in antibody-mediated autoimmune diseases (102). In a murine lupus model, CD19 CAR T cells persistently depleted CD19+ B-cells, eliminated autoantibody production, reduced the clinical manifestations of the disease and prolonged lifespan (103).

Chimeric autoantibody receptor T cells (CAAR T cells) are a modified version of CAR T cells. While CAR T cells recognize a specific surface antigen on the target cells, CAAR T cells contain domains of the antigen of interest. Thus, CAAR T cells recognize and bind to target autoantibodies expressed on autoreactive B-cells *via* the specific antigen and then destroy autoreactive cells. In a murine model of pemphigus vulgaris CAAR T cells expressing the target autoantigen – i.e. desmoglein 3 – specifically and efficiently eliminated desmoglein 3-specific autoreactive B-cells (104).

Another alternative is to induce immunotolerance as autoimmune diseases are characterized by a loss of self-tolerance. Since patients with pMN have a deficiency of regulatory T cells (13, 105, 106), the generation of CAR Tregs is a promising option to suppress autoimmune manifestations. CAR Tregs were effective in mouse models of encephalomyelitis and ulcerative colitis (107, 108). A second strategy to regain immune tolerance through the induction of Tregs is the use of nanoparticles with autoimmune disease-relevant peptides bound to major histocompatibility complex class II molecules. These nanoparticles may induce antigen-specific Tregs. These antigen-specific Tregs then promote the differentiation of B-cells into disease-suppressing regulatory B-cells, suppress autoantigen-loaded antigen-presenting cells and inhibit CD4+ and CD8+ T cells, leading to the resolution of autoimmune manifestations (109).

It would therefore be of interest to evaluate these new biotechnologies in the context of pMN refractory to conventional treatment.

4.3 Cytokine-Regulating Treatment

Cytokines may play a role in the pathogenesis of pMN. We have shown that high serum IL-17A levels were associated with poor prognosis in pMN, defined by more thromboembolic complications and more relapses of nephrotic syndrome. Rituximab treatment induced Th1 and regulatory T cell cytokines but did not impact Th17 cytokines (13). These data raise the question of additional maintenance therapy to block Th17-mediated inflammation. The blockade could be achieved by the use of anti-IL6 (e.g. siltuximab), anti-IL6 receptor (e.g. tocilizumab), anti-IL-17A (e.g. ixekizumab or secukinumab) or anti-IL-17 receptor (e.g. brodalumab) in combination with rituximab. Immunomodulators have the advantage of not inducing immunosuppression and therefore limit the adverse effects. These treatments could be of value for patients who relapse frequently.

Further studies are needed to evaluate their effectiveness on prevention of relapses either alone or in combination with standard immunosuppressive treatment.

4.4 Other Immunosuppressive Treatments

Belimumab is a human IgG1-λ monoclonal antibody that inhibits B-cell activating factor (BAFF). In pMN patients it reduced anti-PLA2R1 antibody levels and proteinuria (110). When comparing this decrease in anti-PLA2R1 antibody levels with belimumab to that reported with rituximab in the GEMRITUX study, the decrease was faster in rituximab-treated patients than in belimumab-treated patients. It has been proposed that the faster effect of rituximab was related to immediate B-cell lysis, whereas the delayed effect of belimumab was related to progressive B-cell "exhaustion" secondary to BAFF binding and inhibition (111). Further studies are needed to assess the effectiveness of belimumab in pMN, alone or in combination with other therapies.

An ongoing clinical trial is also evaluating anti-CD38 antibody treatments to target plasma cells in pMN with anti-PLA2R1 antibodies (NCT0415440).

4.5 New Nephroprotective Therapies

New nephroprotective therapies have been developed in recent years. Sparsentan, a dual endothelin type A and angiotensin II type 1 receptor antagonist, has demonstrated superiority in reducing proteinuria over ibersartan in focal segmental glomerulosclerosis (FSGS) (112).

Preclinical studies suggest that inhibition of glomerular roundabout guidance receptor 2 (ROBO2)/slit guidance ligand 2 (SLIT2) signaling can stabilize podocyte adhesion and reduce proteinuria. A study is underway to evaluate the effectiveness of ROBO2/SLIT2 inhibition with the ROBO2 fusion protein PF-06730512 in patients with FSGS (113).

Sodium/glucose cotransporter 2 (SGLT2) inhibitors in combination with inhibitors of the renin angiotensin aldosterone system have been shown to be of value in preventing kidney failure in patients with chronic kidney disease (CKD) (114, 115).

Treatment with a steroidal mineralocorticoid receptor antagonist in patients with CKD reduces blood pressure and proteinuria (116). Finerenone, a selective non-steroidal mineralocorticoid receptor antagonist, in combination with inhibitors of the renin angiotensin aldosterone system, has been shown to be effective in reducing albuminuria in patients with diabetic nephropathy (117). Recently, finerenone has been shown to result in lower risks of CKD progression and cardiovascular events than placebo in patients with CKD and type 2 diabetes (118).

These treatments could also be of interest in pMN. Further studies are needed to evaluate their effectiveness in this indication.

5 CONCLUSION

Important advances have been made recently in the understanding of the pathophysiology and management of pMN. The KDIGO 2021 guidelines have drastically changed the approach of the disease management model from a homogeneous management of all patients to a personalized management based on the risk assessment of disease progression. Currently, rituximab is one of the first-line treatments for pMN with proven safety and efficacy. Recent advances in the understanding of the mechanisms behind the resistance to rituximab have made it possible to propose alternative and better adapted treatments in order to increase clinical remission rates and reduce the risk of relapse.

AUTHOR CONTRIBUTIONS

The original concept and design of the study was made by BS-P. MT performed a detailed literature search. MT, VE, MC, VB and BS-P drafted and revised the manuscript. All authors contributed to the article and approved the submitted version.

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An Interdisciplinary Diagnostic Approach to Guide Therapy in C3 Glomerulopathy

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Since the re-classification of membranoproliferative glomerulonephritis the new disease entity C3 glomerulopathy is diagnosed if C3 deposition is clearly dominant over immunoglobulins in immunohistochemistry or immunofluorescence. Although this new definition is more orientated at the pathophysiology as mediated by activity of the alternative complement pathway C3 glomerulopathy remains a heterogenous group of disorders. Genetic or autoimmune causes are associated in several but not in all patients with this disease. However, prognosis is poorly predictable, and clinicians cannot directly identify patients that might benefit from therapy. Moreover, therapy may range from supportive care alone, unspecific immune suppression, plasma treatment, or plasma exchange to complement inhibition. The current biopsy based diagnostic approaches sometimes combined with complement profiling are not sufficient to guide clinicians neither (i) whether to treat an individual patient, nor (ii) to choose the best therapy. With this perspective, we propose an interdisciplinary diagnostic approach, including detailed analysis of the kidney biopsy for morphological alterations and immunohistochemical staining, for genetic analyses of complement genes, complement activation patterning in plasma, and furthermore for applying novel approaches for convertase typing and complement profiling directly in renal tissue. Such a combined diagnostic approach was used here for a 42-year-old female patient with a novel mutation in the Factor H gene, C3 glomerulopathy and signs of chronic endothelial damage. We present here an approach that might in future help to guide therapy of renal diseases with relevant complement activation, especially since diverse new anti-complement agents are under clinical investigation.

Keywords: C3 glomerulopathy, membranoproliferative glomerulonephritis, complement, factor H, eculizumab, FHL1

INTRODUCTION

In 2013 a consensus report suggested a re-definition of the heterogeneous group of membranoproliferative glomerulonephritis (MPGN) (1, 2). A new classification according to the immunohistochemical/immunofluorescence findings was recommended, in order to allow better association of pathogenesis of the diseases compared to the pure morphological distinction of MPGN type I, II, or III. For that, C3 glomerulopathy (C3G) should be diagnosed if C3 deposition is clearly dominant over immunoglobulins. This new definition of C3G includes the patterns MPGN I and III, as well as intramembranous glomerulonephritis/dense deposit disease (MPGN type II). Moreover, diagnosis of C3G was not restricted to a membranoproliferative pattern but could be every other form of glomerulonephritis, e.g., mesangioproliferative. This new definition resulted from advances in the understanding of complement-mediated kidney diseases, of which C3G is one prototypical disease (3, 4). In C3G, overactivation of the complement system can be associated with genetic mutations in complement genes, like Factor H, C3 and the *FHR1*, *FHR2*, *FHR3*, *FHR4* and *FHR5* genes. Nephritic factors are antibodies that are capable to stabilize complement activation by binding to the alternative pathway (AP) C3 convertase or the C5 convertase or to single complement proteins such as Factor H, C3, C3b, C3d or Factor B. These diverse antibodies interfere with the alternative pathway activation and cause its overactivation. In healthy individuals the alternative complement pathway is constantly activated by default, due to a spontaneous hydrolysis of C3 and controlled by different complement factors. Complement factor H (Factor H) is the primary regulator of the alternative complement cascade. The Factor H genes encode two mRNAs. One codes for the full length Factor H gene which is composed of 20 repeat domains The second mRNA encodes FHL1 a 42 kDa plasma protein that includes the first seven SCR domains of Factor H. Several other mutations, primarily interacting with the alternative complement pathway, have been described. Mutations in other genes link in FHR1, FHR2, FHR3, FHR4 FHR5, C3 included genes which encode components that form the C3 or C5 convertases or for regulators which define the time and the site of C3 convertase action (5).

However, in a relevant number of patients, a causal genetic alteration or autoimmune factor cannot be found. Despite the new classification and orientation towards pathophysiology, C3G remains a very heterogeneous disease. In consequence, the clinical outcome of the patients is different. While some patients initially present with rapidly progressive glomerulonephritis, others present with albuminuria and have stable renal function. The mean 10-year renal survival rate is approximately 50% (6). Clinicians first have to decide whether or not to treat a C3G patient, but there are only few studies focusing on the therapy of this rare disease group. Some of the studies were performed before reclassification, meaning that these studies do not only include C3G but also immune complex forms. Given the change in terminology and disease characterization and the potential confounding effect on trial stratification, the results of these trials are of limited use in guiding current treatment considerations for C3G. In general, there are different treatment strategies, including immunosuppression, plasma therapy, or complement blockade. A significant dilemma is that clinicians lack data on which therapy might be helpful in which patients. The current diagnostic work-up seems not sufficient to guide treatment of single patients. We want to highlight the importance of an interdisciplinary diagnostic approach to understand an individual patient's form of C3G to guide therapies with this work.

CASE ANALYSIS

We here present a case of a 42-year-old female, Caucasian patient that presented with arterial hypertension, elevated serum creatinine values and albuminuria (up to 500 mg/g creatinine) at her nephrologist. Due to ongoing rise of serum creatinine the patient was sent to our hospital (**Figure 1A**). A kidney biopsy was performed, revealing the diagnosis of mesangioproliferative glomerulonephritis. Immunohistochemical staining for immunoglobulins A and G was negative. In contrast, there was dominant mesangial positivity for C3 (**Figure 1B**), leading to the diagnosis of a mesangioproliferative glomerulonephritis with dominant C3 deposition. Since there was no evidence for an infection-related cause, it was classified as mesangioproliferative C3 glomerulonephritis as a subform of C3G. Due to the ongoing impairment of renal function, we agreed that there was an indication for treatment. Since clinical trials are not sufficient to recommend a specific therapy for these cases, we performed further analysis. Genetic testing revealed a heterozygous mutation of the Factor H gene, introducing a stop codon at p.Pro440 -Stop, in SCR7 (**Figure 1C**). Such heterozygous Factor H mutations in domain 7 are not described so far for C3G and to our knowledge most mutations in the Factor H gene associated with C3G or MPGN type II present as homozygous or compound heterozygous settings. The stop codon in domain 7 affects expression of one allele both of

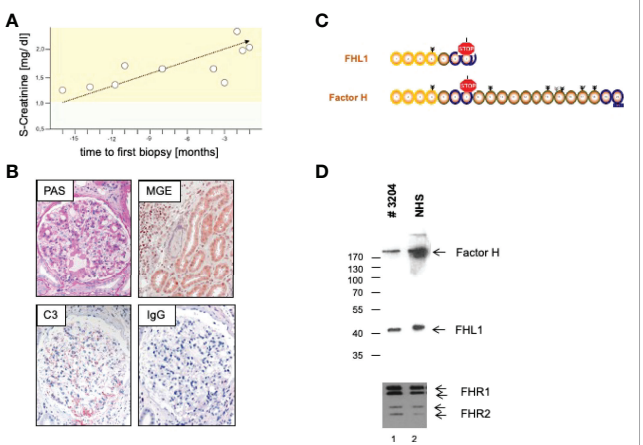

FIGURE 1 | **(A)** Course of S-Creatinine values previous to treatment. **(B)** Representative microphotographs of periodic acid Schiff (PAS) and Masson-Goldner-Elastica (MGE) stained kidney sections show a mesangioproliferative pattern (PAS) and signs of chronic endothelial damage in an arteriole (MGE). Immunohistochemical staining reveal strong positivity for C3, but no deposition of Immunoglobulin G (IgG). **(C)** Genetic analysis revealed introduction of a stop codon in domain 7 with a **(D)** consecutive a lack of Complement Factor H (Factor H) and Factor H like Protein1 (FHL1) in serum of the patient.

Factor H and of the FHL1 protein. Indeed, plasma protein levels of both Factor H and FHL1 were low when compared to healthy subjects (**Figure 1D**). Interestingly, other mutations of Factor H are associated with thrombotic microangiopathies, especially atypical hemolytic uremic syndrome (7). Indeed, the initial biopsy showed some intimal sclerosis without elastosis (**Figure 1B**), which could be regarded as a former or chronic thrombotic microangiopathy, indicating an ongoing damage of endothelial cells. However, the biopsy did not reveal fresh microthrombi. Nevertheless, the genetic analysis linked glomerulonephritis and the (alternative) complement system and so strengthened the diagnosis of C3 glomerulonephritis. To evaluate different therapeutic strategies, we performed further analyses. Using ELISA, a series of complement markers and activation product were analyzed in plasma to follow the complete complement pathways and compared their pattern with a patient with atypical hemolytic uremic syndrome as with other patients and also healthy subjects (**Figure 2A**) (8). C3 consumption combined with increased levels of complement activation products indicates ongoing complement activation, which is also detected in plasma of a patient with genetically mediated atypical hemolytic uremic syndrome. C3 level were reduced in patient serum. In addition C3a, a product of C3 hydrolysis and opsonin, was increased when compared with healthy subject. Moreover, the noncatalytic subunit Ba of complement Factor B was upregulated considerably, further indicating activation of the alternative complement pathway. Since the patient suffered from a mutation that could not control complement overactivation, we assumed activation downstream of the C3 convertase, e.g., the terminal complement pathway. Indeed, we detected the terminal complement cascade products to be elevated (C5a and C5b-9) (**Figure 2A**). The observation of terminal complement activation was not restricted to the serum reflecting defective fluid phase regulation. Analyzing the kidney biopsy, prominent immunohistochemical staining for C3 and, even more impressive, C5b-9 we observed (**Figure 2B**) arguing for activation of the terminal complement pathway also on the surface of the target organ.

Due to complement activation with the apparent involvement of the terminal cascade, signs of thrombotic microangiopathy, and the Factor H/FHL1 mutation, we asked which type of therapy might be best for this patient. Assuming that reduced plasma levels and reduced regulatory Factor H and FHL1 function caused complement deregulation in plasma and on surfaces in the patient, we wanted to test which components, Factor H, FHL1 or complement inhibitors can restore the complement stability and reduce hemolysis. To this end we established *in vitro* hemolytic assays to the show that serum purified Factor H, CPV-101 H, FHL1, Compstatin. sCR1 or also Eculizumab might influence complement mediated hemolysis of sheep erythrocytes, and further more asking whether complement preferred C5 inhibition as the most promising treatment strategy. Both, Factor H, CPV-101, FHL1 and sCR1 reduced hemolysis in this assay and the effects were comparable and dose dependent. The C3 inhibitor (Compstatin) also reduced hemolysis. Moreover, Eculizumab, at that time the only clinically approved inhibitor, also inhibited lyses (**Figure 2C**). Based on these effects we finally decided to start treatment with Eculizumab. After 13 weeks of treatment, again a kidney biopsy was performed, allowing us to reevaluate the inhibitory effect in the target organ. Upon treatment we observed some reduction of C3 and considerable reduction of C5b-9 deposition in the glomeruli (**Figure 3A**). Using a computer-based deep-learning assisted detection of cell nuclei, we quantified mesangial and endocapillary mononuclear cells (**Figure 3B**). With that tool, we found a reduction of both cell types, indicating functional relevance

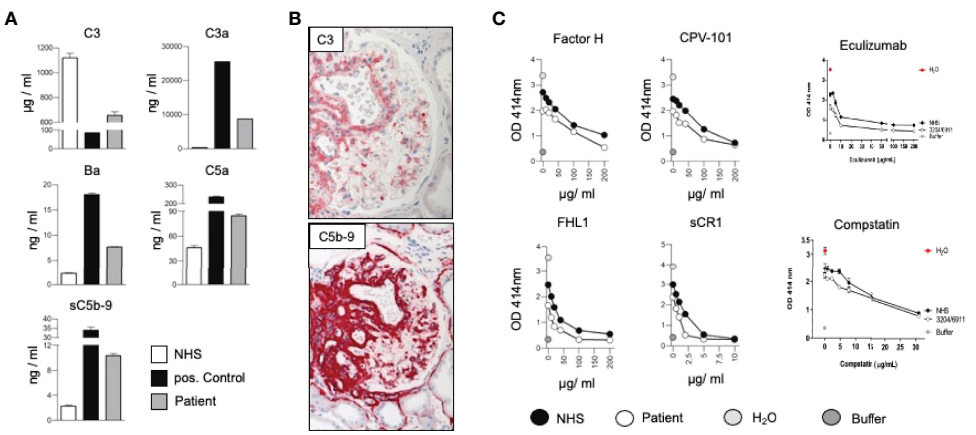

FIGURE 2 | **(A)** Measurement of the complement protein 3 (C3), the cleavage product C3a, Factor Ba, fragment, complement anaphylatoxin C5a, and the soluble form of the membrane attack complex (sC5b-9) in sera of the patient, a healthy subject (NHS) and a patient suffering from hemolytic uremic syndrome induced by deficiency of complement Factor H (positive control) by ELISA. By following the levels of C3, the central complement components indicative of continuous ongoing complement activation at the early levels (8). The consumption of C3 combined with elevated levels of C3a, Ba, C5a and sC5b-9 shows that complement is activated and that activation proceeds to the C3 convertase, as well as C5 convertase level and processes to the terminal pathway. Such strong fluid phase activation is in agreement with reduced Factor H and FHL1 plasma levels. **(B)** Representative immunohistochemical staining for C3 and the membrane attack complex (C5b-9). **(C)** *In vitro* effect of Factor H, CPV-101, FHL1, sCR1 Eculizumab and Compstatin in *in vitro* hemolysis assays using patient serum (white circles) NHS (black circles), H2O (grey circles) or buffer (dark grey circles).

of reduced C5a and C5b-9 deposits. As previously described, we are also able to visualize the alternative and classical C3 convertase using proximity ligation assay (PLA) (**Figure 3C**) (9). Both were unaffected by the C5 blockade, assumably since Eculizumab blocks downstream of the C3 convertases. In line with this argumentation, serum analysis revealed a dramatic reduction in the terminal complement components (C5a, C5b-9). Components upstream of C5 inhibition, e.g. C3 and Ba, were not affected (**Figure 3D**). By including these PLA methods, theoretically it is also possible to distinguish whether the reduction of C5b-9 deposition is a therapy effect or reflects a decreased natural disease activity. In this case, unchanged high signal numbers for the alternative C3 convertase indicates an unchanged natural proximal disease activity, so that the C5b-9 reduction can be interpreted as a therapy effect. However, also C3a was reduced upon Eculizumab treatment, potentially arguing for a feedback regulation on C3 conversion. During treatment the serum creatinine levels stabilized (1.5 – 1.7mg/dl) and the patient presented without significant albuminuria (< 100mg/g creatinine), up to 20 weeks follow-up.

DISCUSSION

C3G is a heterogeneous disease group resulting from defective complement regulation either in the plasma (fluid phase) or on surfaces such as the glomerular basement and cell membranes. Different mechanisms ultimately drive the same pathological principals. The disease ranges from genetic mutations to autoantibody-mediated dysregulation of the alternative complement system. In dense deposit disease, 25 of 32 patients (78%) were positive for an autoantibody stabilizing the alternative C3 convertase (10). Genetic analysis demonstrated several mutations in various genes interacting with alternative complement cascade, including Factor H, FHR1, FHR2, FHR3, FHR4, FHR5 and C3 (1, 3). However, immune-complex mediated MPGN and C3G share pathophysiological aspects and even immunoglobulin dominant MPGN cases can have an underlying, primary dysregulation of the alternative complement pathway (6, 11–13). Thus, also in these cases, if systemic infectious or rheumatological diseases, such as systemic lupus erythematosus, hepatitis C, and cryoglobulinemia are excluded, it is important to consider autoimmune, genetic, and functional plasma analysis for the alternative pathway. The clinical course of C3G is difficult to predict. The Mayo Clinic published its data on 114 C3G patients and concluded variable response to their therapy, meaning that there is no clear data on when therapy is indicated in an individual patient (14). Currently, a kidney biopsy is mostly performed at the initial stage of disease and is primarily of diagnostic relevance. However, endocapillary hypercellularity or necrosis/extracapillary proliferation may serve as criteria for treatment. Up to now, authors recommend unspecific therapy with steroids or the combination with proliferation inhibitors (15). Guidelines and most authors recommend MMF as the first-line therapy and refer to one study if immunosuppression is indicated. In a Spanish cohort, MMF in combination with steroids demonstrated preferable result when 22 patients were compared to 18 patients that received other immunosuppression (mostly steroids alone) (16). This observation was confirmed in another small study in which 67%

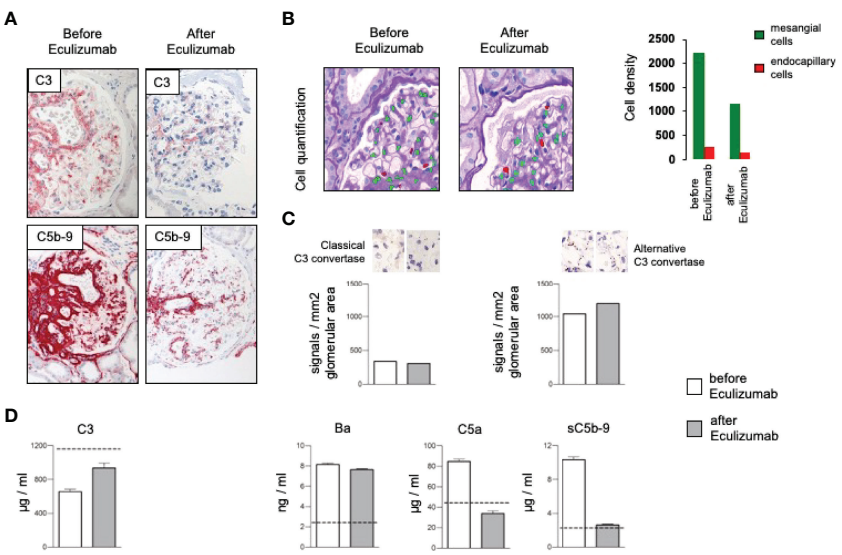

FIGURE 3 | **(A)** Representative immunohistochemical staining for complement C3 with a mild and the membrane attack complex (C5b-9) with a significant reduction after treatment with Eculizumab. **(B)** Analyzing density of mesangial and endocapillary mononuclear cells shows a reduction of both cell types. **(C)** Quantification of the signal density for the classical and the alternative C3 convertase revealed no major changes, especially no reduction of the alternative convertase. **(D)** Complement marker profile before and after Eculizumab treatment. C3 plasma levels increased upon Eculizumab therapy. The proximal activation markers C3a, and Ba did not change. Distal activation markers, e.g. the anaphylatoxin C5a and the soluble membrane attack complex (sC5b-9) showed reduction after treatment with Eculizumab.

of 30 patients treated with MMF were classified as responders to immunosuppression (17). The largest study investigating the therapeutic effect of MMF in C3 glomerulopathy includes 97 patients (C3G n=81; DDD n=16). Here, not only the therapeutic response of MMF and steroids was investigated in comparison to other therapeutic options, but also the different pathogenesis with regard to genetic alterations and antibodies was taken into account. After a follow-up of up to 10 years, a superiority of the group treated with MMF and steroids was shown. This was true for the comparison with other immunosuppressive therapy regimens as well as for supportive therapy alone. However, the therapeutic effect of MMF and steroids seemed to be less pronounced in patients with genetic abnormalities (18). These observations contrasts with other reports which demonstrate no benefit for immunosuppression (19, 20). Given the heterogenous character of the disease some cases of complement-mediated glomerulonephritis seem to benefit from immunosuppression. Such forms might represent the former with complement activation in plasma as indicated by reduced C3 plasma levels as well as elevated levels of the inflammatory anaphylatoxins C3a or C5a. Kidney biopsy from such patients may lack signs of thrombotic microangiopathy, but might show glomerulonephritis with an influx of inflammatory cells and mesangial cell proliferation. This case underlines the importance of establishing multiple complement plasma markers upon time of diagnosis and combine this with genetic testing. We firstly demonstrate a new heterozygous Factor H gene mutation associated with C3G, which affects both Factor H and FHL1 levels in plasma. Since Factor H and FHL1 are strong inhibitors of alternative complement, reduced levels Factor H and FHL1 might force clinicians to treat patients with plasma infusions or exchange. Our analysis gained insights into the therapeutic effect after reconstitution with Factor H and FHL1 by *in-vitro* testing. Indeed, this may be a relevant therapeutic option. Licht et al. also demonstrated the efficacy of plasma infusions in two patients suffering from DDD due to a Factor H mutation and preserved them from disease progression (21). However, even if treatment was well tolerated in this study, plasma infusion risks allergic reactions and might cause volume overload. Moreover, as C3G potentially progresses to end-stage kidney disease, plasma infusion can cause antibody formation, which must be avoided before renal transplantation. Supplementation of Factor H by recombinant proteins might be a therapeutic prospect. Its production in therapeutically useful quantities might be feasible by production in several cellular systems (22). Restoring Factor H activity by human recombinant Factor H in deficient knockout mice led to resolution of glomerular basement membrane lesions in a murine model for dense deposit disease (23). This might be a therapeutic option for patients with complement mediated disease suffering from loss of function Factor H mutations.

The new approach presented here shows that a detailed analysis of the kidney biopsy in combination with extensive complement marker analysis in plasma is helpful at the time of diagnosis, allows to evaluate complement inhibitors for therapy and furthermore allows to follow the complement response upon treatment with complement inhibitors. In addition, repeated biopsies following therapy with complement inhibitors show the effect of treatment in the affected organ, and these effects were combined with inhibition of the activation in plasma, maybe allowing to monitor or even predict clinical response. We detected low levels of C3 and consecutive higher levels of C3a, indicating complement activation. Ba fragment is cleaved from Factor B upon formation of the alternative C3 convertase (C3bBb). Elevated Ba levels strongly argue for alternative activation in plasma since Factor B is only involved in this pathway. A detailed examination of combined complement markers along the cascade allows to follow the complement activation steps and this may help to narrow down the localization of the defect leading to extended complement activation. Given different cause single patients might present with a different pattern of complement activation. With such a detailed analysis of complement components, it might be feasible to choose the right drug for an individual patient. In our case, elevated C5b-9 levels in serum and the kidney biopsy argued for a dominant role of the terminal complement cascade. Despite several successful case reports on Eculizumab in C3G, this treatment seems to have mixed results (24, 25). In a study by Bomback, with six patients (three DDD, three C3G) that presented with albuminuria >1g/d and/or AKI were treated for 12 months (26). During that course of treatment, only three patients had a marked reduction of s-creatinine or albuminuria and one more patient had histopathological evidence of improvement. In the French cohort in a heterogeneous group of 26 patients, including 13 adolescents and 13 children, some of them having CKD, others presenting with progressive disease, and at least two patients requiring dialysis were treated for a median duration of 14 months (27). 54% of these patients had no clinical response. In study of 10 patients that presented with nephrotic albuminuria Eculizumab was used in a single-arm trial in an off-on-off-on design. Patients were treated in two 48-week treatment periods, divided by a 12 week washout period. In this study, only patients with very high levels of sC5b-9 levels were included. Despite this evidence for activation of the terminal complement cascade, only 30% of the patients achieved significant reduction of proteinuria in the first treatment period. All responders showed increase of proteinuria during eculizumab withdrawal (28). However, none of these studies were able to predict the response to the treatment. In this case, we were convinced that C5 inhibition might be the favorable treatment strategy since we found overactivation of the alternative complement pathway and a genetic mutation that cannot control that overactivation. Consequently, we saw the activation of the terminal complement pathway in our patient's sera and kidney. In addition to C3G, the renal biopsy had also shown signs of an old thrombotic microangiopathy, which can occur in atypical hemolytic uremic syndrome. Mutations in Factor H are also present in aHUS, in which eculizumab is an approved therapy. Indeed, the efficacy of eculizumab has been demonstrated in 2 patients with bioptic signs of C3G and thrombotic microangiopathy (29). After *in-vitro* testing the therapeutic potential, we started treatment with Eculizumab. Even if the follow-up period is too short to draw conclusions about the effective efficacy of eculizumab treatment we detected an inhibition of terminal complement activation in the sera and

the kidney. Since Eculizumab acts downstream of the C3 convertase, changes in the concentration of C3a and Ba were less affected under C5 inhibition. However there are signs of a feedback regulation under Eculizumab. Since current recommendations focus on classical risk factors for progressive kidney diseases and do not consider genetic and functional analysis we argue that a combined and detailed analysis of the plasma complement parameters and the kidney might allow guiding therapy in C3G and also in other complement-mediated glomerulonephritis. A more detailed insight into different (alternative) complement activation steps might be more relevant soon since various complement blocking agents are under clinical investigation for renal and extrarenal diseases (30).

In conclusion, we believe that C3G is not only too rare but also too heterogenous for larger, controlled, randomized, prospective therapy studies. Thus, C3G is a perfect example of a disease, in which unravelling the exact pathogenesis by combining morphological *in situ*, genetic, autoimmune, and functional *in vitro* data of single patients likely will be the clue to the best, personalized therapy.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article. The patient's consent is existent *via* the Hamburg Glomerulonephritis Registry approved by the Hamburg Ethics Committee (approval number PV4806).

AUTHOR CONTRIBUTIONS

SA, LP, KH, and PFZ performed functional analyses; CK also contributed to manuscript preparation. CK provided clinical data; SW, TS, and TW performed morphological and *in situ* analyses; TS, PFZ, and TW wrote the manuscript. All authors contributed to the article and approved the submitted version.

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CASE REPORT
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Membranous Nephropathy Secondary to Graves' Disease: A Case Report

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Membranous nephropathy (MN) is a form of kidney disease that is idiopathic in 70%–80% of cases. Glomerular involvement in autoimmune thyroiditis can occur in 10%–30% of patients, and MN manifests in association with Hashimoto thyroiditis in up to 20% of the cases with glomerular involvement. Reports of MN associated with Graves' disease (GD) are extremely rare in the current literature. Herein, we report the case of a 46-year-old man admitted to the hospital with nephrotic syndrome and symptomatic hyperthyroidism due to GD. Kidney biopsy revealed a secondary MN pattern. Immunohistochemical staining for PLA2R was negative, and thyroglobulin showed weak and segmental staining along the glomerular capillary. Anti-thyroid peroxidase (TPO) antibody test was not performed. The patient was treated for GD with methimazole and prednisone, and despite reaching clinical improvement after 8 months, proteinuria remained close to nephrotic levels. In this scenario, the patient was submitted to radioactive iodine, and there was a dramatic reduction in proteinuria levels after treatment. In conclusion, GD association with MN is rare, and when present, diagnosis using PLA2R and immunohistochemistry can be useful in determining association. In addition, radioactive iodine therapy can be an effective treatment modality when preceded with immunosuppressive corticosteroid therapy.

Keywords: Graves' disease, membranous nephropathy, auto-immune thyroiditis, kidney biopsy, renal pathology

INTRODUCTION

Membranous nephropathy (MN) is a glomerulopathy associated with subepithelial immune deposits that induces a spectrum of changes in the glomerular basement membrane (GBM). MN is idiopathic in most cases (70%–80%, primary MN), while the remaining cases are associated with other conditions, ranging from infections, autoimmune diseases, neoplasms, and medications (secondary MN) (1, 2).

Clinical and laboratorial presentation of primary and secondary forms of MN are indistinguishable, with the differential diagnosis relying on an investigation of associated conditions (3). The glomerular involvement in patients with thyroid autoimmune diseases (especially in Hashimoto thyroiditis) can occur in up to 10%–30% of the cases, and MN is present in 20% of these cases. The high prevalence of MN in thyroiditis cases with glomerular involvement suggests a thyroid-associated antigen pathophysiological pathway (4). In contrast to Hashimoto's disease, Graves' disease (GD) association with any kind of glomerular disease is extremely rare.

GD is the main cause of hyperthyroidism worldwide. It is a relatively common disease in the overall population and has an established treatment. Only a few cases have reported the association of GD with glomerulopathies. The present study aims to present a case of MN secondary to GD and to review the current literature involving GD and glomerular diseases.

CASE REPORT

A 46-year-old man with history of hypertension for 4 years and undergoing treatment with Losartan (50 mg/day) and Hydrochlorothiazide (25 mg/day) was referred to the Nephrology Department. He complained of a 3-week history of progressive lower limbs edema evolving to anasarca, fatigue, minimum effort dyspnea, foamy urine, insomnia, and malaise. He had no familial or genetic background of renal diseases.

Upon admission, the patient presented edema, tachypnea, hypertension, and a systolic heart murmur. Previous laboratory tests showed the following: serum creatinine, 1.09 mg/dl; lactic dehydrogenase, 344 U/L; free-T4, 7.63 ng/dl; and thyroid stimulating hormone (TSH), 0.005 mU/L. Serum protein electrophoresis revealed total proteins of 4.0 g/dl and A/G ratio of 0.5, without a serum monoclonal spike. The patient underwent therapy with intravenous furosemide, and the antihypertensive therapy was maintained.

A subsequent laboratory investigation revealed creatinine of 1.09 mg/dl, estimated glomerular filtration rate (by CKD-EPI equation) of 42 ml/min/1.73 m^2, blood urea nitrogen (BUN) of 35 mg/dl, total proteins of 3.5 g/dl, albumin of 1.3 g/dl, lactate dehydrogenase of 244 U/L, total cholesterol of 170 mg/dl, C-reactive protein, 0.61 mg/dl, hemoglobin of 6.3 g/dl, 24-h proteinuria of 11,131 mg, C3 of 90.9 g/dl, C4 of 19.7 mg/dl, anti-TPO > 600 UI/ml (normal range, <15 UI/ml), anti-TG > 4,000 UI/ml (normal range, <40 UI/ml), free T4 of 72.67 ng/dl (normal range, 0.8 to 1.7 ng/dl), and TSH < 0.005 mUI/L (normal range, 0.3–4.0 mUI/L. Serum markers for HIV, hepatitis B and C, venereal disease research laboratory test (VDRL), lupus (ANA, anti-dsDNA, anti-SM, anti-SSA, and anti-SSB), vasculitis (ANCA), and rheumatoid arthirtis (RF). Urinalysis revealed proteins and granular casts. A thyroid ultrasound showed an ecographic pattern consistent with autoimmune thyroiditis, and an echocardiogram showed no relevant heart abnormalities. GD was diagnosed, and the patient was started on methimazole 20 mg day and prednisone 60 mg/day The treatment of anasarca was based on water and salt restriction plus furosemide.

A renal biopsy was performed to investigate nephrotic syndrome. Light microscopy revealed a diffuse GBM thickening (**Figure 1A**) with characteristic deposits ("spikes" and "chain links") under silver stain (**Figure 1B**) and a mild mesangial matrix expansion and focal segmental mesangial proliferation. The tubular compartment showed moderate atrophy, and the interstitial compartment showed focal fibrosis. Immunofluorescence microscopy evidenced strong

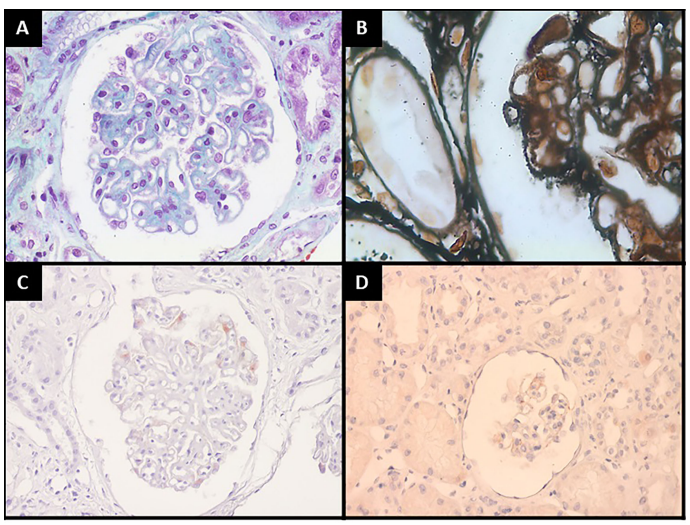

FIGURE 1 | Kidney biopsy findings: **(A)** Global glomerular basement membrane thickening (Masson's Trichrome, 400×). **(B)** Glomerular characteristic deposits forming "spikes" and "chain links" figures (periodic acid methenamine, 1,000×). **(C)** PLA2R immunohistochemical stain revealing no immunoreactivity (400×). **(D)** Some glomeruli show weak and segmental staining for thyroglobulin along the glomerular capillary (200×).

immunoreactivity for IgG (3+/3+), in granular pattern, and global and diffuse distribution along the GBM, in addition to 2 +/3+ staining for IgA, IgM, C3, C1q, Kappa resembling IgG, in a "full house" pattern. Immunohistochemical staining for PLA2R was negative (**Figure 1C**) for thyroglobulin (TG) (**Figure 1D**), showing weak and segmental staining along the glomerular capillary, and anti-thyroid peroxidase (TPO) antibody was not available. The association of histological findings were compatible with a stage III membranous nephropathy, and the immunofluorescence pattern suggested a secondary form of the disease.

The patient was then submitted to colonoscopy, upper digestive endoscopy, computed tomography of the thorax, abdomen, and pelvis, and serum analysis of prostate-specific antigen, carcinoembryonic antigen, and alpha fetoprotein to rule out a non-diagnosed cancer as a secondary cause of MN. All tests showed negative results.

After an 8-month treatment regimen with methimazole and corticosteroids, even with normal serum levels of thyroid hormones, nephrotic proteinuria was maintained despite pharmacological treatment of Graves' disease. In this scenario, the patient underwernt radioiodine thyroid ablation, reaching proteinuria reduction and maintaining stable renal and thyroid parameters since then (**Table 1**). Now, 48 months after thyroid ablation, he currently maintains a serum creatinine of 1.1 mg/dl and 24-h proteinuria of 243 mg.

DISCUSSION

Glomerular diseases associated with GD are rarely described in the literature (1, 2). Membranous nephropathy is the most frequent histological finding in these patients (5–8); however, cases of minimal change disease, IgA nephropathy, and membranoproliferative glomerulonephritis have also been reported (9–11). There have also been reported cases of crescent glomerulonephritis (CG) associated with ANCA-positive vasculitis, as a complication of treatment of GD with propylthiouracil, benzilthiouracil, and carbimazole/methimazole (12–14).

Our literature review identified 18 cases of glomerular diseases associated with GD (**Table 2**)with median age of symptom presentation of 33 years (± 16 years) and no gender predominance. Currently, two non-mutually mechanisms are proposed to explain the association of MN and GD: (1) *in situ* immune response against TG deposition at the subepithelial level and (2) circulating immune complexes that can be trapped at the subendothelial level due to increased glomerular permeability. None of these proposed mechanisms explain how these immune components would transverse the GBM. Most plausibly, the proteins dissociate to pass through the GBM to reassemble inside the sub-epithelial space (4). It is noteworthy that both mechanisms can be associated with radioiodine therapy. A third possible mechanism would be the "epitope dispersion," a phenomenon that involves an inflammatory response to a wider set of epitopes of both the target molecule and a cross-reaction with a myriad of different epitopes due to failure to eliminate the given target molecule. Thus, the glomerular immune-mediated lesion would be caused by a subset of antibodies directed to thyroglobulin or thyroid peroxidase, and glomerular antibodies (4).

Our presented case revealed a histological pattern suggestive of secondary MN based on the mild mesangial matrix expansion, some degree of segmental mesangial cellular proliferation, immunofluorescence microscopy in a "full house" staining pattern, and a negative PLA2R staining and a weak staining for thyroglobulin, which favor a possible association with thyroid disease antigens. In the setting of a secondary MN, the KDIGO Clinical Practice Guideline for the Management of Glomerular Diseases recommends treatment of the associated disease and no specific treatment of MN. In this case, it is unsure if the use of cortiscoteroids as an adjuvant treatment of GD could produce any effect in treating the glomerular disease *per se*, as the isolated use of this drug is not recommended to treat MN (24).

There is no clear guideline for treating patients with MN associated with GD. It is clear that the patient showed substantial proteinuria improvement after ablation; however, there have been reported cases of secondary GMN development after radioiodine treatment, probably due to the exacerbation of the previously discussed immune pathways, with thyroid tissue destruction and the liberation of a large quantity of thyroid antigens in the bloodstream (7, 8, 15). On the other hand, one of five patients described in a study by Weetman et al. (1981) who used radioiodine did reach any reduction in proteinuria (17). This patient was diagnosed with MN 6 years prior to developing GD, constituting a fact that suggests that the glomerular disease reached an advance stage before treatment or that the association is a correlation that does not necessarily imply causation. A cohort study of patients with GD by Weetman et al. (17) revealed that 14 out of 18 patients treated with radioiodine did not show any sign of proteinuria, while 4 showed some degree of urinary protein. Furthermore, 9 out of the 14 patients without prior proteinuria developed some form in a period of 5–10 weeks after radioiodine treatment. Lastly, three of the four patients in the previous proteinuria group showed a reduction in protein excretion after the same treatment.

TABLE 1 | Follow-up of patient's laboratory tests.

	Admission	Pre-ablation (after 8 months)	Follow-up (after 14 months)	Follow-up (after 48 months)
Creatinine (md/dl)	1.09	1.1	1.4	1.1
Albumin (g/dl)	1.3	3.3	4.2	
TSH (mIU/L)	<0.005	0.01	–	–
Free-T4 (ng/dl)	–	13.23	–	–
Proteinuria (mg/24 h)	11,131	2,977	456	243

TABLE 2 | Literature review of cases with association of membranous nephropathy and Graves' disease.

Author	n	Gender	Age	Serum Ab	LM	IM	Atg	Treatment	Diseases associated	Proteinuria follow-up (time)
Ploth et al. (15)	1	M	26	–	MN/ MPGN	IgG, IgM, C3	TG	PTU, ^{131}I	After ^{131}I	16.5 g/day→0.45 g/day (9 months)
Horvath et al. (16)	1	F	60	ANTI-TG, ANTI-TPO	MN	IgG, IgM, C3	TG	MTZ, Thyroidectomy	–	32 g/day→10 g/day (11 months)
Weetman et al. (17)	5	N/A	23	TRAB	MN	NA	NA	CBZ, Thyroidectomy	GD after MN	14 g/day→3 g/day (15 years)
		NA	19	ANTI-TG, ANTI-TPO	MN	IgG, C3	NA	CBZ, I^{131}	GD after MN	8.2 g/day→6.4 g/day (11 years)
		NA	23	ANTI-TG	MN	IgG, C3	NA	NA	GD after MN	7.0 g/day→5.8 g/day (15 years)
		NA	33	ANTI-TG, ANTI-TPO, TRAB	MN	IgG, C3	NA	CBZ, TDX, AIE, AZA	GD after MN	8.0 g/day→0.7 g/day (9 years)
		NA	49	ANTI-TPO, TRAB	MN	NA	NA	CBZ	GD after MN	2.2 g/day→2.6 g/day (9 years)
Jordan et al. (5)	1	F	8	ANA, ANTI-TG	MN/ MPGN	IgG, IgM, C3	TG	PTU→MTZ→ Partial thyroidectomy, T4→ PRED	–	6.0 g/day→1.1 g/day (NA)
Sato et al. (6)	1	M	58	ANTI-TPO, ANTI-TG, TRAB	MN	IgG, C3	TG	MTZ	–	3.69 g→0.2–2.5 g/day (16 days)
Becker et al. (7)	1	M	26	ANTI-TPO	MN	IgG, IgM, C3, C4, κ, λ, Fbr.	–	^{131}I, DTP	After ^{131}I	7.2 g/day→NR (NA)
Grcevska et al. (18)	1	M	25	ANTI-TG	MN	IgG, C3	NA	PTU	–	8.4 g/day→NR (NA)
Shima et al. (19)	1	F	6	ANTI-TPO, ANTI-TG, TRAB	MN	IgG, C3, C1q	TPO	MTZ, ACE	–	4.32 g/day→0.3 g/day (3 months)
Vakrani et al. (8)	1	M	50	ANTI-TPO	MN	IgG, C3	NA	MTZ, ^{131}I → PRED, DTP, ACE, STN	After ^{131}I	3.6 g/day→0.25 g/day (3 weeks)
Sasaki et al. (20)	1	F	40	ANTI-TPO, ANTI-TG	MN	IgG, IgM, IgA, C3, Fib	TPO	PTU, MTZ → ^{131}I	–	4.2 g/gCr→1.24 g/gCr (3 months)
Vanacker et al. (21)	1	F	42	ANA	MN	IgG, IgM, C3, C1q	NA	MTZ→ACE	IgA Deficiency	3,85 g/day→0.58 g/day (NA)
Moniwa et al. (22)	1	F	16	ANTI-TPO, ANTI-TG, TRAB, TSAB	MN	IgG, IgA, C3, κ, λ	TPO	TMZ + ACE	–	3g/g→0.83g/g (12 months after startinf MTZ)
Cakir et al. (23)	1	F	15	ANTI-TPO, ANTI-TG, TRAB, ANA	MN	IgG, IgM, C3, κ, λ	NA	MTZ→RTX (4 doses with 4 weeks interval)	–	1.9g/m^2/day→0.15 g/m^2/day 3 months after RTX therapy
Presented case	1	M	46	ANTI-TPO, ANTI-TG	MN	IgG, IgM, IgA, C3, C1q, κ, λ	TG	PRED, MTZ → ^{131}I	–	11.131 g/day→0.243 g/day (48 months)

Ab, antibody; ACEi, angiotensin-converting enzyme inhibitor; Atg, serum antigens; CBZ, carbimazole; F, female; GD, Graves' disease; ^{131}I, radioiodine; IF, immunofluorescence microscopy; LM, light microscopy; M, male; MN, membranous nephropathy; MPGN, membranoproliferative glomerulonephritis; MTZ, methimazole; n, number of cases; NA, not available; PRED, prednisone; PTU, propylthiouracil; RTX: Rituximab; STN, statin; T4, levothyroxine; TG, thyroglobulin; TMZ, thiamazol; TPO, thyroperoxidase, TRAB, thyroid-stimulating hormone receptor antibody; TSAB, thyroid-stimulating antibody.

We have reported a rare case of MN associated with GD. Further studies researching proteinuria in autoimmune thyroiditis patients can reveal the true prevalence of this association. Radioiodine thyroid ablation seems to be an effective therapeutic option in MN secondary to GD, in association with corticosteroid treatment.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Federal University of Maranhão and conducted according to the Declaration of Helsinki. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

PN: acquired data, analyzed data, and wrote the original manuscript. MM: performed clinical care, acquired data, analyzed data, and wrote the original manuscript. GM: perfomed histological analysis, acquired data, analyzed data, and wrote the original manuscript. EC: performed clinical care, acquired data, analyzed data, and wrote the original manuscript. CM: performed clinical care and wrote the original manuscript. NS-F: performed clinical care and wrote the original manuscript. JL: performed clinical care, analyzed data, and wrote the original manuscript. DB: performed clinical care, analyzed data, and wrote the original manuscript. KC: performed clinical care and analyzed data. GG: performed clinical care, analyzed data, and wrote the original manuscript. GG: perfomed histological analysis, acquired data, analyzed data, and wrote the original manuscript. All authors contributed to the article and approved the submitted version.

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