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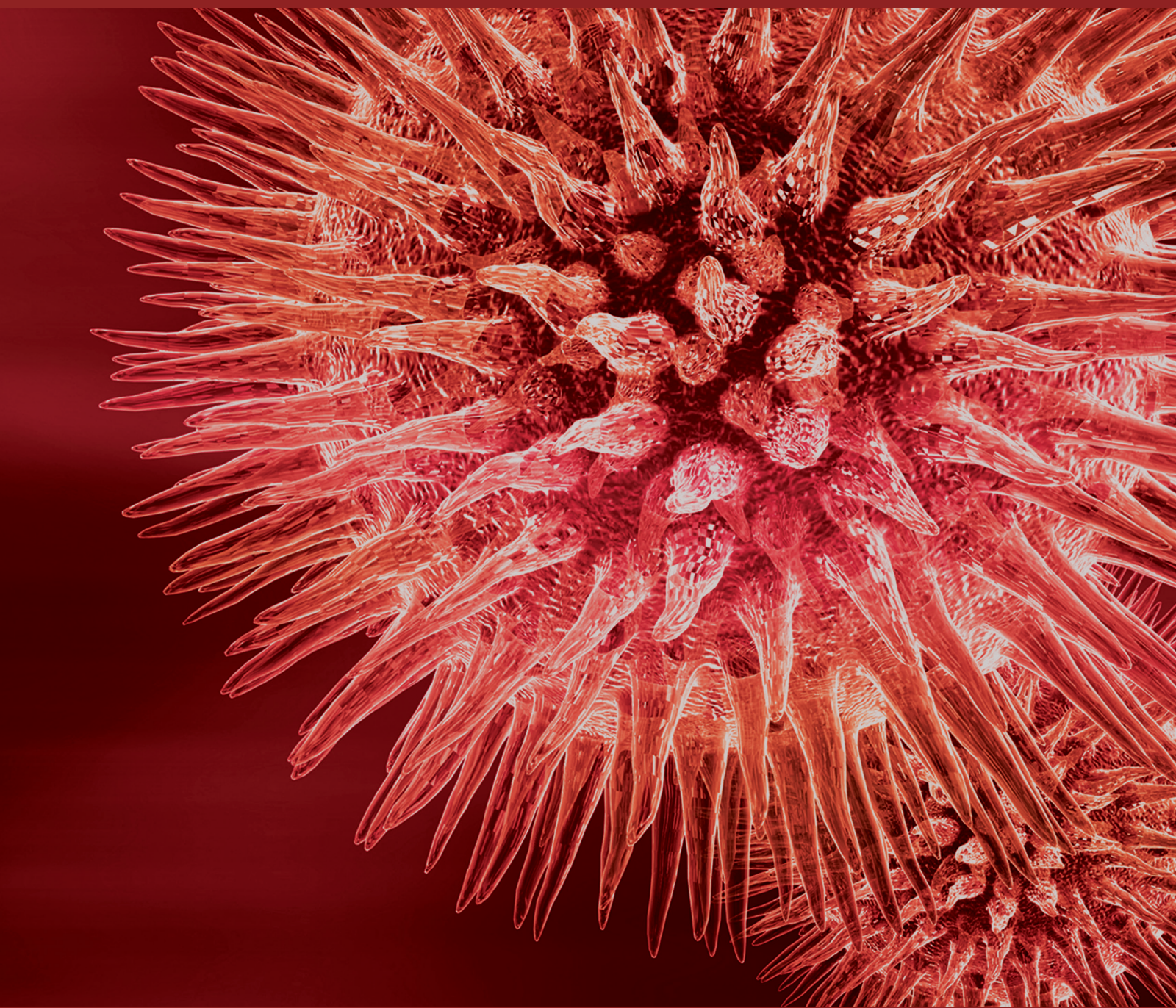
Nephrotic Syndrome: Genetics, Mechanism, and Therapies

Lead Guest Editor: Jae I. Shin
Guest Editors: Andreas Kronbichler, Jun Oh, and Björn Meijers



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Editorial

Nephrotic Syndrome: Genetics, Mechanism, and Therapies

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Nephrotic syndrome (NS) is characterized by massive proteinuria, hypoalbuminemia, and hypercholesterolemia. Minimal change nephrotic syndrome (MCNS) is the most common form of NS in children and membranous nephropathy (MN) is more common in adults. Focal segmental glomerulosclerosis (FSGS) is one of the primary glomerular disorders in both children and adults which frequently shows steroid resistance and can progress to end-stage renal failure. Recently, there have been advances in the fields of pathogenesis and treatment of various kinds of NS. In this special issue, several investigators reviewed the recent advances or presented the experimental works regarding the pathogenesis and treatment of NS.

The etiopathogenesis of NS is incompletely understood. To understand the pathogenesis of primary MN, A. Kronbichler et al. reviewed the discovery of several antibodies and genetics which contributed to an increased understanding of MN, such as antibodies against the M-type phospholipase A2 receptor (PLA2R), several risk alleles related to the PLA2R1 gene, and thrombospondin type 1 domain-containing 7A (THSD7A) antibodies.

To understand the genetic causes of NS, M. S. Guaragna et al. reviewed the list of NPHS2 mutations reported between June 2013 and February 2017, with a closer look to mutations occurring in Latin American countries, emphasizing the importance of implementing the molecular evaluation of NS. To understand the pathogenesis of NS, P. Zapata-Benavides et al. measured different expression levels and protein localization of WT1 in patients with steroid-sensitive and steroid-resistant NS and investigated its relationship with miR-15a, miR-16-1, and miR-193a, which modulate the WT1 expression in other models.

V. J. Savin et al. provide an overview of the circulating permeability factors in primary FSGS that have been implicated in the pathogenesis of the potential recurrence in renal allografts after kidney transplantation, focusing on cardiotrophin-like cytokine factor-1 (CLCF-1); they found and suggested therapies specific to CLCF-1, including potential use of cytokine receptor-like factor (CRLF-1) and inhibition of Janus kinase 2.

Regarding the treatment of NS, S. Thalgahagoda et al. presented their experiences on administering the anticancer drug, vincristine, in patients with steroid-resistant NS and suggested that vincristine could be a potential alternative treatment of steroid-resistant NS patients. Rituximab, a chimeric B-cell depleting anti-CD20 antibody, has emerged in the last decade as a treatment option for patients with primary glomerular diseases and M. Rudnicki introduced the data on the use of rituximab in membranoproliferative glomerulonephritis (MPGN), C3 glomerulonephritis (C3GN), and dense deposit disease (DDD) by reviewing the published case reports and retrospective case series.

To provide insight into potential new targets for the treatment of NS from recent basic science publications, E. Königshausen and L. Sellin reviewed recent advances in the treatment of primary causes of NS (idiopathic MN, MCNS, and FSGS) since the publication of the KDIGO guidelines in 2012 and introduced the ongoing RCTs for these diseases that will provide further information on the effectiveness of different treatment options for the causative disease.

Articles published in this special issue covered the various fields of NS such as pathophysiologic mechanisms and treatment options. Understanding theses various faces of NS will lead to the better patient treatment and outcome.

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Review Article

Recent Progress in Deciphering the Etiopathogenesis of Primary Membranous Nephropathy

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Primary membranous nephropathy (MN) is the leading cause of nephrotic syndrome in adults. Discovery of several antibodies has contributed to an increased understanding of MN. Antibodies against the M-type phospholipase A2 receptor (PLA2R) are present in 50–100% with primary MN and are associated with a lower frequency of spontaneous remission. High levels are linked with a higher probability of treatment resistance, higher proteinuria, and impaired renal function, as well as a more rapid decline of kidney function during follow-up. Immunologic remission precedes reduction of proteinuria by months. Pretransplant evaluation of PLA2R antibodies is warranted to predict recurrence of disease following renal transplantation. Several risk alleles related to the PLA2R1 gene and within the HLA loci have been identified, whereas epitope spreading of PLA2R may predict treatment response. More recently, thrombospondin type 1 domain-containing 7A (THSD7A) antibodies have been discovered in primary MN. Several other rare antigens have been described, including antibodies against neutral endopeptidase as a cause of antenatal MN and circulating cationic bovine serum albumin as an antigen with implications in childhood MN. This review focuses on the progress with a special focus on diagnostic accuracy, predictive value, and treatment implications of the established and proposed antigens.

1. Introduction

Recent research in the field has unraveled important pathogenetic insights in the onset of primary membranous nephropathy (MN). Distinction between primary and secondary cases (mainly autoimmune disorders, infections, medications, and malignancy), characterized by a recognizable etiology, is pivotal. A clear difference from a histopathologic perspective is difficult, but absence of phospholipase A2 receptor (PLA2R) glomerular staining, preponderance of IgG1-3, and presence of extensive mesangial or subendothelial electron dense deposits may have a value in the diagnosis of secondary cases [1, 2]. While IgG1 is the predominant IgG subclass observed in secondary cases (60% of biopsies), IgG4 is predominantly found in subjects with primary MN (76% of biopsies) [3]. The exact role of the least represented IgG subclass is not completely understood, but it may exhibit distinct roles compared to IgG1 in allergy and tumor growth [4], and has attracted considerable interest due to increased incidence and prevalence of IgG4-related disease [5, 6].

Globally, MN represents the leading cause of nephrotic syndrome, with a reported annual incidence ranging from 0.2/100.000/year to 1.4/100.000/year and an overall estimate of 1.2/100.000/year [7]. A male predominance has been reported in most studies [7, 8], prognosis is related to renal function decline, and outcome tends to be better in females [8]. In general, patients present with a greater degree of proteinuria compared to those with focal segmental

glomerulosclerosis (FSGS) and minimal change disease (MCD), but renal survival is assumed to be better compared to those having FSGS [9–11]. In line with FSGS [12], recurrence of disease after kidney transplantation takes place in approximately 35% of patients [13]. Recurrence is supposed to occur due to a yet-to-be-defined circulatory factor in FSGS, while progress in the understanding of MN has led to identification of circulatory factors leading to disease onset. While neonatal disease is associated with antibodies against neutral endopeptidase [14], M-type phospholipase A2 receptor antibody (PLA2R-Ab) [15], and thrombospondin type 1 domain-containing 7A (THSD7A) [16] autoantibodies are responsible for approximately 80% of primary MN cases. The aim of this review is to summarize recent findings in primary MN, focusing on implications of identified antibodies as well as their diagnostic accuracy and therapeutic impact.

2. M-Type Phospholipase A2 Receptor Autoantibodies and Glomerular Phospholipase A2 Receptor in Primary Membranous Nephropathy

PLA2R-Abs were first identified by Western-blotting and subsequent mass spectrometry in 26 out of 37 (70%) patients with primary MN. The 185-kD protein was absent in secondary MN as well as other proteinuric diseases (diabetic kidney disease, FSGS, or other autoimmune disorders). Moreover, samples reacting with the glycoprotein recognized recombinant PLA2R in vitro and PLA2R could be detected in human glomeruli. Glomerular deposits of patients with primary MN had a predominance of IgG4-subclass antibodies, and PLA2R colocalized with IgG4 in glomeruli [15]. A subsequent large multicenter study from Europe including 117 patients revealed presence of PLA2R-Ab in 74% and 72% using an immunofluorescence test (IFT) or ELISA, which increased to 76% when positivity in either test was considered as positive finding. The IgG4 subclass was present in 69% of all patients and correlated best with antibody positivity measured by IFT or ELISA [17]. Analysis of PLA2R staining in MN revealed positivity in 69% of 88 patients and was accompanied by PLA2R-Ab positivity in 60/61 patients (different time points of blood sampling). Enhanced PLA2R staining was exclusively found in primary MN and, of note, IgG4 staining was present in 95% of primary MN and in only 20% with secondary causes [18]. A study from the Netherlands investigated 18 samples during active disease and found a frequency of 77.8% PLA2R-related MN in their cohort [19]. In a prospective study by Hoxha and colleagues, PLA2R-Ab were measured in treatment-naïve patients. PLA2R-Ab were detected in 133/163 (81.6%) patients [20].

Several studies from Asia investigated presence of PLA2R-Ab and glomerular PLA2R staining. In a Korean study, 69/100 patients (69%) had detectable PLA2R-Ab as assessed by Western blot [21]. PLA2-related MN as detected either by IFT, by ELISA, or by glomerular PLA2R staining was reported in 94/114 (82.5%) in a study from India [22]. A study from China including consecutive patients with primary MN reported a frequency of 64.6% (53/82) [23]. In contrast to this low frequency of antibody positivity, another study from China found PLA2R-Ab in 49/60 patients (81.7%). When decreasing the dilution of samples for Western blot analysis, 10 of the remaining samples had low titers, albeit with a weaker strength compared to the other positive samples [24]. A larger study including 572 patients found detectable PLA2R-Ab in 68.5% ($n = 392$) as assessed by ELISA. Among the antibody negative cases ($n = 180$), glomerular staining was present in 70.6% ($n = 127$) [25]. Similar high frequency of PLA2R-MN (92.2%) was reported from another Chinese study analyzing 179 primary MN patients [26]. In Japanese patients, the prevalence of PLA2R-Ab may be lower compared to the rest of the world. A retrospective analysis of patients with 100 primary MN cases (after screening for clinical or laboratory signs of a secondary cause) revealed positivity in 53% of patients, with a higher prevalence observed in these subjects with nephrotic range proteinuria (61% versus 43%) [27]. Another study found PLA2R-Ab in 50% of their patients, whereas similar frequency of positive glomerular PLA2R staining could be detected in two cohorts (52.6 and 52.7%, resp.) [28, 29]. The respective studies reporting adult-onset primary MN are summarized in Table 1.

Analysis of PLA2R-Ab screening during remission showed a limited predictive value to identify PLA2R-related MN, with 8 out of 37 (22%) samples testing positive, whereas PLA2R was found in 22 of 37 (59%) corresponding biopsies [43]. PLA2R-Ab levels have been examined in childhood MN and were present to a lesser extent in a pediatric population, ranging from 2/34 (5.9%) to 10/22 (45.5%) patients reported from Japan and Northern America [44, 45]. In contrast, Kumar and coworkers reported PLA2-related MN in 15/18 (83.3%) of adolescents with primary MN [46].

In general, discrimination between primary and secondary cases is pivotal. In individual studies, a substantial heterogeneity has been reported. To better characterize PLA2R-Ab as a marker for primary MN, two meta-analyses were conducted. The more recent study by Dai and colleagues included 19 studies with 1160 patients. PLA2R-Ab positivity yielded a sensitivity of 0.68, a specificity of 0.97, a diagnostic odds ratio of 73.75, and an area under the curve of 0.82 when comparing primary and secondary MN. The respective values for glomerular PLA2R staining were 0.78, 0.91, 34.70, and 0.84 for the same parameters without heterogeneity [47].

The GEMRITUX trial comparing nonimmunosuppressive antiproteinuric treatment (NIAT) with rituximab against NIAT alone found PLA2R-Ab in three subjects who were initially found to be negative [38]. In line, "seroconversion" has been reported in a recent case series and a "kidney in the sink"-hypothesis has been generated, discussing the role of a PLA2R-Ab buffer capacity exhaustion of the kidney leading to antibody detection at a later stage [48]. In contrast to this assumption, a case report reported presence of PLA2R-Ab in a patient eight months ahead of MN presentation. PLA2R-Ab was measured during a thrombophilia work-up [49].

Taken together, the ideal time point of sample collection is at the time of kidney biopsy and ahead of treatment initiation, which may account for misclassification as PLA2R-Ab negative MN in some cases. Detection of serum PLA2R-Ab by diverse commercial measures is reliable, but cases of

Table 1: A summary of studies investigating the prevalence of either phospholipase A2 receptor antibodies, glomerular phospholipase A2 receptor staining, or both in primary membranous nephropathy. ELISA (Enzyme-Linked Immunosorbent Assay), IFT (Indirect Immunofluorescence Test), PLA2R (phospholipase a2 receptor), PLA2R-Ab (phospholipase a2 receptor antibody), WB (Western blot). *Dilution of serum samples was decreased to 1 : 10, and exposure time was increased to 10 minutes.

Reference	Country	Prevalence (PLA2R-Ab)	Detection method	Prevalence (PLA2R staining)	Predominant IgG subclass
[15]	USA	26/37 (70%)	WB		
[17]	Netherlands, France, UK	87/117 (74%), 84/117 (72%)	IFT, ELISA		IgG4 (81/87, serum)
[18]	Germany	60/88 (68%)	IFT	61/88 (69%)	IgG4 (58/61, biopsy)
[19]	Netherlands	14/18 (78%)	WB		IgG4 (14/14, serum)
[20]	Germany	133/163 (82%)	IFT, ELISA		
[21]	Korea	69/100 (69%)	WB		
[22]	India	75/114 (65%), 76/114 (67%)	IFT, ELISA	86/114 (75%)	
[23]	China	53/82 (65%) 51/82 (62%)	IFT, ELISA		
[24]	China	49/60 (82%) 59/60 (98%)*	WB		
[25]	China	392/572 (69%)	ELISA	514/572 (90%)	IgG4 (97.7% (PLA2R-Ab/R +/+), 98.4% (PLA2R-Ab/R −/+))
[26]	China			165/179 (92%)	IgG4 (167/179, 93%)
[27]	Japan	53/100 (53%)	WB		
[28]	Japan	19/38 (50%) 19/38 (50%)	IFT, ELISA	20/38 (53%)	IgG4 (20/20, biopsy)
[29]	Japan			29/55 (53%)	IgG4 (28/29, biopsy)

PLA2R-related MN may be missed by antibody detection only. Thus, staining for PLA2R antigen in the kidney should be considered to overcome this limitation. A preponderance of IgG4 is found in cases with PLA2R-related MN in serum and kidney specimens. Presence of PLA2R-Ab show a lower prevalence in Japan (around 50%) and in early-onset MN compared to other investigated cohorts (PLA2R-Ab positivity ranging from 65 to almost 100%). While PLA2R-Ab detection and predominant IgG4 presence may be considered a good measure to discriminate primary from secondary causes of MN in Caucasians, this is of lower validity in Chinese patients as shown by the recent meta-analyses.

2.1. Secondary Membranous Nephropathy and PLA2R Ab or PLA2R Staining Positivity. Initial studies have reported PLA2R-Ab positivity occurring in primary MN only. With the increasing body of evidence, secondary MN forms positive for either PLA2R-Ab or glomerular PLA2R staining have been identified.

In a cohort of patients with membranous lupus nephritis (LN), PLA2R-Abs were undetectable [30]. This is in line with Japanese studies reporting PLA2R-Ab positivity exclusively in patients with primary MN [27] or absence of either PLA2R-Ab or glomerular staining for PLA2R [28], whereas a low frequency (5.4%) could be observed in another analysis (one case HBV-associated and one malignancy-related) [29]. Positive PLA2R staining had a sensitivity of 75% and a specificity of 83% for primary MN in a large US study (85 primary and 80 secondary cases). Predominant secondary causes were hepatitis C (7/11, 64%), sarcoidosis (3/4, 75%), and malignancy (3/12, 25%), whereas positive staining was found to a lesser extent in patients with autoimmune diseases (1/46, 2%). Interestingly, all PLA2R positive secondary MN cases showed IgG4-predominant staining [31]. A French nationwide survey retrospectively analyzed kidney biopsies from patients with sarcoidosis and found PLA2R antigen in immune deposits in all patients. Serial serum samples were obtained in two patients and circulating PLA2R-Ab levels were in accordance with sarcoidosis activity and both, PLA2R-Ab and PLA2R, were predominantly related to the IgG4 subclass [50].

In a Chinese study, 84% of 102 tissue samples of patients with primary MN were PLA2R positive. Among secondary causes, one out of 38 patients with class V LN and 64% with hepatitis B virus-associated MN (HBV-MN) showed positive PLA2R staining [32]. Of 22 cases with secondary MN (HBV-MN, LN or both), 36.4% (8/22) had detectable PLA2R-Ab [23]. Analysis of 24 patients with psoriasis and MN revealed PLA2R-Ab and PLA2R positivity in 7 (29.2%) subjects. Among the patients with PLA2R-positivity, a majority showed IgG4 predominance on immunofluorescence (IF) [33]. Secondary causes of MN were accompanied by detectable PLA2R-Ab in a minority of patients in a Chinese study, including 1/20 positive cases with LN, 1/16 with HBV-MN, and 3/10 with malignancy-associated MN. In line with other observations, IgG4 was the predominant PLA2R-reactive subclass [24]. A Korean study found PLA2R-Ab in

Table 2: Secondary causes of membranous nephropathy and the prevalence of either PLA2R-Ab or positive glomerular PLA2R staining are summarized. AID (autoimmune diseases), ELISA (Enzyme-Linked Immunosorbent Assay), IgG4-RD (IgG4-related disease), IFT (Indirect Immunofluorescence Test), LN (lupus nephritis), PLA2R (Phospholipase A2 Receptor), PLA2R-Ab (phospholipase A2 receptor antibody), and WB (Western blot). *Notably, 25/39 (64%) of patients with hepatitis B-associated MN had detectable PLA2R staining, whereas PLA2R positivity was found in 1/38 (2.6%) of lupus nephritis cases. *1By decreasing the dilution, 2 additional cases of hepatitis B-associated MN were considered positive.

Reference	Country	Prevalence (PLA2R-Ab)	Detection method	Prevalence (PLA2R staining)	Predominant IgG subclass	Entities
[15]	USA	0/8 (0%)	WB			LN, hepatitis B
[30]	Sweden	0/25 (0%)	IFT			LN
[27]	Japan	0/31 (0%)	WB			AID, cancer, drugs, hepatitis B, others
[28]	Japan	0/21 (0%)	IFT, ELISA			LN, cancer, hepatitis B
[29]	Japan			2/37 (5%)	IgG4 (2/2, biopsy)	AID, cancer, drugs, hepatitis B, hepatitis C, thyroiditis
[31]	USA			14/80 (18%)	IgG4 (6/6, biopsy)	AID, cancer, hepatitis B, hepatitis C, HIV, syphilis
[32]	China			26/77 (34%)*		LN, hepatitis B
[23]	China	8/22 (36%)	IFT, ELISA			LN, hepatitis B
[33]	China	7/24 (29%)	IFT	7/24 (29%)	IgG4 (5/6, biopsy)	psoriasis
[24]	China	5/46 (11%) 7/46 (15%)*1	WB		IgG4	LN, cancer, hepatitis B
[21]	Korea	2/9 (22%)	WB			LN, cancer, hepatitis B, hepatitis C
[26]	China			2/69 (3%)	IgG4 (2/2, biopsy)	LN, cancer, hepatitis B, IgG4-RD

two out of nine patients with secondary MN (1 HBV and 1 malignancy-associated) and both showed predominant IgG4 immunostaining [21]. Positive PLA2R-staining was found in 2/26 (7.7%) of patients with HBV-MN and 2/2 (100%) presenting with malignancy. All patients with secondary causes and detectable glomerular PLA2R staining had predominant IgG4 deposition [26]. The respective findings are summarized in Table 2.

A preponderance of IgG4 staining on IF is assumed to be present in primary MN. As discussed by De Vriese and colleagues, the question arises whether some of these cases represent true secondary MN or rather PLA2R-related MN with coincident secondary diseases [51]. A higher frequency of secondary causes associated with PLA2R-Ab or PLA2R staining has been reported in Chinese studies, especially cases with hepatitis B-associated MN known to be endemic in China. Among the diseases with antibody positivity or positive staining, hepatitis B- or C-associated MN, malignancy-associated MN, and sarcoidosis emerged to be predominantly found.

2.2. Correlation of PLA2R-Ab Titers with Clinical Characteristics. In a study including 101 primary MN patients, baseline creatinine levels appeared to be higher in PLA2R-Ab positive patients, whereas baseline rate of proteinuria correlated with PLA2R-Ab levels. Spontaneous remission was recorded more often in patients being PLA2R-Ab negative [34]. Analysis of a Dutch cohort ($n = 77$) revealed a correlation of PLA2R-Ab titers with baseline proteinuria, serum creatinine, and baseline estimated glomerular filtration rate (eGFR) after adjustment for fractional IgG excretion (as representative of urinary loss of antibodies). Dividing patients into tertiles based on ELISA titers, renal failure tended to occur more frequently in patients belonging to the group in the highest tertile (PLA2R-Ab titer > 610 U/ml) and these patients had a significantly lower incidence of spontaneous remission compared to the lowest (PLA2R-Ab titer 41–175 U/ml) tertile (4 versus 38%, resp.). Accordingly, physicians were prompted to initiate immunosuppression more often in patients in the highest tertile compared to both other groups, whereas time to treatment response was significantly shorter in patients within the lowest tertile [17]. A single-center study from the same institution including 18 primary MN cases highlighted correlation of PLA2R-Ab with proteinuria, serum ß2-microglobulin (ß2m), urinary IgG excretion, urinary ß2m, and serum creatinine, whereas eGFR was negatively correlated [19]. Another large study including 118 PLA2R-Ab positive patients established different tertile values, with tertile 1 ranging from 20 to 86 U/ml, tertile 2 from 87 to 201 U/ml, and tertile 3 > 202 U/ml. Patients in tertile 3 were older, had a higher baseline systolic blood pressure, and tended to have more severe proteinuria. The proportion of patients reaching the predefined primary end point, namely, serum creatinine increase ≥ 25% and a creatinine value ≥ 1.3 mg/dl, was significantly higher in tertile 3 compared to tertile 1 (69% versus 25%). At the end of follow-up, proteinuria and PLA2R-Ab levels were higher in patients belonging to tertile 3 [35]. In a cross-sectional analysis, risk of progressive kidney function decline over a long observation period (up to 5 years) was increased in patients with higher PLA2R-Ab levels (high titer > 88 μ/ml versus low titer < 24 μ/ml) [36]. In the GEMRITUX study, a titer of < 275 U/ml was significantly associated with achieving the primary end point (complete or partial remission) [38]. A clear association between proteinuria

and serum PLA2R-Ab levels and serum creatinine could be confirmed by a prospective study including 133 patients with detectable antibodies. PLA2R-Ab titers at baseline were significantly higher in those patients who did not experience remission during follow-up. Patients having lower PLA2R-Ab levels reached remission significantly faster compared to those with higher titers at baseline [20]. Another study from India observed a significantly higher proportion of complete remissions in the combined lower and intermediate range PLA2R-Ab tertile compared to those belonging to the highest tertile. Furthermore, resistant disease was common in patients with a baseline titer > 300 RU/ml (55.2%), whereas remission was frequently observed in those with a titer of < 100 RU/ml at baseline (68.2%). In general, detectable PLA2R-Abs were associated with treatment resistance compared to patients with no antibodies [22]. In a large study from China, detectable PLA2R-Abs ($n = 392$) were associated with a higher level of proteinuria and a lower level of eGFR. A weak correlation of antibody levels with proteinuria, serum albumin, and eGFR was shown. Moreover, patients with a positive PLA2R-Ab test and glomerular PLA2R staining were more likely not to achieve remission compared to those with undetectable antibodies and positive glomerular staining. In general, patients with a higher PLA2R-Ab or glomerular PLA2R rate had a higher proportion of failure to achieve remission. Combined positivity was a significant predictive value for no remission [25]. In line with several other reports, PLA2R-Ab positivity in Korean patients was associated with more severe proteinuria and lower serum albumin [21]. Proteinuria and number of patients having nephrotic range proteinuria were significantly higher in PLA2R-Ab positive cases in a Chinese study [23]. In a Japanese analysis including 19 patients with and without detectable PLA2R-Ab in each group, serum IgG was significantly lower in patients with antibodies, whereas proteinuria tended to be higher [28]. Respective associations/correlations have been highlighted in Table 3.

Taken together, based on the available data it is not possible to provide definite PLA2R-Ab cut-off, since different values have been reported in the respective studies. Nevertheless, in several studies, patients within the highest tertile had worse renal outcome and more frequently treatment resistance towards initiated immunosuppression. Notably, two reports have used purified protein to coat ELISA plates [17, 36], whereas the remainder used a commercially available kit [22, 35, 38]. High PLA2R-Ab titers (exceeding 250–300 IU/L) have been associated with more severe proteinuria, higher serum creatinine/lower eGFR, serum/urinary ß2M, and urinary IgG excretion.

2.3. PLA2R-Ab Presence and Decline Predict Treatment Response. PLA2R-Abs were present during clinical disease activity, whereas a decline or complete disappearance of PLA2R-Ab preceded resolution of proteinuria [15]. In a first multicenter retrospective study, the effects of rituximab on PLA2R-Ab levels were tested. 25 of 35 (71%) patients had detectable PLA2R-Ab and these antibodies disappeared or declined in 17 (68%) patients within 12 months following rituximab treatment, accompanied by a higher rate of complete or partial remission compared to those with a persistent antibody titer (59% and 88% versus 0% and 33% after 12 and 24 months). Changes in antibody titers preceded changes in proteinuria [40].

An observational study assigning patients either to oral cyclophosphamide or to mycophenolate mofetil and corticosteroids for 12 months evaluated PLA2R-Ab ($n = 34/48$) in retrospective. By 2 months of treatment, PLA2R-Ab levels declined from 428 U/ml to 24 U/ml. Antibody status at the end of treatment predicted 5-year outcome, with 58% (14/24) of patients with absent PLA2R-Ab achieving persistent remission compared to 0 out of 9 in those with persisting antibodies [41]. Combined treatment with rituximab and cyclosporine for 6 months of revealed PLA2R-Ab negativity in 77.8% after 3 months and in all at least once during follow-up, which was accompanied by remission. In two patients with relapse, reemergence of PLA2R-Ab was associated with re-occurrence of proteinuria [52]. A recent randomized controlled trial comparing NIAT and rituximab against NIAT alone found significantly more PLA2R-Ab negativity and decreasing titers in the NIAT-rituximab group. In the group with immunologic remission, the primary end point (complete or partial remission after 6 months) was achieved in 43% compared to 18% with persistent antibody positivity [38]. In 81 antibody-positive patients receiving a B-cell driven (375 mg/m^2 once and a second infusion when more than five circulating B-cells/mm2 were detected after one week) or a fixed regimen (375 mg/m^2 four times, one week apart), a lower PLA2R-Ab titer and full depletion after six months significantly predicted remission. In general, antibody decline preceded proteinuria and, particularly, a 50% reduction of PLA2R-Ab preceded equivalent proteinuria reduction by 10 months. Reappearance of PLA2R-Ab predicted disease relapse [37]. A similar pattern of PLA2R-Ab course was found in a Dutch study, showing a significant decline in the remission phase and a reappearance of PLA2R-Ab during relapse [19]. In a prospective German study, a decrease in PLA2R-Ab levels was accompanied by a steady increase in serum albumin. After three months of follow-up, a 45% decrease of PLA2R-Ab titers was paralleled by a 25% decrease in proteinuria in a cohort of 133 patients. The changes were even more pronounced in patients receiving immunosuppression, with a decline in PLA2R-Ab levels ranging from 69 to 81% and proteinuria by 38.8% [20]. In another analysis from India including 114 patients, a decline over 90% in PLA2R-Ab decline in PLA2R-Ab titers by 12 months was accompanied by remission in 85.4%, whereas a decline lower than 50% in the same period was associated with persistent nephrotic range proteinuria in 87.5%. A correlation between proteinuria and serum albumin with PLA2R-Ab titers at 6 and 12 months was found [22]. In a recent Chinese study comparing PLA2R-related MN ($n = 91$) with negative patients ($n = 13$), the total remission rate was significantly higher in patients with absent PLA2R-Ag staining [39].

A subanalysis of the prospective study conducted by Hoxha and colleagues analyzed the clinical outcome of included patients with nonnephrotic range proteinuria. During the follow-up period, significantly more patients with detectable PLA2R-Ab developed nephrotic range proteinuria

Table 3: Baseline characteristics and presence of PLA2R-Ab with respective associations and correlations with outcome and variables are highlighted. ß2m (ß2-microglobulin), eGFR (estimated glomerular filtration rate), PLA2R-Ab (phospholipase A2R-antibody). *Analysis of different variables after adjustment of the respective antibody titers for fractional IgG excretion.

Reference	Country	Number of patients	Correlations/associations
[34]	Netherlands	101	Baseline creatinine ($p = 0.03$) Antibody levels and proteinuria ($p = 0.017$) Spontaneous remission in seronegative patients ($p = 0.038$)
[17]	Netherlands, France	77/79	Antibody levels and proteinuria ($p < 0.01$)* Antibody levels and serum creatinine ($p < 0.01$) and eGFR ($p < 0.01$)* Spontaneous remission more frequently in patients within the lowest PLA2R-Ab tertile ($p < 0.01$) Highest tertile more often needed immunosuppression, and higher levels were associated with longer time to remission ($p < 0.01$)
[19]	Netherlands	18	Antibody levels positively correlated with proteinuria ($p < 0.01$), serum ß2m ($p < 0.01$), urinary IgG excretion ($p = 0.03$), urinary ß2m excretion ($p < 0.01$), and serum creatinine ($p = 0.03$) and negatively correlated with eGFR ($p = 0.049$)
[35]	Germany	118	Patients in the highest tertile were older ($p < 0.01$), had lower serum albumin ($p = 0.04$), and higher systolic blood pressure ($p < 0.01$) Antibody level (IgG) is associated with outcome (serum creatinine increase ≥ 25% or creatinine level ≥ 1.3 mg/dl) Patients in tertile 3 reached the outcome parameters more frequently ($p = 0.03$) and significantly faster Proteinuria was higher at the time of last follow-up in patients within tertile 3 ($p = 0.03$)
[36]	UK	90	High PLA2R-Ab levels associated with an increased risk of progression ($p < 0.001$)
[37]	Italy	81	Patients in the lowest tertile antibody group had lower proteinuria compared to both other groups
[38]	France	75	Lower PLA2R-Ab levels associated with achievement of the primary end point (complete or partial remission)
[20]	Germany	133	PLA2R-Ab levels correlated with proteinuria and serum creatinine Lower PLA2R-Ab titers were associated with a faster time to remission and remission was achieved more frequently
[22]	India	114	PLA2R-Ab positivity was associated with treatment resistance ($p = 0.02$) Higher levels were associated with resistant disease (55%); low titers were associated with remission (68%)
[25]	China	392	PLA2R-Ab positivity was associated with a higher level of proteinuria ($p < 0.001$) and a lower level of eGFR ($p < 0.01$) Antibody levels correlated with proteinuria ($p < 0.01$), serum albumin ($p < 0.001$), and eGFR ($p < 0.001$) Patients with glomerular positivity (undetectable antibodies) were more likely to achieve complete remission ($p < 0.001$)
[21]	Korea	69	PLA2R-Ab positivity was associated with lower serum albumin ($p = 0.004$) and higher levels of proteinuria ($p = 0.003$) and nephrotic range proteinuria was more frequently observed ($p < 0.001$)
[23]	China	53	PLA2R-Ab positivity was associated with higher proteinuria ($p = 0.008$) and nephrotic range proteinuria ($p = 0.036$)
[28]	Japan	19	Serum IgG levels were significantly lower in PLA2R-Ab positive patients Serum albumin correlated inversely with serum PLA2R-Ab titers

(13 out of 16 patients) compared to those that tested negative (five out of 17). In accordance with this finding, immunosuppression was initiated more often in patients with PLA2R-Ab (13 versus 2 patients, resp.). At the end of follow-up, persistence of PLA2R-Ab was associated with no remission of proteinuria and a decline in renal function [42]. Findings have been highlighted in Table 4.

Taken together, higher baseline PLA2R-Ab titers are associated with worse renal outcome and resistance towards immunosuppressive therapy. In general, decline in antibody levels precede changes in proteinuria by months, irrespective of the immunosuppressive measure. Moreover, reappearance of PLA2R-Ab is a strong predictor of disease relapse.

2.4. Recurrence of Disease after Transplantation and PLA2R-Ab. In a study by Quintana and colleagues, recurrence was observed in 7 of 21 (33.3%) patients. PLA2R-Abs were measured ahead of kidney transplantation and were positive in 85.7% and 35.7% of the recipients with and without recurrence, respectively. A correlation of PLA2R-Ab positivity at the time of graft biopsy and before transplantation with recurrence of primary MN was found by the authors. A PLA2R-Ab cut-off level of 45 U/ml could predict recurrence, with a sensitivity of 85.3% and a specificity of 85.1%. Presence of *HLA-DQA1* 05:01(05) and *HLA-DQB1* 02:01 was reported in 6 of 7 patients with recurrent MN and was associated with the highest pretransplantation PLA2R-Ab levels [53]. These findings were in part corroborated by another single-center study. Among the six patients (37.5%) with recurrence, a positive ELISA with a titer > 30 RU/ml provided a sensitivity and specificity of 83% and 100%, respectively. The risk alleles were found in 5 out of six patients (83%). Combination of both above-mentioned studies ($n = 37$) yielded a sensitivity of 85% and a specificity of 92% to predict recurrence with a PLA2R-Ab titer of > 29 U/ml [54]. Another study including 10 patients with primary MN reported an association of persistent IgG4 PLA2R-Ab and the respective antibody titer after six months with the recurrence of disease [55]. Moreover, persistence or reappearance of PLA2R-Ab after kidney transplantation was associated with increasing proteinuria and treatment-resistant recurrent MN in another case series [56]. Taken together, a PLA2R-Ab titer before renal transplantation > 30 or > 45 U/ml (depending on the respective publications), persistence, or reappearance during follow-up may predict recurrence of disease. This needs to be confirmed by further studies assessing patients in a prospective manner.

2.5. Immunodominant Epitope Region and Impact of Epitope Spreading on Outcome. Epitopes were identified by SPOT (peptide arrays on membrane) technology and detected by Western blot. Restricting analysis to the IgG4 subclass yielded lower background and stronger signals localized to seven consensus epitopes, which were in the extracellular domain of the PLA2R. Six of them were localized to the C-type lectin-like domains (CTLDs, one in CTLD1, one in CTLD2, two in CTLD6, and two in CTLD8), whereas one was located to the N-terminal cysteine-rich region (C-R) [57]. Further research aimed at deciphering the responsible epitope responsible for PLA2R-Ab binding. Fresquet et al. identified the major epitope within the N-terminus to CTLD3 (comprising a complex of CysR, FnII, and CTLD1–CTLD 3) of PLA2R which was recognized by 90% of human PLA2R-Ab and effectively competed for the binding with full-length PLA2R. Further experiments using mass spectrometry revealed a pivotal role of peptide 1 and peptide 2, representing the ß1 and ß3 strain from the ricin domain. Extension of peptide 1 towards peptide 2 is exerted by a 31-mer within the cysteine-rich (CysR) region, and competitive binding analyses revealed the essential role of 31-mer in the binding of PLA2R-Ab [58]. In line with the results described above, Kao and colleagues reported recognition of patient sera by a protein complex consisting of the CysR, fibronectin-like type II (FnII), and CTLD1 domains of PLA2R. Binding analysis comparing full-length PLA2R and the complex of CysR-FnII-CTLD1 indicated an equally efficacy in the recognition of PLA2R-Ab positive patient sera [59]. Since 10% of patients did not show binding to the described epitopes, it was assumed that a further epitope in the more C-terminal region may exist [60]. Further in depth analyses led to the identification of the three pivotal epitopes responsible for PLA2R-Ab binding. Seitz-Polski et al. described the loss of recognition in all tested patients ($n = 50$) after the deletion of the three epitopes, namely CysR, CTLD1 and CTLD7. Based on their findings, they set up ELISAs to specifically measure presence of antibodies directed against these domains. A total of 69 patients with presence of PLA2R were tested, and CysR as the dominant and most common epitope was recognized in 68 patients, whereas the CTLD1 and CTLD7 domains were recognized in 42 and 32 sera, respectively. Based on these findings, they classified patients according to antibody presence into a CysR (CysR antibodies only, $n = 23$), CysRC1 (CysR and CTLD1 only, $n = 14$) and a CysRC1C7 group (with presence of antibodies against all domains, $n = 32$). The CysR antibodies were significantly higher in the latter two groups compared to the CysR group. Patients in the CysR group were significantly younger and their baseline proteinuria was lower compared to the other two groups, while a proteinuria over 5 g/g creatinine was most frequently found in the CysRC1C7 group. Furthermore, a progressive increase of proteinuria was accompanied by a progressive increase in CTLD7. Assessment of outcome revealed significantly more spontaneous remissions in the CysR group, whereas the occurrence of end stage renal increase was limited to the CysRC1 and CysRC1C7 groups and proteinuria increased from CysR to CysRC1C7. In accordance, the proportion of patients with a 30% increase in serum creatinine was higher in the CysRC1 and CysRC1C7 groups. Multivariate analysis retained CysRC1, CysRC1C7 and high titers of PLA2R-Ab as independent risk factors for poor renal prognosis. Serial samples were available for a subset of patients and an epitope switch was observed in a minority, associated with a change in clinical presentation (i.e., one patients switched from the CysRC1C7 to the CysR group and entered remission following initiation of immunosuppression). ELISA tests were set up for the detection of IgG4 antibodies only. Analysis

Table 4: Effects of treatment on PLA2R-Ab and variables of interest have been highlighted. The study by Wang et al. [39] included nonnephrotic patients only and all patients received nonimmunosuppressive treatment with inhibition of the renin angiotensin aldosterone system.

Reference	Country	Number of patients	Treatment	Effect of treatment
[40]	USA	25	Rituximab	17/25 had undetectable Ab levels within 12 months 59/88% attained remission by 12 and 24 months (compared to 0/33% in those with persistent PLA2R-Ab)
[41]	Netherlands	33	MMF, $n = 15$; CYC, $n = 18$	16/18 became Ab negative during 12 months of CYC versus 8/15 in the MMF group ($p = 0.02$) In general, remission was observed in 22/24 negative patients versus 3/9 positive patients at 12 months Antibody status at the end of therapy predicted long-term outcome (persistent remission and alternative outcome measures)
[38]	France	55	Rituximab, $n = 27$; Placebo, $n = 28$	PLA2R-Ab negativity: 14/25 and 13/26 at months 3 and 6 in the rituximab group; 1/23 and 3/25 at months 3 and 6 in the placebo group In the rituximab group, antibody depletion at month 3, 6 patients (43%) were associated with the primary end point (complete/partial remission), with 2/11 patients (18%) without antibody depletion
[37]	Italy	81	Rituximab	Low titer of PLA2R-Ab predicted achievement of primary end point (complete or partial remission, $p = 0.001$) PLA2R-Ab promptly decreased and preceded a comparable proteinuria response by approximately 2 years Depletion of PLA2R-Ab after 6 months predicted primary end point Reappearance of antibodies was associated with relapse ($p < 0.001$)
[19]	Netherlands	18	MMF or CYC	PLA2R-Ab decreased significantly during remission and increased again during relapse ($p < 0.01$)
[20]	Germany	133	CNI, alkylating agents or rituximab	Decrease in proteinuria was accompanied by an increase in serum albumin ($p < 0.001$) At 3 months, proteinuria fell by 25%, whereas PLA2R-Ab levels decreased by 45% PLA2R-Ab levels as a risk factor for not achieving remission
[22]	India	76	CNI or CYC	>90% reduction in PLA2R-Ab titer at 12 months from baseline, accompanied by remission in 85% (<50% Ab reduction, 88% had persistent nephrotic range proteinuria) Proteinuria ($p > 0.0001$, each) and serum albumin ($p = 0.001$ and $p < 0.0001$) correlated with PLA2R-Ab titer at 6 and 12 months A greater proportion of subjects in the first two tertiles had complete remission compared with patients in the highest tertile ($p = 0.04$)
[42]	Germany	16		In initially nonnephrotic patients, nephrotic range proteinuria developed more often in PLA2R-Ab positive patients ($p < 0.005$) Significantly more PLA2R-Ab positive patients received immunosuppressive therapy ($p < 0.001$)

of other subclasses highlighted that anti-CTLD7 antibodies were exclusively restricted to the IgG4 subclass [61].

The identification of the immunodominant epitope regions of PLA2R have further provided insight into pathogenesis. Moreover, the CysRC1, CysRC1C7 and higher titers of circulating PLA2R-Ab may be considered as independent risk factors for poor prognosis. Moreover, epitope spreading has been observed, indicating changes in both directions (high risk phenotype to the CysR group and vice versa). It is tempting to speculate that specific developments (i.e., commercially available ELISA kits) will further help to facilitate identification of patients bearing a high risk of treatment resistance or progressing to end stage renal failure.

2.6. Identification of Susceptible Loci Associated with Primary Membranous Nephropathy. A genome-wide association study (GWAS) was employed to identify risk alleles responsible for the onset of primary MN. Three cohorts (with Dutch, French and British patients) were analyzed and independently displayed a significant association with *HLA-DQA1* on chromosome 6p21. In a joint analysis, comprising 556 patients with primary MN and 2338 controls, the authors identified the most significant association within *HLA-DQA1* and *PLA2R1* on chromosome 2q24 [62]. A subsequent study from Spain including 89 primary MN patients and 286 controls confirmed genetic associations with *HLA-DQA1* and *PLA2R1*. Interestingly, combination of the susceptibility genotypes (A/A and A/G for *HLA-DQA1* and A/A for *PLA2R1*) significantly predicted response to treatment, which became even more apparent after adjustment for baseline proteinuria. Moreover, carriers of the A/A or A/G genotype for *HLA-DQA1* had a longer mean doubling of serum creatinine-free survival [63]. A study from Germany observed an association between classical HLA alleles (*DRB1**0301-*DQA1**0501-*DQB**0201 haplotype) and primary MN. Whereas *PLA2R1* was exclusively identified in primary MN, the *HLA-DQA1* risk allele was associated with LN, diabetic nephropathy and FSGS as well [64]. In a cross-sectional study from the UK, *HLA-DQA1**05:01 and *HLA-DQB1**02.01 were associated with higher PLA2R-Ab levels [36]. In a large monocentric study, analysis of the risk alleles revealed that homozygous carriage of the *PLA2R1* A allele was associated with a 2-fold higher PLA2R-Ab level compared to A/G and G/G carriers [37].

Similar results were reported in non-Caucasian patients with primary MN. A study from India compared 114 cases and 95 healthy controls and observed differences in the *PLA2R1* and *HLA-DQA1* SNPs which became more evident when studying PLA2R-related cases only [22]. In a large Northern American analysis, *PLA2R1* SNPs were associated with PLA2R-related MN in Caucasians only. A strong epistasis between *PLA2R1* and *HLA-DQA1* was found [65]. In African Americans, 120 PLA2R-related MN biopsies were screened for the presence of *APOL1* risk alleles, identifying 46 cases with 0, 51 cases with 1 and 23 cases with 2 *APOL1* risk alleles. Presence of two risk alleles was associated with histologic features implicated in worse renal outcome [66].

Analysis of 1112 Chinese patients and 1020 healthy controls revealed a strong association between three SNPs within *PLA2R1* and one SNP within *HLA-DQA1* and a potentiation when risk alleles were combined [67]. Restricting analysis to the MHC region in PLA2R-positive primary MN, *HLA-DRB1**15:01 and *HLA-DRB3**02:02 were strongly associated. Whereas *HLA-DRB3**02:02 was found in a comparable number of PLA2R-negative MN, *HLA-DRB1**15:01 was found in 81.8% of patients with PLA2R-related MN compared to 16% of PLA2R-negative MN and 21% of the healthy controls. A replication cohort confirmed the findings of both HLA loci. Interestingly, in a joint analysis, 387 patients (98.7%) carried at least one of the proposed HLA risk alleles [68]. Another Chinese study included 261 cases and 599 healthy controls. *HLA-DRB1**15:01 and *HLA-DRB1**03:01 were independently associated with primary MN. PLA2R-Ab positivity was associated with the carriage of one of the risk alleles, whereas association remained at a lower degree for *DRB1**15:01, while *DRB1**03:01 lost its effect [69]. In a Japanese cohort comprising 183 patients with primary MN and 811 healthy controls, several tested *PLA2R1* SNPs were associated. Moreover, the association of *HLA-DRB1**15:01 could also be observed, whereas *HLA-DQB1**06:02 emerged as a second independent risk allele [70]. To identify rare genetic variants in *PLA2R1*, all 30 exons of *PLA2R1* were sequenced. Although the general association between *PLA2R1* and primary MN could be confirmed, no evidence was provided that rare variants within the coding region cause the proposed association between primary MN and the *PLA2R1* gene [71].

Despite progress in the understanding of genetics related to primary MN, there is a lack of heritability. One report from 1984 highlighted occurrence of MN in three pairs of brothers, with one pair being monozygotic (HLA-identical) twins [72]. In general, the presence of *HLA-DQA1* and *PLA2R1* risk alleles confers a significant association with primary MN and the combination of risk phenotypes potentiates the risk to develop MN in most ethnicities. Particularly, MHC class II molecules play a pivotal role in MN. In Chinese patients with primary MN, carriage of *HLA-DRB1**15:01 confers a significant risk to develop PLA2R-related MN, whereas *HLA-DRB1**03:01 or *HLA-DRB**06:02 may carry a risk to develop either PLA2R-related or unrelated MN.

3. Thrombospondin Type 1 Domain-Containing 7A in (Primary) Membranous Nephropathy

Since about 20–30% of cases with primary MN may exhibit other antigens, a similar approach as with the discovery of PLA2R-Ab was chosen to identify further antigens. Thrombospondin type 1 domain-containing 7A (THSD7A) was identified by Western-blotting and subsequent mass spectrometry and has a molecular size of approximately 250 kDa. Among patients with PLA2R-Ab negative primary MN, THSD7A was found in 6 out of 44 patients in a European and in 9 out of 110 patients in a US cohort. In accordance with PLA2R-Ab, IgG4 was the predominant IgG subclass of anti-THSD7A, and colocalization of IgG4 and THSD7A was observed in renal tissue specimens. Staining for nephrin colocalized with staining for THSD7A, suggesting expression

of THSD7A in proximity to the podocyte foot processes. Interestingly, anti-THSD7A antibodies were more frequently observed in women (65% versus 27%) [16]. A single-center study from Japan including consecutive patients with MN found THSD7A-related MN in five out of 55 patients (9.1%). Again, total numbers were low, but the authors found that patients with THSD7A-related MN were significantly younger compared to PLA2R-related MN and a female predominance (three out of five), which was in contrast to patients being PLA2R-positive [29]. Lower prevalence was reported in a Chinese cohort (three patients with primary MN), and additionally two patients had secondary causes (one tumor-associated and one hepatitis B-associated, overall 3.7%). Importantly, IgG4 staining was positive in primary and secondary causes [73]. Analysis of consecutive biopsies from the Boston cohort revealed a positive staining for THSD7A in 7/258 (3%) patients, whereas two cases presented with dual (PLA2R and THSD7A) positivity (1%) [74]. Concomitant occurrence of THSD7A-related MN and mixed adenoneuroendocrine carcinoma of the gallbladder with positive staining in the kidney, the gallbladder and an affected lymph node highlighted a potential link between THSD7A-positivity and malignancy. Screening of 1009 patients with MN revealed further 25 patients with positive anti-THSD7A antibodies. Of these, seven had a malignancy [75]. Further analysis of consecutive MN patients revealed a prevalence of 2.6% out of 345 patients, with a majority being female. The association between THSD7A-related MN and malignant disease exceeded the number of PLA2R-related MN and malignancy. After inclusion of the retrospective cohorts and analysis of samples derived from 1276 MN patients, 8 out of 40 patients with detectable anti-THSD7A antibodies presented with malignancy and the median time from diagnosis of MN and development or detection of malignancy was 3 months. Again, a female predominance was reported by the authors. Achievement of complete remission was accompanied by undetectable anti-THSD7A antibody titers, whereas a majority of those with partial remission and one patient with no remission remained positive [76]. In contrast to PLA2R1, THSD7A is expressed on both murine and human podocytes, and administration of human anti-THSD7A antibodies bind to murine THSD7A, leading to proteinuria and a histopathology resembling MN [77]. This finding confirmed the assumption that antigen-antibody interaction is pivotal in the onset of MN.

Taken together, anti-THSD7A antibodies are a novel antigen which is more frequently present in Japanese patients with primary MN. In particular, research needs to decipher its association with malignancy and intensive screening may be warranted in positive patients, since the number of observed malignant diseases may be underestimated by the current reports due to a short follow-up period.

4. Rare Forms of Childhood Membranous Nephropathy and Potential Ways to the Detection of Novel Antigens

MN is a common cause of nephrotic syndrome in adults; it is rare, however in children [78, 79]. Since PLA2R and THSD7A do not seem to play any major role in infants and young children, other auto- or allo-antigens must looked for, after secondary causes have been excluded thoroughly, particularly malignancy. Recently, rare causes of MN were identified in children, such as antibodies to neutral endopetidase (NEP).

A case of antenatal MN with a severe, unusual histopathological presentation was further investigated, since the transplacental passage of nephritogenic antibodies was suspected to be causative. Sequential serum samples from the mother (before and after delivery) and serum of the infant after birth showed reactivity with glomerular capillary walls and the brush border. A 90 kDa antigen was found, and further experiments identified NEP as the antigenic fraction. Kidney biopsy of the infant and of rabbits after incubation with maternal IgG revealed colocalization of IgG antibodies and NEP in subepithelial immune deposits. The mother's granulocytes exhibited a lack of NEP expression, thus an alloimmunization process was discussed as responsible pathogenetic step [80]. Truncating mutations in the metallomembrane endopeptidase *(MME)* gene were found to be causative of alloimmunization during pregnancy, leading to the production of anti-NEP antibodies. Whereas infants of four mothers with mutations had oligoanuria, nephrotic syndrome or renal failure, all children of one mother had no overt renal disease. Analysis of anti-NEP antibodies revealed an approximately 10-fold lower titer for IgG as compared to those of the remaining mothers. Rapid improvement of kidney failure and nephrotic syndrome was observed in affected infants [14]. Two mothers were followed during their second pregnancy. One mother developed a marked increase in anti-NEP IgG1 and IgG4 antibodies. Severe disease occurred despite treatment strategies aiming at reducing its levels. The other mother produced anti-NEP IgG4 antibodies exclusively and delivery of a healthy newborn was possible [81]. More recently, two additional mothers (from Germany and Italy) bearing a truncating deletion in the *MME* gene were identified. The German mother produced predominantly anti-NEP IgG4 antibodies, whereas the Italian mother mainly produced complement-fixing anti-NEP IgG1 antibodies, the latter able to inhibit NEP enzymatic activity. Whereas the German child had rapid regression of all symptoms, the Italian child required treatment with intermittent peritoneal dialysis and an impaired renal function at the time of last follow-up was reported. These findings highlight that complement-fixing anti-NEP IgG1 antibodies are necessary to develop a severe disease phenotype [82].

Various reports have already indicated a role for allergens, including dietary allergens in the pathogenesis of renal glomerular diseases. Early studies demonstrated that some patients develop relapses after cow's milk exposure and elimination of milk from the diet could prevent relapses of lipoid nephrosis [83]. This has been explained by a dysregulation of the IL-13 system [84], leading to overproduction of IgE-specific antibodies of food compounds, including cow's milk and theoretically also bovine serum albumin (BSA). Anti-BSA antibodies, commonly prevalent in the general population, have been detected in high levels in 4 of 5 consecutive children (age < 5 years) and 7 of 41 consecutive adults with MN. Elevated levels of circulating BSA were

also present in those children with high levels of anti-BSA antibodies. As shown in various animal models, the cationic form of BSA is able to induce MN. Subepithelial deposits of BSA were detected in children with both, high levels of circulating cationic BSA and antibodies, and in the absence of PLA2R [85].

Gel electrophoresis identified a few proteins which were repetitively recognized by MN sera. Among these, antibodies against aldose reductase (AR), manganese superoxide dismutase (SOD2) were elevated in MN compared to FGSG and normal controls. Analysis of kidney specimen revealed marked expression of AR and SOD2 in glomeruli of patients with MN, and colocalization of both, AR and SOD2, with IgG4 and the terminal complement complex [86]. A proteomic approach found three immune proteins in glomeruli of patients with MN, namely α-enolase (αENO), elongation factor 2 and glycyl aminoacyl-tRNA synthetase. One of the factors, αENO, fulfilled the criteria and was considered as autoantigen, colocalized with IgG4 in deposits, and was found to be elevated in 25% of 131 MN patients [87]. Moreover, PLA2R Ab levels correlated with antibodies against AR, SOD2 and αENO [88]. Recently, Kimura found no difference in the frequency of αENO antibodies between primary and secondary MN, although IgG4 antibodies were exclusively found in primary MN [89].

Taken together, rare antigens have been observed in antenatal or early-onset membranous nephropathy. In cases with anti-NEP antibodies, preponderance of IgG4 antibodies was associated with a good prognosis, whereas a predominance of IgG1 was accompanied by a severe disease phenotype. Moreover, a role for the cationic form of BSA to cause membranous nephropathy in children has been described and circulating antibodies have been observed. Rarer antigens have been discovered by gel electrophoresis. One can speculate that more antigens will be discovered in near future.

5. Conclusions and Outlook

The aim of the present review was to summarize recent literature related to the immunology of primary MN. While PLA2R-Ab represents the leading circulatory antibody in primary MN with a presence of 70–80%, the role of antibodies to THSD7A needs to be better defined, since malignancy-associated THSD7A-related MN has recently been reported to occur in a significant proportion of patients. Other antibodies leading to secondary forms of MN have not been described so far, and there is still room for further antibody discovery. Moreover, primary MN has a clear genetic basis, but reports related to genetic heritability are widely lacking. In addition, novel ELISA kits detecting different epitopes of PLA2R-Ab may pave the way to distinguish PLA2R-associated MN patients with a predictable poor and favorable outcome. Rare antibodies have been identified in childhood or antenatal MN. With this increasing research in mind, it is likely that we will learn more about the etiopathogenesis of primary and secondary MN in near future.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Review Article

Multiple Targets for Novel Therapy of FSGS Associated with Circulating Permeability Factor

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A plasma component is responsible for altered glomerular permeability in patients with focal segmental glomerulosclerosis. Evidence includes recurrence after renal transplantation, remission after plasmapheresis, proteinuria in infants of affected mothers, transfer of proteinuria to experimental animals, and impaired glomerular permeability after exposure to patient plasma. Therapy may include decreasing synthesis of the injurious agent, removing or blocking its interaction with cells, or blocking signaling or enhancing cell defenses to restore the permeability barrier and prevent progression. Agents that may prevent the synthesis of the permeability factor include cytotoxic agents or aggressive chemotherapy. Extracorporeal therapies include plasmapheresis, immunoadsorption with protein A or anti-immunoglobulin, or lipopheresis. Oral or intravenous galactose also decreases P_{alb} activity. Studies of glomeruli have shown that several strategies prevent the action of FSGS sera. These include blocking receptor-ligand interactions, modulating cell reactions using indomethacin or eicosanoids 20-HETE or 8,9-EET, and enhancing cytoskeleton and protein interactions using calcineurin inhibitors, glucocorticoids, or rituximab. We have identified cardiotrophin-like cytokine factor 1 (CLCF-1) as a candidate for the permeability factor. Therapies specific to CLCF-1 include potential use of cytokine receptor-like factor (CRLF-1) and inhibition of Janus kinase 2. Combined therapy using multiple modalities offers therapy to reverse proteinuria and prevent scarring.

1. Introduction

Nephrotic syndrome is defined by loss of protein in the urine, decreased plasma albumin, fluid retention resulting in edema formation, and, in many cases, hyperlipidemia. The discovery of mutations in nephrin as the genetic cause of proteinuria that characterizes Finnish nephropathy [1] led to the understanding that nephrotic syndrome is the result of glomerular podocyte dysfunction. Persistent proteinuria is a precursor of renal scarring and decline in glomerular filtration and may, indeed, be a cause of progression of renal disease. Mutations affecting the structure or function of podocytes or their response to injury may lead to proteinuria and to glomerular scarring. However, currently identified monogenic disorders account for a minority of cases [2], and the cause of podocyte dysfunction or loss leading to nephrotic syndrome is not known in most patients. Glomerular scarring may affect only certain segments of a minority of glomeruli in a pattern termed focal and segmental glomerulosclerosis (FSGS). FSGS is an orphan disease representing a syndrome with many causes [3] and is the cause of about 3.3% of end-stage-renal disease in the United States [4]; it occurs worldwide and affects all races and ethnic groups. The frequency of FSGS appears to be increasing worldwide [5]. Certain patients with renal failure due to FSGS experience recurrence of proteinuria and of progressive renal dysfunction after renal transplantation [6]. This review will focus on the rationale for therapies and on trials that address mechanisms that may reverse proteinuria and prevent or delay renal failure in patients with nephrotic syndrome and features of FSGS. Such

therapies may be useful in treatment of idiopathic nephrotic syndrome (INS) and in the treatment of recurrent FSGS in renal allografts.

2. Mutations That Point to Therapeutic Targets

Analysis of the mutated proteins in familial or monogenetic FSGS indicates several podocyte functions that render them vulnerable to injury or loss. Some are structural podocyte proteins including those of the slit-diaphragm (nephrin, Neph1), actin associated proteins (alpha-actinin 4) [11], CD2AP [12], and podocin [13]. Others are components of the glomerular basement membrane (GBM) [14, 15] and of the attachment complex including integrins [16] that provide physical adherence as well as "outside-in" signaling that maintain differentiated function. Still others are channels such as TRPC6, a gated calcium channel [17], mutations of which may predispose to injury mediated by increased magnitude and duration of signals by ANG II and other humoral responses. Finally, mitochondrial disorders lead to impaired energy availability and cellular damage [18]. APOL 1 risk mutations lead to increased incidence and rapid progression of FSGS in certain African Americans [19]. Each of these mutations leads to potential for therapies to protect or stabilize podocyte function and to minimize injury to the filtration barrier and consequent glomerulosclerosis. Current technology permits testing for these and other mutations [20]. The study of a cohort of patients with nephrotic syndrome is ongoing [21] and new point mutations associated with FSGS continue to be identified from whole genome sequencing [2].

3. Clinical and Experimental Evidence for a Circulating Factor

Evidence for the presence of a circulating factor that causes glomerular or podocyte injury and proteinuria includes early onset of proteinuria after renal transplantation in patients with FSGS [22], occurrence of proteinuria in infants of mothers with FSGS [23], transmission of proteinuria to rats or mice by FSGS plasma or fractions thereof [24, 25], remission after plasmapheresis [26, 27] or immunoadsorption with protein A [28] or anti-human IG [29] columns, and recovery of proteinuria in a transplanted kidney after retransplantation into a patient without FSGS as his underlying disease [30]. NS in each of these settings is initiated and perpetuated by factor or factors that are extrinsic to the kidney but intrinsic to blood that is perfusing the kidney. The identity of this substance has not been proven.

4. Role of Lymphocytes in NS

Lymphocytes have been implicated in the etiology of INS/FSGS on the basis of clinical observations and response to therapy and several experimental models in mice and rats. The initial postulate that T lymphocytes are the source of a substance causing proteinuria in INS was put forward by Shalhoub in 1974 [31]. This theory had no experimental basis but was derived from clinical observations including the lack of evidence of a humoral antibody response and remission induced by measles, steroids, or cyclophosphamide. The observations that lymphoma was sometimes accompanied by NS and that therapy resolved proteinuria reinforced the potential causal relationship. Studies of lymphocyte-derived products have failed to identify any unique etiological substance in INS. Glucocorticoids remain in use as first-line agents in INS and the severity of renal disease in INS is often defined by the responsiveness to corticosteroids. Steroid resistant or steroid dependent INS is more likely to progress to pathological FSGS and to renal failure than is steroid sensitive INS. ACTH, first introduced in the 1950s, has returned as ACTH gel (H.P. Acthar Gel, Mallinckrodt Pharmaceutical) [32, 33] It appears to have efficacy that may exceed that of glucocorticoids, perhaps because of interaction of the melanocortin receptor, MC1R [33–35]. B cells have also been implicated by the efficacy in some human trials of rituximab or similar monoclonal antibodies that target CD40 on B cells and podocytes

A model of FSGS in which glomerular damage appears to be mediated by abnormal T cells is that of the Buffalo/Mna rat. Rats develop proteinuria by 8 weeks of age. There is accompanying macrophage activation and expression of Th2 lymphocyte markers. Kidneys transplanted into affected rats develop glomerular injury. Investigators postulated that a circulating proteinuric substance might be present in both native and transplanted kidneys but have not identified this substance [36, 37]. An additional model of MCNS was derived by injecting CD34(+) stem cells or CD34(−) peripheral blood mononuclear cells from afflicted patients into immunocompromised mice. Both CD34(+) and CD34(−) cells lead to engraftment of human CD45(+) leukocytes but only CD34(+) cells induced albuminuria. Glomeruli from proteinuric mice showed podocyte foot process effacement. Authors concluded that cells responsible for INS are likely to be immature differentiating cells rather than mature peripheral T cells [38]. No follow-up studies using this model have been published.

Participation of B cells in FSGS is suggested by the success of anti-B cell antibodies such as Rituximab in FSGS therapy as well as by clinical responses to immunoadsorption using protein A [39] or anti-human IgG [40]. Although there is no antibody staining in FSGS or its recurrence, a survey of sera from patients with recurrent FSGS contained a large number of autoantibodies compared to non-FSGS patients (7800 antibodies versus 780 in patients with other renal diseases). Ten proteins were selected from those represented in the kidney to make up a panel for testing as a marker for recurrence of FSGS after transplantation [41]. Antibodies included anti-CD40, PTPRO, CGB5, FAS, P2RY11, SNRPB2, and APOL2. Positive results using this panel had a high predictive value of posttransplant recurrence in a retrospective cohort of 64 transplanted FSGS patients. Anti-CD40 was the most highly related to recurrent FSGS. Authors presented evidence that suPAR and anti-CD40, given together in mice, produced significant proteinuria and that blocking activation of $\alpha v\beta 3$ integrin interfered with this proteinuria. No further

studies regarding this interaction have been published but its relevance and the potential for future clinical trials has been proposed in a recent review [42]. We have observed that a monoclonal antibody to PTPRO increases P_{alb} during in vitro testing [43].

Bone marrow transplantation has been used to decrease the incidence of recurrence after renal transplantation; the optimal use of this strategy has not been established [44]. We have reported that P_{alb} activity and proteinuria were abolished for more than 10 years in a patient who was treated with standard therapy for Hodgkin's lymphoma [45]. This case report illustrates the potential role of aggressive cytotoxic therapy in eliminating circulating permeability factor or factors.

5. Lessons from In Vitro Studies of Isolated Glomeruli and Cultured Podocytes

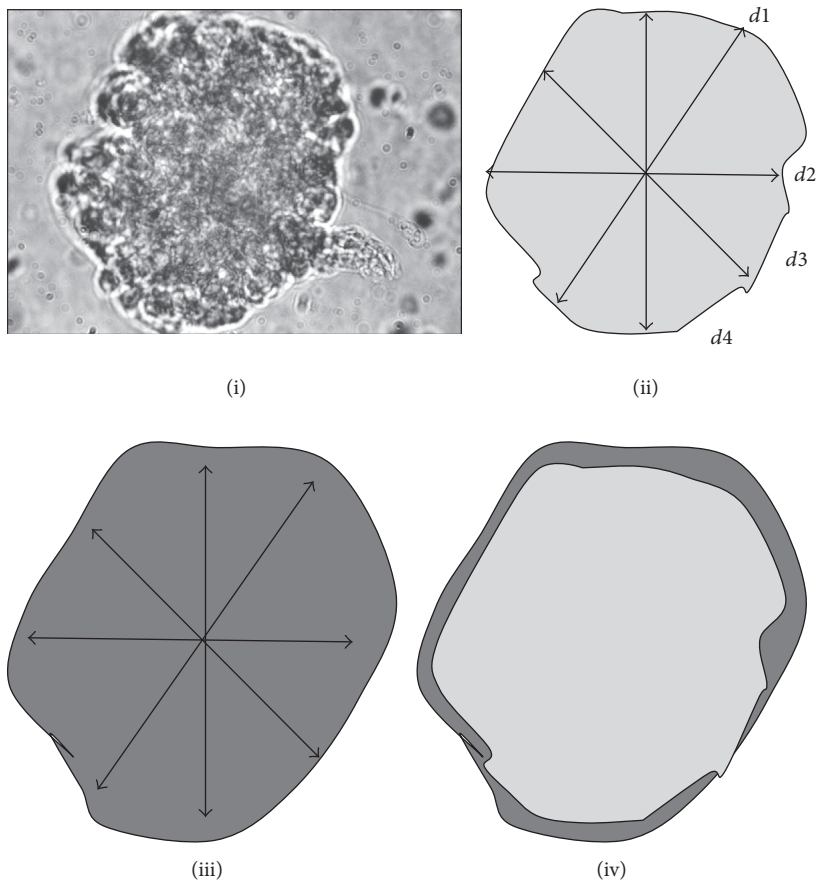
Additional information about renal responses in FSGS has been derived from studies of isolated glomeruli or of cultured podocytes. We and others have incubated glomeruli from experimental animals with plasma or sera from individuals with INS or FSGS or with other noninflammatory renal diseases [8, 46, 47]. The original assay is based on measurement of capillary expansion after filtration across the capillary wall in response to an albumin oncotic gradient. Convectional albumin permeability of the glomerular capillary barrier is calculated from the increase in glomerular volume as shown in Figure 1(a). Calculation is as follows [7]. Glomerular volume in each condition is calculated from the geometric mean of 4 diameters at 45° angles as indicated using the formula: $V = (4/3)\pi(d/2)^3$. Volume increase is calculated as follows: $\Delta V = V_{1\%} - V_{5\%}$. Since $\Delta V \propto \Delta$oncotic pressure $\times$ σ_{albumin}, $\sigma_{\text{albumin control}} = 1, \sigma_{\text{albumin experimental}}$ can be calculated as follows: $\Delta V_{\text{experimental}}/\Delta V_{\text{control}}$. We have defined albumin permeability after experimental treatment from the formula: $P_{\text{albumin experimental}} = (1 - \sigma_{\text{albumin experimental}})$ or $(1 - \Delta V_{\text{experimental}}/\Delta V_{\text{control}})$. P_{albumin} or P_{alb} is a dimensionless parameter which ranges from zero in the normal glomerular capillary to a maximum of 1.0 after injury.

We have used the assay to define an activity level, termed "P_{alb} activity" or simply "P_{alb}" [48]. We and our colleagues have recently reported the use of a comparable method in which fluorescent albumin is used as a marker for filtration and for measuring P_{alb} [49]. P_{alb} testing has been standardized using normal or patient plasma in a 1 : 50 dilution and an oncotic gradient of 16–20 mm Hg [8]. Sera of children with INS cause moderate damage to the glomerular protein filtration barrier [47] while sera of individuals with collapsing FSGS [46] or from patients with posttransplant recurrence of FSGS caused a profound loss of barrier function [8]. The assay is reliable across a range of observers. The correlation coefficient between values of replicate samples from 35 patients was 0.72 ($p < 0.001$) [8] and have been stable and reproducible in studies of human samples and the original assay and its modifications have been employed in a wide range of experimental protocols for more than 30 years in several centers [49–51].

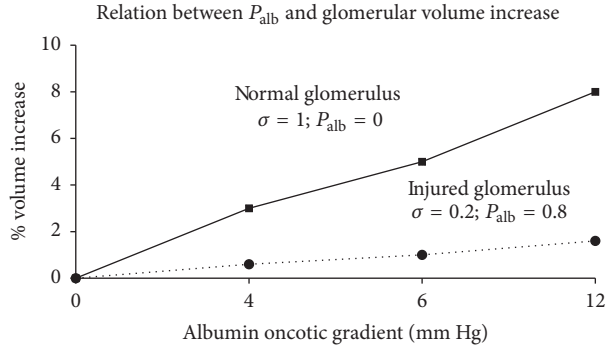
Studies of the effects of plasma or serum from patients with FSGS on isolated glomeruli have documented a profound and nearly immediate increase in P_{alb}. The increase is accompanied and is dependent on phosphorylation of JAK2 and STAT3 [9]. Comparable activation of the JAK/STAT pathway occurs in cultured immortalized podocytes incubated with FSGS serum [9]. The effect of patient serum is greatest in samples from patients with the most aggressive renal disease as indicated both by a more rapid course to ESRD [52] and by comparison of the very high P_{alb} activity of sera from patients with collapsing FSGS [46] or recurrence after transplantation versus those with MCNS or transplantation without recurrence [8]. P_{alb} activity predicts posttransplant recurrence in patients with podocin mutations [53]. P_{alb} activity is decreased or abolished by PP [8, 54] or IA with protein A [40]. Activity is blocked by JAK or STAT inhibitors [9], by the addition of galactose or adsorption of sera on a galactose column [55], or by a variety of potential therapeutic agents including indomethacin [56], cyclosporine A [57], NO, acting as a superoxide scavenger [58], antibody to CLCF-1 or by CRLF-1 [9], extract of Tripterygium wilfordii [59], and eicosanoids 20-HETE [60] and 8,9 EET [61]. One or more components from plasma of humans and other mammals [62, 63] and urine from patients with recurrent FSGS also block P_{alb} activity [64].

6. Proposed Candidates for the FSGS Permeability Factor

We and others have attempted to identify substance or substances in the plasma of patients with FSGS, especially those who are resistant to therapy or who progress to ESRD and experience recurrent nephrotic syndrome and FSGS after transplantation [25]. Historical candidates as proteinuric factors include vascular permeability factor (VPF) that is synthesized by T cells [65] and hemopexin [66]. More recently, elevated concentrations of soluble urokinase-type plasminogen activator receptor (suPAR) have been reported in patients with FSGS. This finding and results of studies of cultured podocytes have led to the proposal of suPAR as a lead candidate as an etiological factor in FSGS. Studies of cultured podocytes have been interpreted as supportive [67]. SuPAR effects include altered cytoskeleton in immortalized podocytes, altered podocyte attachment with activation of β3 integrin [68], and activation of STAT1 in vascular smooth muscle cells via a PDGFR receptor [69]. SuPAR has numerous other effects on several cell types including alteration of mobilization and migration and of mesenchymal stem cells [70]. Other investigators have disputed the interpretation of the experimental and clinical findings regarding suPAR [71, 72]. Active investigations are ongoing, but suPAR is elevated in many inflammatory states and in diabetes mellitus. SuPAR appears to be associated with progression of renal disease regardless of its etiology [73, 74]. Circulating suPAR is increased in renal failure in part because of diminished loss in urine [75]. Understanding of the role that suPAR plays in the initiation and progression of renal disease, including INS and FSGS, is incomplete.



(a) (i) Video micrograph of a glomerulus isolated from a normal rat. (ii) Cartoon of planar area traced from micrograph in medium containing 5% BSA. Diameters used to calculate glomerular volume are indicated as $d1$, $d2$, $d3$, and $d4$. (iii) Area after exposure to medium with 1% albumin concentration. (iv) Difference between images in 5% and 1% BSA. Increase in glomerular size is consequent to distension of glomerular capillaries. Calculations used to determine $P_{albumin}$ are detailed in the text



(b) An example of the increase in glomerular volume in an untreated control glomerulus (solid line) and in a glomerulus after treatment with an injurious agent such as protamine (dotted line) as described previously [7, 8]. Here P_{alb} of the injured glomerulus is about 0.8. The figure has been redrawn from prior published figures

Figure 1

A role for lipoproteins in FSGS is suggested by reports of successful therapy using lipopheresis and by the presence of abnormal lipids in proteomic analyses. In addition, lipoproteins have been proposed both as inhibitors of FSGS permeability activity and as candidates for the active factor. Specific apolipoproteins that act as inhibitors of P_{alb} activity of FSGS sera include APO-E2, APO-E4, and fragments of APO-A iv [76]. Genotyping of apolipoproteins did not reveal

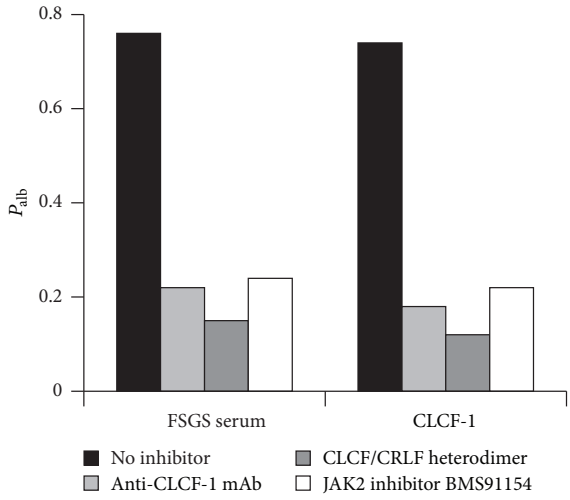
differences between FSGS patients who were sensitive to steroids and those who were resistant to therapy [77]. In contrast, a form of APO-A1 was identified in plasma only in recurring FSGS patients and was absent from those without recurrence [78]. Additional observations that implicate lipids in proteinuria are derived from work indicating the role of rituximab as an inhibitor of downregulation of podocyte sphingomyelinase-like phosphodiesterase 3b (SMPDL-3b) [79].

We have recently performed studies to enrich P_{alb} activity of recurrent FSGS plasma and have identified cardiotrophin-like cytokine-1 (CLCF-1) in the active fraction of FSGS plasma using tandem LC-MS/MS [10, 80]. CLCF-1 is best known for its action through the JAK/STAT pathway. CLCF-1 is a member of the IL-6 family of cytokines. CLCF-1 was initially cloned from T cells [81, 82] and is a neurotrophic and B cell stimulating factor that increases expression of immunoglobulins. CLCF-1 acts through a complex receptor composed of CNTFR, LIFR, and gp130 [83]. Gp130 defines the IL-6 family of cytokines while, in the case of CLCF-1, CNTFR and LIFR confer specificity [84]. The IL-6 family includes a number of pleiotropic cytokines which are expressed by both bone marrow derived and somatic cells. The IL-6 family includes erythropoietin, Leukemia Inhibitory Factor (LIF), and Ciliary Neurotrophic Factor (CNTF), as well as IL-6 itself. CLCF-1 has 3 potential binding sites and we have shown that activation of podocytes is prevented interaction with CRLF-1 [9], a related and cosecreted cytokine [85]. Receptor-ligand binding initiates signaling by the JAK/STAT pathway. We have reported that podocytes express primarily JAK2 and STAT3 [10] and each is activated by tyrosine phosphorylation by CLCF-1 at specific sites. STAT3 activation of peripheral blood cells is immediate and returns to normal within 24 hours [10]. In contrast, STAT3 phosphorylation in the renal cortex persists for at least 72 hours [10]. pSTAT3 is present in glomeruli at a location consistent with podocytes as well as in vascular smooth muscle within the kidney and proximal tubule cells [10]. CLCF-1 also activates STAT3 in cultured murine or human podocytes and simultaneously alters the actin cytoskeleton [10]. These changes may be analogous to those required for foot process retraction in vivo during proteinuric states.

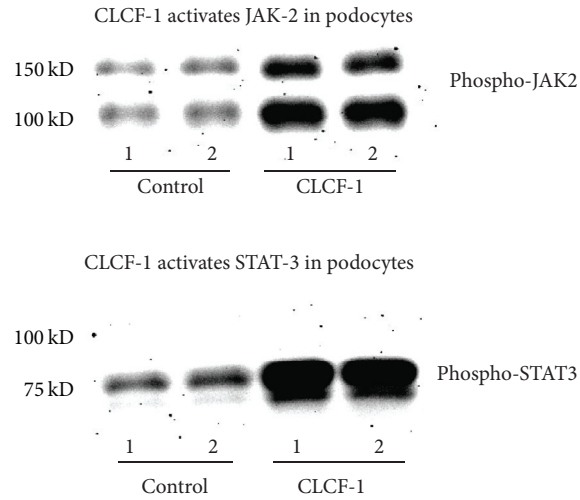
We have shown that P_{alb} activity is removed by plasmapheresis (PP) with a kinetic pattern comparable to that of immunoglobulins and that activity can be recovered from discarded plasmapheresis fluid [8]. We have followed P_{alb} activity during sequential fractionation of FSGS plasma obtained by plasmapheresis [25, 86]. The active component of the plasma fraction is soluble in ammonium sulfate at a concentration of 70% but precipitates at 80%. Injection of this fraction into rats results in proteinuria [25]. In additional studies, we found that P_{alb} activity is retained on a column of galactose-coated agarose beads and can be recovered by elution using a galactose solution [55]. Activity is retained by a filtration membrane with molecular weight cut-off of 30 kDa. We have performed liquid chromatography/tandem mass spectrometry (LC/MS/MS) on the plasma fraction obtained by galactose affinity chromatography of FSGS plasma and have identified a candidate cytokine, cardiotrophin-like cytokine factor-1 (CLCF-1) [80]. This cytokine mimics the P_{alb} activity of FSGS plasma [9, 10] and a monoclonal antibody to CLCF-1 markedly decreases the activity of FSGS sera [9]. In addition, both FSGS serum and CLCF-1 activate the JAK2/STAT3 pathway [9]. P_{alb} activity of FSGS serum as well as that of CLCF-1 is inhibited by specific inhibitors of JAK2 and STAT3 [9] and by FDA approved JAK inhibitors ruxolitinib (Incyte), by tofacitinib (Pfizer), and by baricitinib (E. Lilly), which is in review by the FDA. P_{alb} activity and podocyte STAT phosphorylation are also inhibited by cytokine receptor-like factor-1 (CRLF-1), a cytokine which dimerizes with CLCF-1 [9]. The effects of CLCF-1 on P_{alb} and on JAK2 and STAT3 activation are shown in Figures 2(a) and 2(b). Figure 2(c) illustrates that JAK2 inhibition blocks the effect of FSGS sera on P_{alb} activity of sera from FSGS patients (Figure 2 depicts data previously published [9, 10]). We are continuing to investigate the role of CLCF-1, CRLF-1, and related molecules in the genesis of proteinuria in FSGS in native kidneys and in posttransplant recurrence of FSGS using isolated glomeruli, cultured podocytes, and responses in intact rodents.

The question of design and interpretation of animal models for recurrent FSGS mediated by a circulating molecule or molecules has not been resolved. Proteinuria is a multistep process. The initial change in permeability is the result of podocyte signaling and a functional increase in permeability. Filtered albumin is reabsorbed by the proximal tubule and reabsorption may delay recognition of the altered filtration barrier. Visual confirmation of proximal tubule reabsorption is provided by 2-photon imaging of rat kidneys in several models of proteinuria [87]. Anatomical changes follow and include impaired podocyte architecture and attachment and to podocyte loss. Glomerular segmental scarring is evident only in later phases and leads to altered hemodynamics and rheology. It is proposed that loss of podocytes exposes glomerular basement membrane and permits movement of parietal epithelial cells to vacant basement membrane where they form an attachment [88] that alters the geometry of both the capillary and Bowman's space. As the disease advances, there is scarring in the tubular interstitial compartment with capillary loss, tubular atrophy, and collagen accumulation. Late in the process, local or circulating cells and soluble substances alter the interstitium leading to accumulation of extracellular material and interstitial fibrosis. The role of the circulating permeability factor in each of these processes is not known. In our opinion, many of the later events that lead to progressive renal failure are shared with other renal diseases.

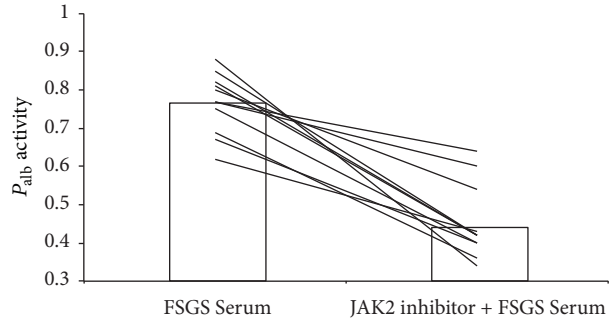
We propose that models that are based on effects of toxins such as puromycin, adriamycin, bisphosphonates, or reduced nephron number [89] or on podocyte death induced by diphtheria toxin in genetically modified mice [90] or by antibodies to podocytes [91] are not suitable models for the early and reversible proteinuria of recurrence FSGS after transplantation. We have focused on the initiating steps of podocyte signaling and altered attachment rather than on processes that determine subsequent glomerular scarring and interstitial fibrosis. We believe that this focus may permit the design of preemptive treatment. To date, no animal model of



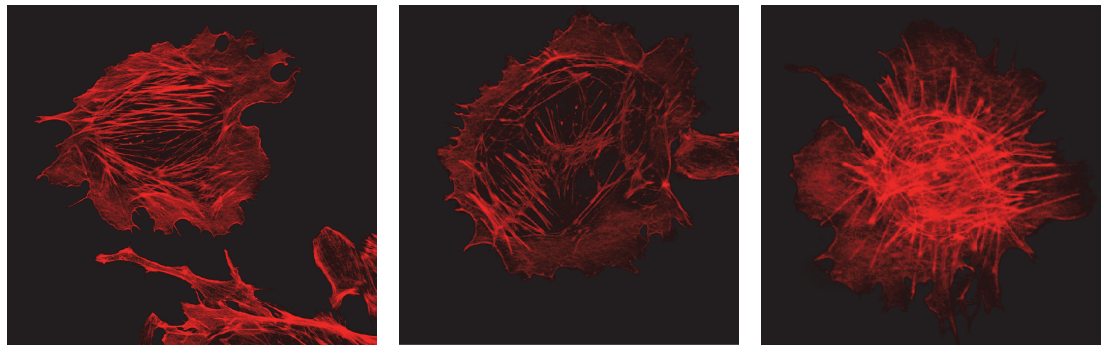
(a) Effect of FSGS serum or CLCF-1 on P_{alb} of glomeruli isolated from normal rats. The increases by FSGS serum and CLCF-1 were comparable in magnitude. Each was prevented by a monoclonal antibody to CLCF-1, by the heterodimer CLCF-1/CRLF-1, or by the specific JAK2 inhibitor, BMS91154. Data from Sharma et al. [9]



(b) Western blots showing JAK2 and STAT3 phosphorylation in cultured immortalized murine podocytes after incubation with CLCF-1. CLCF-1 increased both JAK2 and STAT3 phosphorylation. Data from Savin et al. [10]



(c) Effect of the specific JAK2 inhibitor BMS91154 on the increase in P_{alb} induced by serum of 11 individual patients with FSGS. Glomeruli were incubated with FSGS serum only or with BMS91154 for 15 minutes prior to the addition of FSGS patient plasma, 5% vol/vol. Values represent P_{alb} activity after incubation with FSGS serum only and P_{alb} activity after preincubation with inhibitor and then with FSGS sera. In this experiment, the P_{alb} activity of FSGS serum was diminished from 0.77 to 0.44 [9] by preincubation of glomeruli with the inhibitor. Bars show averages and lines depict sera of individual patients. Data from Sharma et al. [9]



(d) Cytoskeleton of immortalized murine podocytes after incubation with CLCF-1 for up to 60 minutes. (i) Prior to incubation, there were many nearly parallel actin filaments near the base of the cell. Cells had filopodia that extended in several directions and were tightly adherent to the coverslip on which they were grown. (ii) After 15 minutes of incubation, the number and thickness of basal actin filaments decreased, filopodia retracted, and the number and extent of lamellipodia increased markedly. (iii) By 60 minutes, nearly all large actin filaments had been lost and the cell had assumed a rounded configuration with extensive formation of lamellipodia with actin arcs. These confocal micrographs are representative of those obtained in during performance of published studies [10]

Figure 2

glomerular dysfunction that arises directly from responses to a plasma substance and leads to renal failure has been defined. Targeted therapy may require knowledge of cell signaling during exposure to plasma of patients with recurrent FSGS or to the components of such plasma. Human trials will be required to prove the relevance and efficacy of proposed therapies.

Cultured immortalized murine and human podocytes have also been used in functional assays to define plasma activity. Assay parameters include alterations in the pattern of actin cytoskeleton [68]. Activation of $\beta 3$ integrin, as evidenced by its phosphorylation, has recently been proposed as an indicator of podocyte injury and a high throughput assay presented as a model for discovery of novel therapeutics [92]. Increased podocyte motility manifest as migration into a "scratch" defect in a nearly confluent cell layer has also been used to measure activity [68, 93]. We and others have used alterations in cytoskeleton configuration as a measure of the effects of FSGS samples and confirmed that cytoskeleton is reorganized after incubation in FSGS serum or a candidate cytokine [10]. Figure 2(d) illustrates cytoskeleton responses of podocytes during incubation with CLCF-1. During incubation with CLCF-1, filopodia retract and basal parallel actin filaments are attenuated and decrease in number. Simultaneously, lamellipodia become more prominent, the cells assume a more rounded state, and short nonparallel actin filaments and actin arcs predominate [10].

7. Targets for Therapy in FSGS and Its Recurrence

Trials in FSGS and its posttransplant recurrence have focused on agents that may remove or neutralize the injurious factors themselves (PP, IA [28, 94–96], and galactose [55, 97–99]), modify the immune response by interacting with glucocorticoid and/or melanocortin receptors (prednisone, dexamethasone, and ACTH as Acthar Gel), alter cellular immunity (calcineurin inhibitors including tacrolimus, cyclosporine A, humanized antibodies to components of B cells (rituximab), and T cells (abatacept)), and attempt to limit fibrosis by antibodies to the cytokines TGFβ and TNFα. A number of agents, including glucocorticoids and calcineurin inhibitors, exert protective effects on podocytes by changing protein expression profiles [100–103] and stabilizing actin cytoskeleton [104]. The antiproteinuric effects of angiotensin converting inhibitors and angiotensin receptor blockers (ARBs) are well known and appear to occur regardless of the etiology of proteinuria. Results of a randomized controlled trial of a blocker both of angiotensin and of endothelin receptor have been reported in abstract form H. Trachtman. Am Soc Nephrol, Annual Meeting, November, 2016, HI-OR06, and showed marked reduction of proteinuria in about 28% of FSGS on sparsentan versus 9% in those treated with irbesartan. Clinical trials related to FSGS are listed in https://ClinicalTrials.gov and include therapies related to corticosteroids and ACTH gel, inhibitors of angiotensin and endothelin receptors, immunosuppressive agents including calcineurin inhibitors and monoclonal antibodies, extracorporeal interventions using PP, IA, and lipopheresis, an inhibitor of sodium/glucose transporter 2 (SGLT2), and treatment with vitamin D and retinoids. Results of most of these trials have not yet been published. Others have shown a positive effect on proteinuria or disease progression in only a minority of subjects [105].

8. Completed Trials in Patients with FSGS in Native Kidneys

Large-scale consortia are necessary to the study of orphan diseases such as FSGS. *The Toronto Registry* has followed patients with common glomerular diseases, FSGS, membranous nephropathy, and IgA nephropathy and has generated a rich experience in regard to natural history and therapy. Studies established that partial as well as complete remissions lead to more stable renal function and prolonged renal survival. Cyclosporine decreases proteinuria in glomerular disease of several etiologies; CsA-induced nephrotoxicity can be minimized by use of lower doses. Mycophenolate (MMF) also induces remission in many patients with FSGS. In a trial comparing CsA to no CsA in FSGS in native kidneys, CsA was better in reducing remissions [106]. Serum P_{alb} activity measured in our lab was not reduced even during clinical remission of proteinuria [107]. This suggests that CsA directly protects glomeruli from the effects of continued presence of the FSGS permeability factor. Additional studies also showed clinical benefit from CsA treatment [108, 109]. Results of studies of isolated glomeruli and podocytes confirm that CsA protects the permeability barrier from the effects of FSGS serum by nonimmune mechanisms [57]. Protection of synaptopodin and of the actin cytoskeleton [104] may be consequent to inhibition of calcineurin activity.

The NIH-funded *FSGS Clinical Trial* examined the relative efficacy of 2 accepted therapies in 138 subjects randomly assigned to CsA or MMF plus dexamethasone. The relevant outcome was complete or partial remission of proteinuria within 1 year. The patients were selected from those with steroid resistant FSGS with relatively well preserved renal function at the time of recruitment. Each treatment regimen induced remission in about 30% of patients. About 10% patients in each treatment group developed renal failure or died during the 78-week study. MMF plus dexamethasone had more side effects while CsA transiently decreased GFR. GFR did not differ between the 2 groups at the end of the study [110]. The trial was limited by relatively advanced disease and small number of subjects and the short duration of therapy.

The Phase 2 *FONT (Novel Therapies for Resistant FSGS)* trial compared usual care to oral administration of galactose or injections of anti-TNF alpha (adalimumab, "Humira®"). As in the prior FSGS trial, patients had steroid resistant FSGS with GFR $\geq$ 40 ml/min/1.75 m^2. The study was limited by the small number of enrolled subjects (21 subjects). No subject in the usual care arm had remission of proteinuria; 2 patients in each experimental treatment arm had remission. The effect of galactose was more prolonged and had higher patient acceptance than was seen with adalimumab treatment [99, 110]. An additional trial of galactose in glomerular disease

[105] has been published as have several case reports [97, 98]. The trial was marred by heterogeneity of diagnosis and small number of subjects [105]. Case reports may have been subject to positive publication bias.

9. Trials to Prevent or Treat Transplant Recurrence of FSGS

A single center study of the use of plasmapheresis to prevent of delay FSGS recurrence after transplant was carried out in patients judged to be at high risk because of prior recurrence or rapid course to renal failure. There was an apparent benefit in preventing recurrence and early transplant loss [111]. The small size of the study and the lack of a control group limit the strength of the findings. Therapies used in other small series have failed to prevent recurrence.

10. Rituximab to Prevent Recurrence of Proteinuria in Patients Receiving Kidney Transplant for FSGS

An NIH sponsored single center clinical trial in preventing recurrence by the use of rituximab is being conducted. The underlying hypothesis is that rituximab will be protective because of its role in control of activity of podocyte sphingomyelinase-like phosphodiesterase 3b [79]. Rituximab partially prevented SMPDL-3b and ASMase downregulation that was observed in podocytes treated with the sera of patients with recurrent FSGS. Overexpression of SMPDL-3b or treatment with rituximab was able to prevent disruption of the actin cytoskeleton and podocyte apoptosis induced by patient sera. Subjects have been recruited and randomized but study results are not available at this time.

11. Individualized Immunoadsorption (IA) for Posttransplant Therapy

Reversal of recurrence and long-term graft survival has been reported in 66% of 18 renal transplant recipients with ESRD due to idiopathic FSGS. This report includes noteworthy details regarding the time course of recurrence. 66.7% of patients experienced disease recurrence in a mean time of 0.75 months after transplantation (KTx), with a mean proteinuria of 8.9 g/day at the time of recurrence. The majority of patients were adults (mean age, 30.8 years). Both cadaveric and living related donors were included. Four of the patients received therapy with rituximab in addition to IA. During a mean time of follow-up of 48.3 months, about 60% of patients achieved complete remission, and about 40% achieved partial remission during average follow-up of over 4 years. At the end of follow-up, 67% had functioning grafts and were in sustained remission, while 33% progressed to ESRD because of FSGS recurrence [95]. These results reinforce the notion that use of current therapies in an individualized and persistent manner can result in marked improvement in allograft survival.

12. Glomerular Disease after Kidney Transplant and Current and Future Clinical Trials

Although several agents have been proposed as mediators of FSGS recurrence after transplantation, none have been definitively proven as etiologic agents. Most studies regarding recurrence in this orphan disease have been descriptive. A study of 1435 adult kidney transplants at the Mayo Clinic showed that as many as 26% of recipients developed glomerular disease by 10 years. FSGS was the diagnosis in 38% of these while other diagnoses included IgA nephropathy, membranous nephropathy, and mesangial proliferative glomerulonephritis. The risk of glomerular disease was increased with younger age, females, and steroid free induction and lower pretransplant serum albumin. Recurrent FSGS increased the risk of graft failure ($R = 2.82$ $p < 0.0001$). Overall glomerular disease caused 22% of allograft losses. These findings emphasize the important impact of glomerular disease in the overall success and benefit of renal transplantation [112].

An antibody to CD80, abatacept, has been used to treat FSGS recurrence and some success was initially reported [113] but others have failed to confirm this effect [114]. Thus, the utility of this agent is not clear.

We have recently proposed a trial of the JAK inhibitor ruxolitinib (Jakifi, Incyte, Corp) (REFOCUS, Rescue FSGS). The decision to emphasize study patients with recurrent proteinuria and FSGS in renal allografts is based on the potential to identify the initial stages of injury at a time when there is little anatomic distortion and to provide intervention when the injury is most likely to be reversible. The strategy of enrolling only patients with recurrence will also minimize the likelihood of confusion arising from the presence of mutations of podocyte proteins and of inclusion of renal disease secondary to hypertension, obesity, or environmental toxins. The choice of ruxolitinib is based on its relative specificity for JAK1 and JAK2 [115]. As an FDA approved drug, its pharmacokinetics and toxicity have been well defined in both preclinical and clinical studies [116, 117]. In addition, it has been used in thousands of patients with hematological disorders including myelofibrosis [118] and polycythemia vera and it is under investigation in other hematological malignancies and solid tumors. Ruxolitinib and several other JAK inhibitors block the effects of FSGS sera on pSTAT in cultured podocytes.

We also plan to propose a formal trial of oral or intravenous galactose in a sample of FSGS patients that is sufficiently large to confirm or deny general efficacy in FSGS after transplantation and/or idiopathic FSGS in native kidneys. The selection of galactose is based on in vitro studies, on pharmacological effects of galactose on plasma activity, and on several case reports in pre- and posttransplant patients [55, 97–99, 105].

The available data regarding idiopathic FSGS is consistent with the concepts shown in Figure 3. As illustrated, a circulating substance, which has not been definitively defined and is labelled here as "cytokine," is present in the circulation. This substance appears to interact with specific receptors on

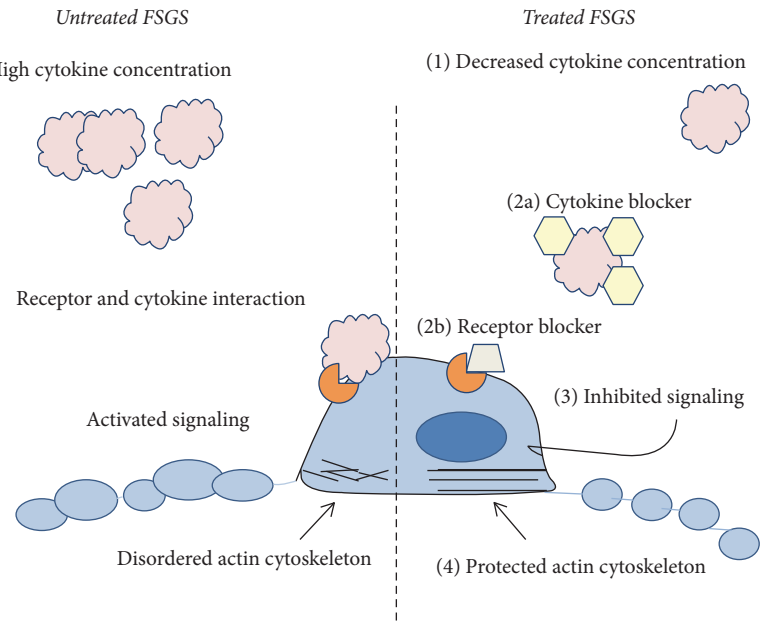


Figure 3: *Scheme showing potential targets for therapy in glomerular injury caused by circulating factor or cytokine.* The left panel shows the milieu that favors podocyte dysfunction and proteinuria. Excess activity of an injurious factor or cytokine permits its interaction with receptors on podocytes which, in turn, activates signaling via JAK2/STAT3 and other pathways. Actin cytoskeleton becomes disordered and podocyte architecture and function is altered. The right panel shows some potential treatment goals including the following: (1) decrease factor synthesis or remove it by plasmapheresis, immunoadsorption or other extracorporeal methods; (2a) administer blocker such as galactose or (2b) receptor blocker such as antibody to specific component or receptor; (3) inhibit intracellular signaling by JAK or STAT inhibitor or inhibitor of other essential cell pathways; (4) protect actin cytoskeleton by calcineurin inhibitors such as CsA or by a sphingomyelinase inhibitor such as rituximab. Identification of multiple targets will permit concurrent use of several modalities that may increase effectiveness while limiting side effects.

the podocytes, activating intracellular signaling pathways and leading to alterations in cytoskeleton, adhesion, and motility. Decreasing its total concentration by inhibiting its synthesis or increasing catabolism, blocking its activity by antibodies or inhibitors of receptor-ligand interaction, interrupting intracellular signaling, or enhancing cell mechanisms that protect cytoskeleton and adhesion may be developed as therapeutic interventions. Multicenter collaborative studies are essential since no center will have enough patients to permit meaningful analysis of treatment effect. Early intervention will provide an opportunity to arrest the disease in its initial stages. New therapies offer to dramatically improve the lives of patients with FSGS in native kidneys and permit successful renal transplantation in those who have progressed to end-stage-renal disease.

13. Summary

Studies of genes expressed in podocytes and the glomerular capillary wall point to cytoskeleton, cell junctions, cell attachment, and metabolism as potential targets for intervention. We and others have demonstrated that FSGS serum or plasma induces increased glomerular capillary permeability in vitro and alters cytoskeleton and signaling responses of cultured podocytes. These responses may be used to screen potential therapeutic agents prior to or simultaneously with conduct of clinical trials. A number of trials are ongoing in patients with FSGS in native kidneys or in recurrence after transplantation while others have been proposed and await implementation. Problems that must be overcome include heterogeneity of patients, short duration of studies, small sample size, and late initiation of therapy. Careful design and participation of consortia will be required to attain clinical and statistical significance.

Abbreviations

8,9 EET:	8,9-Epoxyeicosatrienoic acid
20 HETE:	20-Hydroxyeicosatetraeonic acid
ACTH:	Adrenocorticotrophic hormone
CLCF-1:	Cardiotrophin-like cytokine factor-1
CNTF:	Ciliary Neurotrophic Factor
CNTFRα:	Ciliary Neurotrophic Factor Receptor α
sCNTFRα:	Soluble Ciliary Neurotrophic Factor Receptor α
CRLF-1:	Cytokine receptor-like factor-1
CsA:	Cyclosporine A
FSGS:	Focal segmental glomerulosclerosis
gp130:	Glycoprotein 130
IA:	Immunoadsorption
IL-6:	Interleukin-6
INS:	Idiopathic nephrotic syndrome
JAK:	Janus kinase
LIFRα:	Leukemia inhibitory factor receptor α

NS: Nephrotic syndrome
P_{alb}: Glomerular albumin permeability, measured during in vitro studies
PP: Plasmapheresis
STAT: Signal transducer and activator of transcription
suPAR: Soluble urokinase-type plasminogen activator receptor
TGFβ: Transforming growth factor-β
TNFα: Tumor necrosis factor-α
Tyk: Tyrosine kinase
VPF: Vascular permeability factor.

Disclosure

The views expressed in this article are those of the authors and do not necessarily reflect the position or policy of the Department of Veterans Affairs or the United States government.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

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Review Article

NPHS2 Mutations: A Closer Look to Latin American Countries

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Nephrotic syndrome is one of the most common kidney pathologies in childhood, being characterized by proteinuria, edema, and hypoalbuminemia. In clinical practice, it is divided into two categories based on the response to steroid therapy: steroid-sensitive and steroid resistant. Inherited impairments of proteins located in the glomerular filtration barrier have been identified as important causes of nephrotic syndrome, with one of these being podocin, coded by *NPHS2* gene. *NPHS2* mutations are the most frequent genetic cause of steroid resistant nephrotic syndrome. The aim of this review is to update the list of *NPHS2* mutations reported between June 2013 and February 2017, with a closer look to mutations occurring in Latin American countries.

1. Introduction

In the high-throughput sequencing era, new candidate genes associated with monogenic and genetic heterogeneous diseases such as steroid resistant nephrotic syndrome (SRNS) are piling up [1–6]. However, mutations in the three main genes (*NPHS1*, *NPHS2*, and exons 8 and 9 of *WT1* gene) are still the most frequent molecular cause of SRNS in childhood and adolescence. More than 200 *NPHS2* (OMIM *604766) gene mutations are registered in HGMD Professional 2017.1 (http://www.hgmd.cf.ac.uk) and 127 in the HGMD Public 2017.1 associated with familial and sporadic forms of SRNS.

SRNS is one of the most common kidney pathologies in childhood, being characterized by proteinuria, edema, and hypoalbuminemia. The most frequent renal histological feature associated with SRNS is focal segmental glomerulosclerosis (FSGS). Almost 40% of SRNS/FSGS children develop end-stage renal disease (ESRD) before adulthood and may receive a kidney transplant, with a 10 to 50% risk of recurrence of FSGS in the allograft kidney [7–9]. Although the pathogenesis of NS is not yet completely understood, much has been learnt about the glomerular filtration barrier (GFB) which is composed of three layers: the fenestrated capillary endothelial cells; the glomerular basement membrane (GBM); and the podocytes, specialized cells with interdigitating foot processes that are interconnected to form a slit diaphragm (SD) membrane, a multiprotein signaling complex that controls the ultrafiltration in this dynamic structure [10]. Nephrotic protein leakage may occur as a result from damage in one of these GFB components [11], although functional pathways specifically in the podocyte have revealed this cell as the key component of the pathogenesis of SRNS [5, 6, 12, 13].

More than 50 genes have been identified so far, associated with SRNS of congenital (0–3 months), infantile (4–12 months), childhood/adolescence (1–18 years), or adult onset [6]. In 2007, Hinkes et al. [14] screened the four genes *NPHS1* (OMIM *602716), *NPHS2* (OMIM *604766), *WT1* (OMIM *607102), and *LAMB2* (OMIM *150325) in a large European cohort of 89 children from 80 families with NS manifesting in the first year of life. They detected disease-causing mutations in one of the four genes in 66.3% of the families, 84.8% of congenital onset, and 44.1% of infantile onset. Seven years

later, in 2014, Sadowski et al. [5] screened 27 genes associated with SRNS in 1783 families from an international cohort study (107 members from 30 countries). The eight larger contributing centers were Germany, Switzerland, Turkey, Egypt, Saudi Arabia, Los Angeles, Ann Arbor, and India but many other centers participated, including Argentina representing South America with 16 families. The main conclusion was that a monogenic cause was detected in 29.5% of the SRNS cases (0–25 years) in one of the 27 genes analyzed. After a detailed analysis of mutation distribution by gene and by age of onset, *NPHS2* mutations were the most frequent (5.7% to 12.7%) in patients with SRNS onset between 1 and 18 years old.

Mutations in *NPHS2* gene, located at 1q25-31, are the most common cause of SRNS in childhood and were first described by Boute et al. (2000) [15]. *NPHS2* coding region encompasses 1,149 bp, has 8 exons, and encodes a 383-amino-acid protein with 42 kD, called podocin, which is expressed in fetal and mature kidney [15]. Podocin is predicted to have a hairpin-like structure, with both C- and N-terminal domains facing cytosol and one short transmembrane domain [16]. In addition to its role in anchoring nephrin and CD2AP (OMIM *604241) to the SD, podocin forms homoligomer complexes that bind with cholesterol in lipid rafts, where it may act as a scaffolding/targeting/signaling protein [17–19]. *NPHS2* mutations initially found in autosomal-recessive inheritance familial cases [15] and further in sporadic SRNS cases [20] as well represent 40% and 6–17% of these cases, respectively [12, 21–23]. In 2013, Bouchireb et al. [24], in a detailed and complete review, presented a list of *NPHS2* mutations, polymorphisms, and variants of unknown significance published from October 1999 to September 2013. In that review they identified 25 novel pathogenic mutations in addition to the 101 already registered in the mutation database at that time. The mutations were distributed along the entire *NPHS2* gene, with no preferential hotspot.

The aim of this review is to update the list of *NPHS2* mutations reported in the last few years around the world, with a closer look to mutations occurring in Latin American countries.

2. *NPHS2* Mutations Overview

We searched for articles reporting *NPHS2* mutations associated with SRNS in childhood and adolescence that were published from June 2013 to February 2017. The key words "*NPHS2*", "*NPHS2* mutations", "podocin" and "steroid resistant nephrotic syndrome genetics" were used in PubMed databank. We further looked for variants/mutations that were not annotated in open-access databases such as public HGMD (http://www.hgmd.cf.ac.uk/ac/index.php) and GnomeAD Browser (http://gnomad.broadinstitute.org) or in Leiden Open Variation Database (https://www.lovd.nl/NPHS2). For nonsynonymous variants, we classified them as deleterious or benign according to in silico prediction tools (PolyPhen-2 and SIFT) [25, 26]. For splicing variants, we performed splice-site prediction by BDGP neural network [27].

Thirty-nine variants, among them 25 missenses, four nonsenses, three splice-sites, four frameshifts, and three in the promoter region were published from June 2013 to February 2017 in a total of 109 out of 829 SRNS patients in many countries: China (Wang et al., 2017) [28]; India (Jaffer et al., 2014; Dhandapani et al. 2017; Ramanathan et al. 2017) [29–31]; Italy (Benetti et al., 2013) [32]; Iran (Basiratnia et al., 2013) [33]; United Kingdom (Jain et al., 2014) [34]; United States of America (Laurin et al., 2014; Phelan et al., 2015) [35, 36]; Poland (Kuleta et al., 2014) [37]; Finland (Suvanto et al., 2016) [38]; Saudi Arabi (Kari et al., 2013) [39]; Japan (Ogino et al., 2015) [40]; Mexico (Carrasco-Miranda et al., 2013) [41]; Chile (Azocar et al., 2016) [42]; and Brazil (Guaragna et al., 2015) [43]. Ten out of those 39 mutations were unique and had not been annotated in public HGMD (http://www.hgmd.cf.ac.uk/ac/index.php) or in GnomeAD Browser (http://gnomad.broadinstitute.org) or in the Leiden Open Variation Database (https://www.lovd.nl/NPHS2): six were missenses, three were located in splice-site regions, and two were frameshifts (Table 1). We searched GnomaAD Browser for all the variants compiled in Table 1 as well as for other variants such those reported in Mexico (p.Leu142Pro), Chile and Brazil (p.Ala284Val), and Brazil (p.Val260Glu), observing frequency, racial ethnicity, and geographical provenience. Only p.Ala284Val and p.Val260Glu were registered at GnomaAD Browser, but no allele counted was from Latin America population for both of them.

3. Missense Mutations

Four out of the five missenses were described in homozygosity in South Indian SRNS patients (p.Ser46Pro, p.Leu167Pro, p.Pro175Ser, and p.Pro316Ser) [29, 30]. The fifth missense, p.Leu139Arg, was identified in two Mexican children with NS, one SRNS, and one SSNS [41]. As in silico predictions were not performed for those variants in their original publications, we investigated their pathogenicity by predictive tools available, such as SIFT and PolyPhen-2. Both p.Leu139Arg and p.Pro316Ser variants were predicted as damaging by SIFT and PolyPhen-2 (Table 1). At the moment, these five missense variants should be considered as variants of unknown significance and only after proper functional studies they can be associated with SRNS.

4. Splice-Site Mutations

Three splice-site mutations that were not registered in any of the three searched databases have been identified (Table 1). The homozygous splice-site mutation c.451+3A>T whose effect on podocin protein was evaluated by renal mRNA analysis demonstrated exon 3 skipping that led to a premature termination codon (p.Val128Phefs*28). This mutation was originally identified by Benetti et al. [32] in an Italian girl with SRNS. Either c.535-1G>A or c.738+2T>C were described by Wang et al. [28] in compound heterozygosis with another known *NPHS2* mutation in two nonrelated SRNS Chinese children. They evaluated the conservation of variant sites using PhyloP Primates tool that resulted in scores of 4.481 and 3.839 for c.535-1G>A and c.738+2T>C, respectively, indicating high degree of conservation at these sites. They also evaluated those variants using Mutation Taster that classified

Table 1: *NPHS2* variants described from June 2013 to February 2017*.

Exon/intron	Nucleotide change	Amino acid change	In silico prediction consequence		Ref.	Country
			PolyPhen/score[a]	SIFT/score[b]		
			Missense			
1	c.133T>C	p.Ser46Pro	B/0.0	T/0.28	[29]	India
3	c.415T>A	p.Leu139Arg	P/0.967	D/0.0	[41]	Mexico
4	c.500T>C	p.Leu167Pro	P/1.0	D/0.0	[29]	India
4	c.523C>T	p.Pro175Ser	B/0.0	D/0.0	[29]	India
8	c.946C>T	p.Pro316Ser	P/0.976	D/0.02	[30]	India
Exon/intron	Nucleotide change	Amino acid change	Splice-site prediction by neural network		Ref.	Country
			Score normal sequence	Score mutant sequence		
			Splice-site			
Intron 3	c.451+3A>T	—	0.54	0	[32]	Italy
Intron 4	c.[53]5-1G>A	—	0.99	0.54	[28]	China
Intron 5	c.738+2T>C	—	0.90	0	[28]	China
Exon/intron	Nucleotide change	Amino acid change	Consequence		Ref.	Country
			Frameshift			
5	c.714delG	p.Lis239Argfs*13	D		[43]	Brazil
8	c.988_989delCT	p.Ser329 = fs*14	D		[38]	Finland

*The variants presented in this table were not annotated in the public HGMD (http://www.hgmd.cf.ac.uk/ac/index.php) (access date May 31) or in the GnomeAD Browser (http://gnomad.broadinstitute.org) (access date May 31) or in the Leiden Open Variation Database (https://www.lovd.nl/NPHS2) (access date May 31); [a]PolyPhen: 1.000 = probably damaging (P); 0.5000 = possibly damaging (PO); 0.000 = benign (B). [b]SIFT: ≤0.05 = damaging (D); >0.05 = tolerated (T).

them as damaging. Nevertheless, they did not use the BDGP Splice Site Prediction by Neural Network in silico tool to predict the splicing recognition sites; therefore, we performed this analysis and the resulting prediction is shown in Table 1.

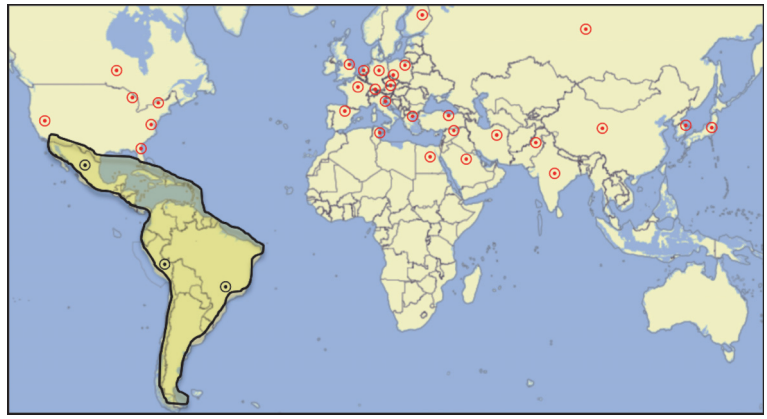
5. Frameshift Mutations

Two small deletions were described: one (p.Ser329 = fs*14) was found in heterozygosis in five SRNS individuals from the same Finnish family, with early-onset, slow progression, and dominant inheritance pattern [38]; the other (p.Lis239Argfs*13) was identified by our group in two Brazilian sisters with early-onset SRNS in association with the p.Val260Glu missense [43].

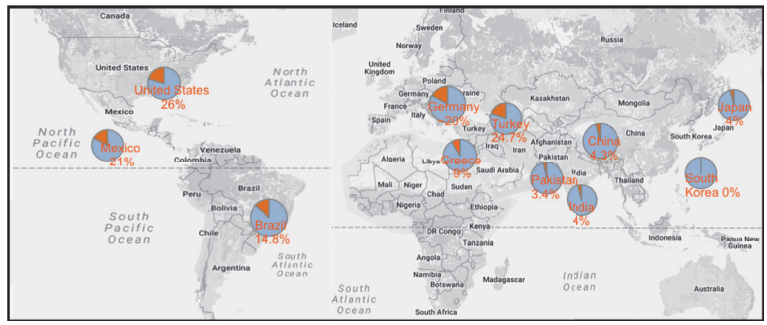
6. *NPHS2* Mutations in the World with a Closer Look to Latin America

Population studies from different countries, mainly from Europe, South Asia, and North America, revealed that the prevalence of *NPHS2* mutations in children with SRNS may vary according to ethnicity. It appears to be frequent among Americans and Turkish [21, 44] (26% and 24.7%, resp.) but not as frequent among Greek [45], Chinese [28, 46], Indian [47], Japanese [40, 48], Pakistani [49], and Korean [50] patients (9%, 4.3%, 4%, 4%, 3.4%, and 0%, resp.). Recently a large multicentric study was performed with samples from 1783 SRNS families from eight contributing centers in which twenty-seven SRNS associated genes were sequenced. Disease-causing mutations were identified in different genes; however, mutations in *NPHS2* were more frequent [5, 13]. Some of them, with a high frequency in particular geographical regions, are considered as founder alleles for *NPHS2*: p.Arg138Gln and p.Gly140Aspfs*41 are predominant in Europe; p.Pro118Leu in Turkey; p.Val180Met in North Africa; p.Arg138* in Israel and Arabian countries; p.Val260Glu in Oman, Arabia; and p.Met1? and Asn199Lysfs*14 in Egypt [5, 15].

The contribution of Latin American countries to genetic studies in SRNS is scarce. Searches for *NPHS2* mutations had been performed mainly in three countries: Mexico, Chile, and Brazil (Figure 1). In Mexico, only the 3rd exon of *NPHS2* was sequenced in eight SRNS and five SSNS children [41]. The heterozygous p.Leu139Arg variant was identified in two patients, one SRNS and one SSNS; therefore, it was considered as a variant of unknown significance. In Chile, Azocar et al. [42] performed a molecular study in SRNS children and found *NPHS2* mutations in 21%. The mutations identified were homozygosis for p.Pro341Ser in one patient and compound heterozygosis for p.Arg229Gln and p.Ala284Val in six patients [42, 51]. In Brazil, our group performed the molecular analysis of *NPHS2* in 27 SRNS children and identified disease-causing mutations in 14.8%. We identified the following associations: the [p.Ala284Val];[p.Arg229Gln] and [p.Ala284Val(;)p.Arg229Gln] in two sporadic unrelated patients with late-onset SRNS; the [p.Glu310Lys];[p.Arg229Gln] association in one sporadic patient with early-onset SRNS, and the [p.Lis239Argfs*13(;)p.Val260Glu] in a familial case also with early-onset SRNS [43]. Although performed in small samples, those studies suggest that the [p.Ala284Val];[p.Arg229Gln] association is frequent in South American countries. Actually, 13 out of 14 South American families evaluated by Machuca



(a)



(b)

Figure 1: (a) Map of the main countries where *NPHS2* mutations have been studied so far in SRNS cohorts around the world (red circle marks). Latin America is highlighted in darker yellow, with the three main centers (Mexico, Chile, and Brazil), where *NPHS2* mutations had been published represented by black circle marks. (b) Pye charts representing the percentage of *NPHS2* mutations (in orange) found in some countries from South Asia, East Asia, Europe, North America, and South America.

et al. [52] also carried the [p.Ala284Val];[p.Arg229Gln] association, with one-half presenting the adult onset form of the disease. The p.Val260Glu variant is worth mentioning in this group, which is already considered as a founder allele in Oman, Arabia [5], and also identified in one of our familial cases. We are not aware of an Arabian ancestrality of this family, but given the highly miscegenated nature of the Brazilian population, we cannot exclude this possibility.

This review aimed to give a new perspective to Nephrotic Syndrome in Latin American countries, emphasizing the importance of implementing the molecular evaluation of NS, especially investigating mutations on those genes more frequently associated with SRNS in this region. The molecular characterization of SRNS in childhood and adolescence is relevant to guide further treatment, since patients bearing *NPHS2* mutations may be spared of the undesired side effects of corticosteroids. Additionally, living donor transplantation might be considered since SRNS patients with homozygous or compound heterozygous mutations in *NPHS2* have reduced risks for recurrence of FSGS after renal transplant compared with children without mutations.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Research Article

Pulsed Vincristine Therapy in Steroid-Resistant Nephrotic Syndrome

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Steroid-resistant nephrotic syndrome (SRNS) poses a therapeutic challenge for the paediatric nephrologist. As relentless progression to renal failure occurs with continued proteinuria, such patients will be treated with different cytotoxic medications with variable success rates and side-effects. We present here our findings on administering the anticancer drug vincristine for SRNS patients at a single centre in Sri Lanka. *Methods*. Between 2002 and 2007, fifty-four children presenting with steroid and cyclophosphamide resistance were treated with vincristine at 1.5 mg/m^2 in weekly intravenous pulses for 8 weeks along with a tapering steroid regimen of 6 months. All patients were closely followed up for 5 years. *Results*. Of the 54 patients 39 were males and 15 were females (age range 3.5–11.6 years, median 6.1 years). At the end of the treatment course, 21 patients achieved complete remission while 7 had partial remission and no response was seen in 26 patients. Sustained remission at 6, 12, 24, and 60 months were 15 (27.78%), 11 (20.37%), 9 (16.67%), and 7 (12.96%), respectively. Most side-effects observed were reversible and no serious side-effects were noted during vincristine therapy. *Conclusion*. Although its therapeutic mechanisms in nephrotic syndrome are still not elucidated, vincristine appears to be a potent alternative that could be considered for treating SRNS.

1. Introduction

Nephrotic syndrome (NS) is the commonest paediatric glomerular disorder with an annual incidence of 2–7 per 100,000 [1]. While 80–90% children with NS achieve remission with initial corticosteroid therapy, the remaining 10–20% do not respond, thus being classified as steroid-resistant nephrotic syndrome (SRNS). A patient is considered to have steroid resistance if there is lack of remission despite treatment with prednisolone at a dose of 2 mg/kg/day (60 mg/m^2/day) for 4 weeks [2]. Due to the complications of unremitting proteinuria and progressive renal disease and the side-effects of treatment with immunosuppressive medication, the management of SRNS is difficult and challenging. Failure to induce remission carries a significant risk of progression to end-stage renal disease (ESRD) within 15 years in about 50% [3].

A renal biopsy is usually undertaken in all children with SRNS before starting specific treatment. Though the renal histology of most patients with steroid sensitive nephrotic syndrome (90%) reveals minimal change nephropathy (MCN), the renal histology in SRNS is different, with up to 30–40% of patients showing focal segmental glomerulosclerosis (FSGS) [4]. The histologies in the remaining patients with SRNS include minimal change disease (30–40), mesangial proliferation, membranoproliferative glomerulonephritis, membranous nephropathy, and IgA nephropathy [2].

Children with SRNS have been treated with immunosuppressive agents such as cyclophosphamide (CYC), chlorambucil, and cyclosporine A (CYA) and more lately with mycophenolate mofetil. In those who do not respond or respond only partially, nonimmunosuppressive agents such as ACE inhibitors and angiotensin receptor blockers are employed to reduce the proteinuria.

Cyclophosphamide, although widely used in the past to treat SRNS, is now thought to have little therapeutic efficacy in the treatment of this condition. Its efficacy seems to be more in those with minimal change disease and late steroid resistance and those with a partial response to steroids. It also possesses a sinister adverse effect profile including leucopenia, haemorrhagic cystitis, reversible alopecia, gonadal toxicity, and oncological risk [5, 6]. At the time of the current study CYC was the primarily used agent for the treatment of SRNS.

CYA, a calcineurin inhibitor, has largely replaced CYC as the agent of choice for the treatment of SRNS, achieving significant complete remission rates [7]. It is effective in both minimal change disease and FSGS. This agent was however not freely available in Sri Lankan hospitals at the time of this study. Therefore other means of therapy had to be sought when patients presented with resistance to both corticosteroids and CYC. In this study we assess the efficacy of vincristine sulphate, a vinca alkaloid used in cancer therapy, in inducing and sustaining remission in SRNS.

2. Patients and Methods

This single-centre study was conducted at the Paediatric Nephrology Unit, Teaching Hospital Peradeniya, Sri Lanka. Children who failed to enter remission with prednisolone prescribed at a dose of 2 mg/kg/day (60 mg/m^2/day) for 4 weeks were referred for further management. In all patients the same steroid dose was continued for additional 2 weeks during which a renal biopsy was performed. Patients who had a renal histology of either minimal change disease, idiopathic mesangial proliferation, or focal and segmental glomerulosclerosis were treated with oral cyclophosphamide (CYC) prescribed at a dose of 3 mg/Kg/day for 8 weeks along with 60 mg/m^2 of alternate day steroids. The steroids were tapered over a period of 6 months. If remission was not achieved by 6 weeks of CYC therapy then CYC therapy was discontinued. These patients received vincristine at 1.5 mg/m^2 in weekly intravenous pulses for 8 weeks along with a tapering course of steroids. The tapering steroid course consisted of 60 mg/m^2 every other day for 2 weeks and then was tapered by 10 mg/m^2 every 2 weeks over a period of 12 weeks. During therapy patients were reviewed on a weekly basis with full blood counts, urine protein excretion, serum protein and cholesterol levels, renal and liver function tests, and a full clinical examination focusing on the potential side-effects of vincristine. All possible side-effects were documented. Once they completed vincristine therapy, these patients were reviewed on a monthly basis.

We analysed the number of patients treated with vincristine from 2002 to 2007 who had complete, partial, or no remission along with the duration of sustained remission. We also analysed the adverse event profile during therapy. The collected data was entered in SPSS software version 16 and analysed using descriptive statistics and Mann–Whitney U test.

Table 1: Baseline characteristics at the beginning of the study.

Characteristics	Value
Number of patients	54
Median age (years)	6.1
Gender:	
Male	39 (72.2%)
Female	15 (27.8%)
Biopsy histology: FSGS	32 (59.3%)
MCN and mesangial proliferation	22 (40.7%)

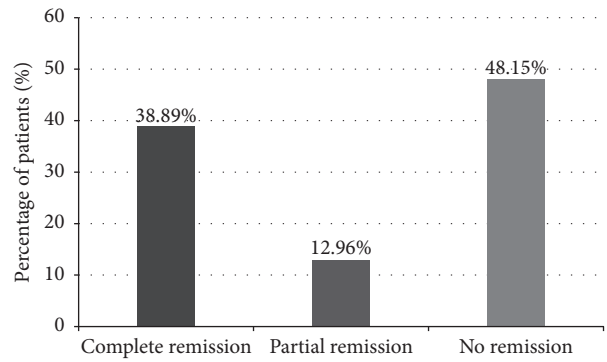

Figure 1: Patients achieving complete, partial, or no remission after vincristine therapy.

3. Results

The outcome of fifty-four children who received vincristine during this period was analysed. The ages ranged from 3.5 years to 11.6 years with a median of 6.1 years. Thirty-nine were males (72.2%) and 15 were females (27.8%). The baseline characteristics at the beginning of the study are shown in Table 1.

At the end of the course of vincristine, 21 patients out of the 54 achieved complete remission. Seven achieved partial remission and remission was not achieved in 26 patients (Figure 1).

Out of the patients who achieved complete remission at the end of vincristine therapy, 6 (11.11%) patients relapsed during the first 6 months. Sustained remission at 6 months was seen in 15 (27.78%) patients. Eleven (20.37%), 9 (16.67%), and 7 (12.96%) patients had sustained remission at 12, 24, and 60 months, respectively (Figure 2). The number of patients having MCNS with mesangial proliferation (32/54) who achieved remission was significantly higher than that with FSGS (22/54) ($p = 0.009$).

The most frequently observed side-effects were abdominal distension and cramps, constipation, and change in the sense of taste. The occurrence of hair loss could be partly due to previous CYC therapy. None of these side-effects led to the discontinuation of treatment. The side-effects are indicated in Table 2.

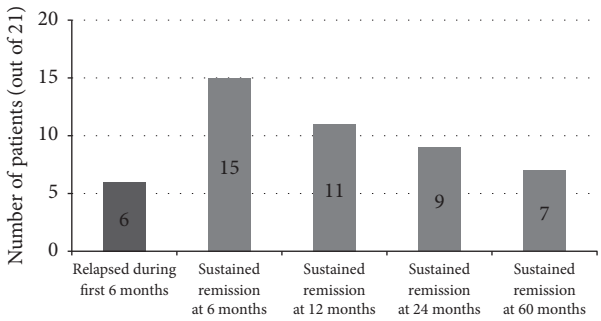

Figure 2: Patients who relapsed and remained in sustained remission for the first 60 months.

Table 2: Occurrence of side-effects.

Side-effect	Number of patients
Vomiting	7
Weight loss	4
Diarrhoea	6
Bloating, abdominal pain, or cramps	21
Mouth ulcers	3
Headache	4
Hair loss	38
Constipation	13
Loss of appetite	11
Changes in sense of taste	17
Numbness and tingling in the hands and feet	8
Reversible bilateral ptosis	3

4. Discussion

In a review of randomized controlled trials on treatment strategies in SRNS, Hodson et al. conclude that calcineurin inhibitors such as cyclosporine increase the likelihood of complete or partial remission compared with placebo/no treatment or CYC [8]. Even though we used CYC as the first-line treatment for steroid resistance, treatment options for CYC resistance were limited due to the unavailability of CYA used in developed countries for such cases. This encouraged us to use vincristine as an alternative after CYC in the present series of patients.

Vincristine is a vinca alkaloid that has played an important role as a chemotherapeutic drug for malignant diseases. It exerts antitumor activity by preventing spindle microtubule formation to disable the aligning and moving of chromosomes. Composed of two multirings, vindoline and catherantine, it interacts with β-tubulin at a region adjacent to the GTP-binding site known as vinca domain [9, 10]. Vincristine also is a potent inhibitor of Topoisomerase II [11].

The few previous studies done regarding vincristine and its effect on NS point towards the importance of vincristine use especially when the patient becomes resistant to steroids and second-line immunosuppressants. In 1994 Almeida et al. administered 1.5 mg/m^2 of intravenous vincristine weekly for 8 weeks with simultaneous daily prednisolone for 4 weeks to children who were steroid resistant. With only 2 children out of 7 achieving complete remission, they concluded that their results do not encourage the use of vincristine [12]. However, Goonasekera et al. in 1998 highlighted the importance of reevaluating vincristine therapy as a potent alternative drug in patients with FSGS, based on their success with two children suffering from primary steroid- and cyclophosphamide-resistant FSGS who achieved complete remission with vincristine therapy [13]. A recent study by Kausman et al. where SDNS patients were treated with a longer regimen of vincristine reported significant reduction of relapse frequency and minimal side-effects. In addition, vincristine was also successful during subsequent relapses [14]. To add to these numbers, two children with SRNS and one child with SDNS out of 17 children achieved complete remission as reported by Krishnan et al. in 2006 [15].

The results of this study are more encouraging than the previously published literature in terms of the number of patients who achieved complete and partial remission. Most of the side-effects observed were transient and did not warrant discontinuation of the treatment. However, due to the use of steroids and CYC course prior to the vincristine therapy it is unclear if the achieved remission can be solely attributed to the action of vincristine. In spite of this, being inexpensive and reliable in patients who are noncompliant with oral medication and having fewer reversible side-effects can be stated as advantages of vincristine use [15].

How vincristine exerts its effects in nephrotic patients is still not elucidated. In an attempt to understand its action in adriamycin- (ADR-) induced nephropathy Yin et al. reported that vincristine can stabilize the actin cytoskeleton in the ADR-injured podocyte at a low dosage that does not disrupt microtubules. Their data also suggested that vincristine exerted this by suppressing overexpression of $\alpha 3\beta 1$ integrin and focal adhesion kinase (FAK) [16]. This is important since inhibition of FAK activation or their deletion in podocytes has been found to protect against proteinuria and foot process effacement induced by glomerular injury [17]. Although this information provides an insight to vincristine's influence on nephropathy, further clarification of its therapeutic mechanism will definitely shed more light in this area. Thus it would be helpful to attract more attention to use it as a cheap and effective alternative treatment for NS.

5. Conclusions

In conclusion, it appears that vincristine is a potent and safe alternative treatment and should be considered in the treatment of SRNS. However a randomized controlled trial is required to ascertain whether it should be used in combination or as a single agent when treating SRNS.

Disclosure

The results of the initial 14 patients have been presented as an abstract at the International Pediatric Nephrology Association (IPNA) held in Adelaide, Australia, in 2004.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Review Article

Rituximab for Treatment of Membranoproliferative Glomerulonephritis and C3 Glomerulopathies

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Membranoproliferative glomerulonephritis (MPGN) is a histological pattern of injury resulting from predominantly subendothelial and mesangial deposition of immunoglobulins or complement factors with subsequent inflammation and proliferation particularly of the glomerular basement membrane. Recent classification of MPGN is based on pathogenesis dividing MPGN into immunoglobulin-associated MPGN and complement-mediated C3 glomerulonephritis (C3GN) and dense deposit disease (DDD). Current guidelines suggest treatment with steroids, cytotoxic agents with or without plasmapheresis only for subjects with progressive disease, that is, nephrotic range proteinuria and decline of renal function. Rituximab, a chimeric B-cell depleting anti-CD20 antibody, has emerged in the last decade as a treatment option for patients with primary glomerular diseases such as minimal change disease, focal-segmental glomerulosclerosis, or idiopathic membranous nephropathy. However, data on the use of rituximab in MPGN, C3GN, and DDD are limited to case reports and retrospective case series. Patients with immunoglobulin-associated and idiopathic MPGN who were treated with rituximab showed partial and complete responses in the majorities of cases. However, rituximab was not effective in few cases of C3GN and DDD. Despite promising results in immunoglobulin-associated and idiopathic MPGN, current evidence on this treatment remains weak, and controlled and prospective data are urgently needed.

1. A Brief Introduction on Membranoproliferative Glomerulonephritis

From a traditional perspective membranoproliferative glomerulonephritis (MPGN) has been defined by a morphological pattern of glomerular injury, in which electron-dense immunoglobulins and/or complement components are deposited between endothelial cells and the basement membrane [1]. Increased matrix deposition and hypercellularity lead to typical changes seen by light microscopy: thickening of the capillary wall often appears as a double contour ("tram track," "membrano-") and mesangial cells interpose in the newly formed second layer of basement membrane ("proliferative"). Based on these morphological features MPGN has historically been classified into three types: in the most frequent MPGN type 1 light microscopy typically shows double contours of the capillary walls and mesangial proliferation, and electron microscopy reveals subendothelial electron-dense deposits [2]. These deposits can be positive for either immunoglobulins or complement factor C3, or for both. On electron microscopy MPGN type 2 reveals a distinctive feature of extremely electron-dense material deposited throughout the whole basement membrane, which on immunohistology stains positive for C3 but usually not or only sparsely for immunoglobulins [3]. A typical feature of MPGN type 3 is the presence of subepithelial immune deposits in addition to subendothelial and mesangial immune deposits, which can be positive for immunoglobulins or C3 like in type 1 MPGN [4].

In the last decade novel insights in pathogenic mechanisms have changed our understanding and the classification of MPGN [5]. It has been increasingly recognized that in some cases the deposition of immunoglobulins in the first place is followed by a secondary complement activation (i.e., "immunoglobulin-associated MPGN"). Immunoglobulin-associated or immune-complex mediated

MPGN is often secondary to infections (viral such as hepatitis B or C; bacterial such as endocarditis, atrioventricular shunts, visceral abscesses, mycoplasma, or protozoal such as malaria or schistosomiasis), can be caused by cryoglobulinemia (with or without hepatitis B or C), or represents a poststreptococcal glomerulonephritis. Other causes are autoimmune diseases including systemic lupus erythematosus, or malignancies such as lymphoproliferative disorders including monoclonal IgG gammopathies. If no obvious cause can be identified then the case is termed idiopathic MPGN, although some authors argue that such rare cases might represent a C3 glomerulonephritis with some immunoglobulin deposits, and therefore an underlying pathology of the complement system has to be excluded.

In other cases a primary pathology of complement control results in the deposition of C3 without a significant deposition of immunoglobulins (i.e., C3 glomerulonephritis-C3GN, or dense deposit disease, DDD). The difference between DDD and C3GN is represented by the fact that DDD is characterized by extremely electron-dense deposits in the glomerular basement membrane, while the glomerular changes of C3GN are more heterogenous. Although the most frequent histologic pattern identified by light microscopy is MPGN other glomerular changes such as mesangial proliferative glomerulonephritis and endocapillary proliferative GN with or without crescents have also been described in patients with DDD [6].

However, both pathologies are a consequence of abnormal glomerular accumulation of C3 due to acquired or genetic disorders of complement regulation [7]. Hyperactivation of the alternative pathway of complement as seen in C3GN and DDD can be associated with the presence of C3 nephritic factors (C3Nefs), which stabilize C3 convertase or its components against complement factor H- (CFH-) mediated decay, thus leading to prolonged and dysregulated activation of the complement system. Although C3Nefs are found in 40–80% of patients with C3GN/DDD their correlation with disease course and outcome has been questioned [8]. Antibodies against other components of the complement system have also been identified, such as anti-CFH, anti-complement factor B (CFB), anti-C4, or anti-C3b [9].

Multiple genetic causes have been identified for C3GN and DDD. These include loss of function mutations of CFH and CFB, or gain of function mutations of C3. Furthermore, mutations of the CFHR5 gene or copy number variations of the CFHR gene cluster have been reported [10].

2. Clinicopathological Features and Treatment of MPGN

Clinical presentation of patients with MPGN may be highly variable and similar to that in other types of glomerulonephritis. Patients can present with microscopic hematuria with or without mild proteinuria to nephrotic range proteinuria or even full blown nephrotic syndrome with or without hypertension and renal function decline. Renal prognosis is usually determined by the degree of proteinuria and reduction of eGFR at time of presentation and during follow-up, like in most proteinuric renal diseases [11]. Another important adverse prognostic factor is the degree of tubulointerstitial fibrosis on renal biopsy rather than the disease type or severity of glomerular changes [12].

There are several issues which have to be taken into account when treating patients with MPGN, C3GN, or DDD. In immunoglobulin-associated MPGN at least partial resolution of MPGN occurs when the primary cause is successfully treated, for example, antiviral therapy in hepatitis B or C [13], antimicrobial therapy in infectious diseases [14], or chemotherapy in chronic lymphocytic leukemia [15] and multiple myeloma [16]. In the case of MPGN secondary to a monoclonal gammopathy the term MGRS (monoclonal gammopathy of renal significance) should be used [17]. Although the optimal treatment in these cases is uncertain, some authors suggest treating this condition like multiple myeloma [18].

In patients with a HCV-related renal disease (presumably MPGN) and cryoglobulinemia with nephrotic proteinuria and evidence of progressive kidney disease, treatment with plasmapheresis, rituximab, or cyclophosphamide in conjunction with steroids and antiviral therapy may be most useful, but data are limited and treatment approaches have to be individualized [19].

There are no randomized controlled trials upon which treatment decisions for idiopathic MPGN can be based, once secondary causes have been excluded. Patients with nonnephrotic proteinuria and stable renal function may be treated with supportive measures such as renin-angiotensin-aldosterone (RAS) blockade and consequent blood pressure control alone, since these patients have a favourable long term renal outcome [20]. According to recent KDIGO glomerulonephritis guidelines immunosuppressive treatment should only be started in those patients with nephrotic range proteinuria and renal function decline, and also in this setting quality of evidence is poor [21]. Furthermore, in most and particularly in the early reports patients were classified by light and electron microscopy into MPGN type 1 and type 3, while patients with type 2 were often discussed separately. Since classification has changed in recent years, the results of these case reports and case series have to be interpreted with caution.

The efficacy of glucocorticoids has been tested in 80 children with MPGN, nephrotic range proteinuria, and preserved renal function. Although therapy with 40 mg/m^2 prednisone up to 41 months showed a lower rate of treatment failure and a borderline significant stabilization of renal function at 10 years of follow-up, steroid therapy was associated with substantial toxicity [22]. Data on the efficacy of cyclophosphamide are conflicting. Cattran et al. did not show any difference in patient survival, renal function decline, and proteinuria at 2 years as compared to a control group [23]. On the contrary the rate of complete remission after 10 months was 79% in an (uncontrolled) observational study of 19 patients by Faedda et al. [24]. Only limited data from small case series are available for mycophenolate mofetil [25], cyclosporine A [26], and tacrolimus [27] for treatment of immunoglobulin-associated MPGN. Both C3GN and DDD are extremely rare diseases and data on treatment are even more limited than for immunoglobulin-associated or

Table 1: *Studies of rituximab treatment in idiopathic MPGN*. RTX: rituximab, CreaCL: 24 h creatinine clearance, NA: not applicable or not reported, and CR and PR: complete and partial remission (as defined by the authors).

Authors	Study design	*n* (MPGN)	RTX protocol	Renal function	Proteinuria	Outcome
Sugiura et al. [37]	Prospective single-arm ($n = 24$)	1 (idiopathic)	1×375 mg/m^2	Creatinine 0.51–1.95 mg/dl (whole cohort)	9.8 g/day	Proteinuria decreased from 9.8 → 1.8 g/day
Dillon et al. [36]	Prospective uncontrolled open-label ($n = 6$)	6 (4 idiopathic, 2 with cryoglobulinemia)	1000 mg on day 1 and on day 15	CreaCl 48 ± 13 ml/min/1.73 m^2	3.9 ± 2.0 g/day	Proteinuria 2.1 ± 2.3 g/day CR in patients with cryoglobulinemia Stable renal function
Kong et al. [38]	Retrospective case review	2 (idiopathic)	$1 \times$ and $2 \times$ 375 mg/m^2	NA	NA	CR and PR

idiopathic MPGN. On the level of case reports therapies including plasma infusion, plasmapheresis, glucocorticoids, cyclophosphamide, mycophenolate mofetil, eculizumab, or calcineurin inhibitors have been reported with varying degrees of efficacy [28]. In summary, the overall benefit of standard immunosuppressive therapy in the setting of immunoglobulin-associated MPGN, C3GN, and DDD might be very limited and quality of evidence is very weak. Therefore, the recent KDIGO glomerulonephritis guidelines state that progressive renal function decline remains the only indication for (intensive) immunosuppressive treatment [21].

3. Rituximab in Immunoglobulin-Associated MPGN

Rituximab is a chimeric mouse/human monoclonal antibody targeting the CD20 surface antigen on B-lymphocytes, selectively depleting these cells. It has to be noted that CD20 is expressed on most types of B-lymphocytes (e.g., pre-B-cells, immature B-cells, naïve B-cells, germinal-center B-cells, and memory B-cells) but not on pro-B-cells, plasmablasts, and plasmacells [29]. Furthermore, rituximab seems to have a direct protective effect on podocytes. It has been shown that rituximab regulates the sphingomyelin phosphodiesterase acid-like 3b protein and acid sphingomyelinase activity, thus stabilizing the actin cytoskeleton and preventing apoptosis of podocytes [30]. Since in immunoglobulin-associated MPGN the deposition of immunoglobulins is probably the primary event and complement activation as well as unspecific glomerular and tubular changes are a consequence thereof, depletion of B-cell autoantibody production by rituximab represents a reasonable approach.

In fact, treatment with rituximab has been effective in MPGN caused by chronic lymphocytic leukemia, but it is unclear if the beneficial effect was due to its immunosuppressive characteristics (i.e., antibody depletion) or primarily due to an effect on leukemic cells [15, 31, 32]. Also in MPGN associated with mixed cryoglobulinemia with or without HCV infection rituximab has been shown to be effective in conjunction with glucocorticoids, although severe adverse effects such as fatal infections have been reported [33–35].

Since truly idiopathic MPGN can only be established after exclusion of secondary causes such as mentioned above, its prevalence is decreasing. Nevertheless, some reports have been published on the efficacy and safety of rituximab in this setting (Table 1). In an open-label prospective trial in 6 patients with type 1 MPGN (4 idiopathic and 2 with cryoglobulinemia) who did not receive any immunosuppressants prior to the study 1000 mg rituximab was administered on day 1 and day 15 and outcome was change in proteinuria [36]. These patients had slightly reduced renal function with a creatinine clearance of 48 ± 13 ml/min/1.73 m^2. B-cells were effectively depleted and 24 h proteinuria was significantly reduced from 3.9 ± 2.0 g to 2.1 ± 2.3 g after 12 months. Interestingly those 2 patients with cryoglobulinemia showed the best response with complete remission after 12 months. Renal function remained stable in all 6 patients and no adverse events were noted.

In two reports on treatment of subjects with primary glomerular diseases with rituximab MPGN patients were included. In a retrospective report of 24 patients with various primary glomerulopathies one patient had MPGN. Treatment consisted of a single dose of rituximab of 375 mg/m^2 (maximum of 500 mg), which was associated with a complete B-cell depletion. Urinary protein excretion decreased from 9.8 g/day to 1.8 g/day after 6 months [37]. In another single-center retrospective case review of 24 patients with primary glomerulonephritides 2 patients with MPGN were included. One patient achieved a complete and the other a partial remission after one or two doses of rituximab (375 mg/m^2). Interestingly one of these patients presented with a crescentic rapid-progressive glomerulonephritis (RPGN) and progressed to dialysis after treatment with rituximab. However, 5 months later he was able to discontinue dialysis and was even in complete remission after 14 months [38].

In summary, rituximab seems to be effective in immunoglobulin-associated MPGN caused by lymphoproliferative disorders, in cryoglobulinemia with or without viral infections, or in truly idiopathic MPGN. However, the level of evidence is extremely low for all of these indications and caution is warranted due to several limitations: (1) the effect of various concomitant immunosuppressive medications such

Table 2: *Reports on rituximab treatment in C3 glomerulopathy and dense-deposit disease*. RTX: rituximab, C3GN: C3 glomerulopathy, DDD: dense-deposit disease, MMF: mycophenolate mofetil, RAS: renin angiotensin system, C3Nef: C3 nephritic factor, TCC: terminal complement complex, CFH: complement factor H, CFI: complement factor I, MCP: membrane cofactor protein, and CR and PR: complete and partial remission (as defined by the authors).

Authors	Diagnosis	Follow-up	Other therapy	RTX protocol	Laboratory parameters	Outcome
Daina et al. [41]	DDD	5 months for RTX 48 months for eculizumab	Steroids Eculizumab RAS blockade	1×375 mg/m^2	C3Nef positive C3 < 10% TCC high Genetics (CFH variants V62 and H402)	No effect of RTX on renal function or proteinuria PR with eculizumab
Rousset-Rouvière et al. [42]	DDD	21 months	Steroids MMF Eculizumab RAS blockade	2×375 mg/m^2	C3 < 0.04 g/L C4 normal C3Nef positive Genetics: normal (CFH, CFI, MCP) Anti-CFH negative	Acute renal failure after RTX CR with eculizumab
Giaime et al. [40]	DDD	30 months	RAS blockade	4×700 mg weekly repeated after 18 months	Creatinine 235 μmol/L Proteinuria 6 g/day C3 0.12 g/L (low) C4 normal C3Nef positive	Creatinine 200 μmol/L Proteinuria < 0.5 g/L C3 remained low C3Nef remained positive
Payette et al. [43]	C3GN	72 months	Steroids MMF Eculizumab	4×375 mg/m^2	Proteinuria 1–5.3 g/day C3Nef positive CFI mutation (I398L) anti-CFH positive low C3	No effect of RTX on proteinuria PR with eculizumab Normal renal function

as glucocorticoids has not been evaluated; (2) a comparison with plasmapheresis, cyclophosphamide, or any other intensive immunosuppressive protocol is lacking, particularly in cryoglobulinemia; (3) the effect of modern anti-HCV therapy on MPGN (with or without cryoglobulinemia) has not been studied so far, but a favourable effect and an improvement of the safety profile of a concomitant therapy with rituximab and other immunosuppressants seems likely; (4) the regimen of rituximab is not clear and it is also not clear if rituximab should be repeated upon B-cell repopulation.

4. Rituximab in C3GN and DDD

In contrast to immunoglobulin-associated MPGN the primary pathology of C3GN and DDD is an excessive activation of the alternative complement pathway with glomerular deposition of C3 without a significant deposition of immunoglobulins. It may be hypothesized that in the presence of autoantibodies such as C3Nefs, which lead to uncontrolled activation of the complement cascade and finally in end-organ damage, a B-cell depletion with rituximab may be effective [39]. However, there are few case reports published on the use of B-cell depleting therapy to decrease production of C3Nef and subsequently to reduce proteinuria and stabilize or even improve renal function (Table 2). In one report a 34-year-old patient with DDD was treated solely with rituximab [40]. Low C3 and normal C4 indicated an activation of the alternative complement pathway, and C3Nef was found to be positive. CFH and complement factor I (CFI) were normal, and anti-CFH was negative. Serum creatinine was 235 μmol/L. After initial treatment with ACE-inhibitors and good blood pressure control, the patient remained nephrotic and received 4 weekly doses of 700 mg rituximab as the only immunosuppressive therapy. Within 6 months a complete remission of the nephrotic syndrome was achieved, which lasted until 18 months. At this timepoint B-cell counts began to rise and the initial course of rituximab was repeated. After 30 months of follow-up the patient had a stable renal function and proteinuria remained < 0.5 g/day. Remarkably, C3Nef remained positive throughout the study, and C3 levels were always low, which questions the pathogenetic role of C3Nef/the complement system, at least in this patient.

In an 11-year-old girl with DDD, nephrotic syndrome, normal renal function, C3Nef positivity, and low C3 a single dose of 375 mg/m^2 rituximab was administered which resulted in complete B-cell depletion [41]. After 5 months proteinuria did not resolve and serum creatinine increased. Genetic analysis revealed factor H variants which have previously been associated with DDD, and the terminal complement complex showed a high level of activity, further supporting the hypothesis of activation of the alternative complement pathway. Finally, a therapy with eculizumab over

48 weeks resulted in stabilization of renal function and partial remission of proteinuria.

In a similar report an 8-year-old boy was diagnosed with DDD with signs of activation of the alternative complement pathway (low C3, normal C4, genetics of CFH, CFI and MCP normal, and anti-CFH negative) and positivity of C3Nef [42]. Initial treatment with RAS-blockade, steroids and MMF resulted in complete remission, and after 11 months steroids were withdrawn. Four months later a relapse of the nephrotic syndrome occurred which did not respond to steroid reinitiation, and 2 doses of rituximab (375 mg/m^2) were given. However, this therapy resulted in acute renal failure which required dialysis treatment. Renal biopsy revealed active DDD with extracapillary crescents which underlined the ineffectiveness of rituximab in this patient. Finally, treatment with eculizumab resulted in a quick and complete response.

A very similar case was reported by Payette et al. [43]: a 5-year-old boy with a C3GN was treated unsuccessfully with steroids, MMF, and also rituximab but responded quickly to a therapy with eculizumab with a decrease of proteinuria from 5.3 g/day to 1.7 g/day. In addition to a positive C3Nef and low C3, also anti-CFH was found to be positive. Interestingly, anti-CFH was reduced by rituximab without any effect on proteinuria.

In summary, the available evidence from single case reports does not support rituximab as an effective treatment for patients with C3GN or DDD, and the role of C3Nefs as a potential target for B-cell depleting therapy remains to be further elucidated. It has to emphasized that the activity of C3Nefs varies during the course of disease without any association to clinical presentation or treatment. Further, C3Nefs are heterogenous and detection might be challenging, and finally C3Nefs have also been found in other renal diseases [44]. In the majority of cases the underlying pathology of C3GN and DDD is an excessive activation of the alternative complement pathway. Therefore, it is reasonable that treatment with eculizumab, a humanized monoclonal antibody that binds to C5 and inhibits activation of the terminal complement complex, may provide a more targeted therapy than rituximab for patients with these diseases. However, complement inhibition in C3GN and DDD does not translate into clinical improvement in all patients, but again quality of evidence is limited. Bomback et al. reported on 6 adult patients with either DDD or C3GN (including 2 with recurrence of the disease after kidney transplantation) treated with eculizumab for 12 months [45]. Two patients showed improvement in serum creatinine, one patient had a remission of nephrotic range proteinuria, and one patient showed less endocapillary proliferation on a repeat kidney biopsy. However, two patients had a decline in renal function during treatment with eculizumab. Functional assays of the complement pathway may present a predictor of response to treatment. On the other hand Oosterveld et al. reported on the efficacy of eculizumab in 5 pediatric patients with DDD with either nephrotic syndrome or severe acute kidney injury who showed an activation of the alternative complement pathway. Treatment with eculizumab in these 5.9- to 13-year-old patients resulted in reduction of proteinuria and improvement of renal function in all 5 patients [46]. The variability of response to eculizumab in patients with DDD and C3GN suggests that pathophysiology of these diseases is more complex than pathophysiology of other complement-mediated diseases such as atypical hemolytic uremic syndrome. Age at presentation, duration of disease, and genetics of complement components are all likely to be important predictors of response to therapy with eculizumab, and these patients may be identifiable by a well-defined clinical, functional, and genetic characterization.

5. Rituximab for Recurrent MPGN after Kidney Transplantation

The recurrence rate of MPGN in complement-mediated disease or due to monoclonal gammopathy is higher than MPGN which is secondary to infection or autoimmune disease, and overall recurrence rates vary between 19 and 48 percent [47, 48]. A diagnosis of recurrent MPGN is strongly suspected in patients with a history of MPGN in their native kidneys who present after transplantation with new-onset proteinuria, hematuria, or renal failure. A kidney biopsy is the gold standard to establish the diagnosis of recurrent MPGN, although transplant glomerulopathy may be difficult to distinguish from MPGN. Electron microscopy helps to differentiate between those two pathologies, as does work-up for donor specific antibodies and C4d-positive staining of peritubular capillaries. Further, secondary causes including complement-mediated disease should be excluded as in MPGN of the native kidneys.

There is no evidence for an effective treatment of recurrent MPGN, although it appears reasonable to treat an underlying cause of MPGN. In a very recent paper Schrezenmeier et al. reported on the successful treatment of acute renal graft failure due to a recurrence of hepatitis C virus-associated MPGN with direct-acting antiviral agents (DAAs) daclatasvir and simeprevir [49]. Progressive disease, that is, presenting with nephrotic range proteinuria and worsening of renal function, can be treated with high doses of steroids, cyclophosphamide, and plasmapheresis, although outcome is uncertain [50, 51]. Few case reports suggest efficacy of rituximab treatment with or without plasmapheresis, but the level of evidence is very weak [52–54]. Rituximab is not an effective treatment of recurrent DDD after renal transplantation [55].

6. Summary and Conclusion

MPGN is a rare disease and current classification based on pathogenesis (i.e., histology, immunofluorescence, and analysis of the complement components) divides this disorder into immunoglobulin-associated MPGN (with or without secondary causes) and into complement-mediated disease which is termed C3GN and DDD. The optimal therapy for all varieties of MPGN is not known, and current KDIGO guidelines recommend treatment with steroids, cyclophosphamide with or without plasmapheresis only for subjects with progressive disease. Rituximab seems to be effective in immunoglobulin-associated MPGN, particularly in those cases associated with monoclonal gammopathy, chronic

lymphocytic leukemia, and cryoglobulinemia with or without HCV, but severe adverse effect such as infections may limit its applicability. It remains to be shown how recently introduced DAAs will change also the course of HCV-associated renal diseases such as MPGN. On the other hand in complement-associated C3GN or DDD several case reports have shown no effect of rituximab on the course of these diseases.

In summary, the level of evidence for efficacy of any kind of treatment in a heterogenous and rare disease such as MPGN is very weak. Therefore, progress in determining the optimal therapy can only be achieved in large collaborative studies including patients with a clear diagnosis of a MPGN subtype in which treatment is tailored according to the subtypes using predefined study protocols.

Conflicts of Interest

The author declares that there are no conflicts of interest regarding the publication of this paper.

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Review Article

Recent Treatment Advances and New Trials in Adult Nephrotic Syndrome

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The etiology of nephrotic syndrome is complex and ranges from primary glomerulonephritis to secondary forms. Patients with nephrotic syndrome often need immunosuppressive treatment with its side effects and may progress to end stage renal disease. This review focuses on recent advances in the treatment of primary causes of nephrotic syndrome (idiopathic membranous nephropathy (iMN), minimal change disease (MCD), and focal segmental glomerulosclerosis (FSGS)) since the publication of the KDIGO guidelines in 2012. Current treatment recommendations are mostly based on randomized controlled trials (RCTs) in children, small RCTs, or case series in adults. Recently, only a few new RCTs have been published, such as the Gemritux trial evaluating rituximab treatment versus supportive antiproteinuric and antihypertensive therapy in iMN. Many RCTs are ongoing for iMN, MCD, and FSGS that will provide further information on the effectiveness of different treatment options for the causative disease. In addition to reviewing recent clinical studies, we provide insight into potential new targets for the treatment of nephrotic syndrome from recent basic science publications.

1. Introduction

Nephrotic syndrome is characterized by gross proteinuria, hypoalbuminemia, hyperlipidemia, and peripheral edema [1]. The etiology of nephrotic syndrome in adults is complex and ranges from primary glomerulonephritis to secondary forms [1]. This review will focus on new therapeutic advances in treating primary forms of nephrotic syndrome in adult patients. Primary forms of nephrotic syndrome in adults are comprised of three histological disease entities: idiopathic membranous nephropathy (iMN), minimal change disease (MCD), and focal segmental glomerulosclerosis (FSGS).

Primary nephrotic syndrome often affects younger patients. In this young population, primary glomerulopathies are the most frequent cause of end stage renal disease (ESRD) [2]. The incidence of primary forms of nephrotic syndromes in adults however is low (0.6–1.2 cases/100.000 adults depending on the underlying histological disease) [2]. Hence, little is known about the therapeutic strategies that can impact this adult population. Therefore, the identification of novel therapeutic strategies for nephrotic syndrome in adults is essential to prevent or delay ESRD.

The basis for therapy of primary nephrotic syndrome is mostly of supportive nature. Supportive strategies include antihypertensive and antiproteinuric therapy and dietary recommendations [3]. Patients with nephrotic syndrome are also at increased risk to develop thromboembolism. In patients with membranous nephropathy, the adjusted hazard ratio for thromboembolism was 10.8 compared to patients with IgA nephropathy [4]. In contrast, for patients with FSGS the hazard ratio was 5.9 [4]. Hence, anticoagulant therapy is recommended in patients with a primary nephrotic syndrome, especially in iMN and serum albumin < 2,5 mg/dl [1]. In 2014, Lee et al. proposed a practical approach to prophylactic anticoagulation therapy in patients with iMN [5]. The presented model takes into account the serum albumin concentration, the individual patient's bleeding risk, and the risk tolerance as reflected by the selected benefit-to risk ratio (http://www.gntools.com) [5].

We will focus on major therapeutic advances in iMN, MCD, and FSGS as causes for primary nephrotic syndrome in adults. Due to space limitations, we had to focus on selected references in this review.

2. Major Therapeutic Advances

2.1. Idiopathic Membranous Nephropathy (iMN). IMN was recently identified as an autoimmune disease against the M-type phospholipase A2 receptor (PLA2R) in 70–80% of the white population [6]. As for most autoimmune diseases, genetic polymorphisms predispose to the disease. Polymorphisms of HLA-DQA1 were associated with polymorphisms in the PLA2R in a genome wide association study (GWAS) [7]. Even more recently, thrombospondin type-1 domain-containing 7A (THSD7A) was established as a second although less common autoantigen in iMN [8]. The fact that 10–20% of patients are seronegative for PLA2R and THSD7A indicates that there are probably more yet unknown autoantigens. Spontaneous remission of iMN is observed in up to 30% of the patients within 14 months [9]. Indications for immunosuppressive therapy for iMN therefore depend on the level of proteinuria (>4 g/24 h), rate of GFR decline, and complications of the nephrotic syndrome [1]. Drop of PLA2R antibody titers usually precede reduction in proteinuria [2, 10, 11]. Recently, De Vriese et al. proposed a serology based approach to diagnosis, prognosis, and treatment monitoring of patients with iMN [12]. This review takes into account that the traditional proteinuria based approach to treatment decisions potentially lags months behind a change in immunological activity and that otherwise proteinuria may reflect an irreversible damage to the glomerular filter without active disease [12]. As changes in PLA2R antibody titers (and potentially THSD7A antibody titers) tightly correlate with disease activity, this approach may help to increase diagnostic and prognostic accuracy and reduce unnecessary immunosuppressive therapy [12]. However, the serology based approach needs to be validated in a randomized controlled trial (RCT) compared to the traditional approach before replacing it in clinical practice.

2.1.1. Guideline Recommendations for Therapy of iMN. If the criteria for immunosuppressive therapy are met, KDIGO guidelines recommend to primarily use alkylating agents (cyclophosphamide) in combination with corticosteroids for 6 months [1]. Alternatively, calcineurin inhibitors are also recommended in addition to corticosteroids for 6 months [1].

2.1.2. Clinical Advances and Randomized Controlled Trials (RCT) in iMN. IMN shows a significant amount of spontaneous remission (see above). A Dutch RCT investigated 26 patients with nephrotic syndrome who had a normal glomerular filtration rate (GFR). One group received early immunosuppression (cyclophosphamide) while the other group was followed until their serum creatinine values increased by at least 25% [13]. They reported that there was no difference in remission rates, the course of GFR loss, or complications after 72 ± 22 months. A retrospective analysis of 254 patients with iMN revealed that after ten years seven patients (3%) developed ESRD, 25 (10%) died, 52 (20%) achieved full remission, and 90 (35%) achieved partial remission if immunosuppression was given only if GFR declined or serious complications of the nephrotic syndrome occurred [14]. These studies therefore support more limited application of immunosuppressive treatment in patients with iMN and normal GFR.

In patients with iMN and reduced GFR at diagnosis, chlorambucil and prednisolone were superior in delaying GFR loss compared to treatment with cyclosporine or supportive measures alone [15]. However, patients treated with chlorambucil and prednisolone experienced a significantly higher rate of adverse events (AEs) [15]. Recent data indicates that the cancer incidence in patients treated with alkylating agents triples compared to that in the nonexposed population [16]. In addition, a systematic review and meta-analysis of 36 trials concluded that alkylating agents in combination with corticosteroids protect from GFR loss [17]. In addition, cyclosporine and mycophenolate mofetil were not superior to alkylating agents (mostly cyclophosphamide) and prednisolone [17].

In search for new therapeutic approaches to treat iMN and other autoimmune diseases, researchers have become interested in the potential of the adrenocorticotropic hormone (ACTH) because only minor side effects were reported in an earlier study [18]. A more recent prospective, open label cohort study with synthetic ACTH (Synacthen®) compared 20 patients with iMN and high risk for progression with a historical control group treated with cyclophosphamide and prednisolone [19]. This study found that synthetic ACTH is less effective in inducing remission in high-risk patients with iMN compared to cyclophosphamide (cumulative remission in ACTH 55% and cyclophosphamide plus corticosteroids 95%) [19]. In addition, AEs in the ACTH cohort were very high (95%, 25% needed hospitalization). Therefore, the authors advised against treatment with ACTH in iMN. In contrast, a dose escalation study using natural ACTH gel (HP Acthar® gel) found a significant reduction in proteinuria with an acceptable AE profile [20]. A RCT testing ACTH versus placebo in patients with iMN at high risk of progression is in progress (clinicaltrials.gov identifier: NCT01386554) and will clarify the role of ACTH in the treatment of iMN in this patient group.

Recent pathophysiological advances in understanding the role of autoimmunity in iMN have resulted in new therapeutic studies targeting B-cells in iMN. Observational studies in the past had indicated that rituximab (375 mg/m^2 weekly for 4 weeks or 1 g every 14 days ×2 or 1 g single dose) was effective as conventional treatment with reduced AEs in short observational periods [21–23]. However, a dose of 375 mg/m^2 only once or twice showed a poor outcome in inducing remission (<50%) with preserved renal function (GFR > 60 ml/min) [24]. So far only one published RCT (Gemritux) has investigated conservative therapy (nonimmunosuppressive antiproteinuric therapy) in comparison with rituximab (375 mg/m^2 at days 1 and 8 in patients with nephrotic proteinuria, normal range creatinine) [25]. In the Gemritux trial, the rituximab cohort showed a remission

rate of 35% versus 21% in the control group after 6 months (p = nonsignificant). In the follow-up period, the remission rates increased to 65% (rituximab) and control (34%) with a significant difference favouring the rituximab group while AEs were comparable. Further RCTs investigating the role of rituximab in the treatment of iMN are ongoing, for example, the MENTOR trial (persistent proteinuria > 5 g/day comparing cyclosporine monotherapy for 12 months with rituximab 1 g on days 1 and 15 repeated at 6 months (clinicaltrials.gov NCT01180036)) [26]. Furthermore, the STARMEN trial is comparing corticosteroids and cyclophosphamide for 6 months with 6 months of tacrolimus followed by a single dose of rituximab (1 g at days 1 and 15, repeated at 6 months independent of CD19 count) (clinicaltrials.gov NCT01180036) [27]. A pilot study using a combination of rituximab and cyclosporine, with the latter tapered off after 6 months while giving repeated rituximab infusion at that point, is also ongoing (clinicaltrials.gov NCT00977977).

2.1.3. Potential New Targets and Therapeutic Strategies in iMN. Unfortunately, current treatment options for iMN have a decent percent of nonresponsive patients and posttreatment relapse rates of 15–30% [1]. To reduce relapse rates, Cattran and Brenchley suggest investigating maintenance therapy with azathioprine or mycophenolate mofetil after induction therapy with alkylating agents or rituximab, comparable to ANCA-associated vasculitis [28]. An ongoing trial is examining the effectiveness of Chinese herbal medicine (Quing-ReMoShen granules) in combination with RAAS-blockers on the reduction of proteinuria and T-cell function in iMN (clinicaltrials.gov NCT01845688). Epitope mapping of the PLA2R and THSD7A autoantibodies will potentially help to develop immunoabsorption columns to deplete autoantibodies [28]. In a retrospective case series, plasma exchange (4 PE against albumin) in combination with intravenous immunoglobulins (20 g) and rituximab (375 mg/m^2) was able to induce partial remission in 10 high-risk patients (more than 3 relapses, proteinuria > 3.5 g/g creatinine, and anti-PLA2R antibody titer > 1 : 500 besides treatment with immunosuppressive therapy for more than 12 months) [29].

Furthermore, a role for T-cells in iMN has been proposed [30]. In patients treated with rituximab, regulatory T-cells (Tregs) increased up to 10-fold, an increase that persisted in responders after 12 months [31]. Therefore, modulation of T-cell function with, for example, soluble T-cell peptides to tolerize T-cells and/or enhance Tregs to downregulate the anti-PLA2R/THSD7A response is promising a new treatment strategy for iMN [28].

2.2. Minimal Change Disease. Minimal change disease (MCD) accounts for 10–15% of patients with idiopathic nephrotic syndrome in adults [32]. Pathologically, it is characterized by minimal changes on light microscopy and foot process effacement on electron microscopy [1]. With regard to the pathogenesis of the disease, it is still debated whether MCD and focal segmental glomerulosclerosis (FSGS) are separate disease types or a disease continuum [2, 32]. For both, circulating factors have been proposed to cause MCD and FSGS [33–36]. Particularly a causative role for T-cells has long been postulated for the disease progression of MCD [32, 37–39]. In addition, the role of B-cells in the pathogenesis of MCD is under debate as depletion of B-cells via rituximab has positive effects on nephrotic syndrome including MCD [38, 40, 41] but there has also been other data that suggests B-cells do not have a causative role in MCD [42, 43].

2.2.1. Guideline Recommendations for MCD Therapy. KDIGO guidelines recommend the usage of corticosteroids to induce remission in adults with MCD [1]. However, the evidence for corticosteroids comes from several large RCTs in children [44] and only small RCTs and observational studies in adults [45–48]. The recommended dosage of prednisone or prednisolone is 1 mg/kg per day (maximum 80 mg) or 2 mg/kg every other day (maximum 120 mg) for 4–16 weeks, tapered slowly over 6 months [1]. In adults, more than 50% of patients will relapse and one-third will become frequent relapsers [1]. 10–20% of the patients are steroid resistant, defined as no response to 16 weeks of oral prednisolone [32]. For patients with frequent relapses and steroid resistance, KDIGO guidelines suggest alkylating agents (oral cyclophosphamide 2–2,5 mg/kg per day for 8 weeks) [1]. In addition, if there are contraindications for alkylating agents, calcineurin inhibitors could be used [1]. As described above, only observational studies support this recommendation as of today.

2.2.2. Clinical Advances and RCTs in MCD. New RCTs on nephrotic syndrome in children have been published since the publication of the KDIGO guidelines in 2012 (for review see [32, 49]). In contrast, not many new studies, especially RCTs, have been conducted in adults since 2012. In children with steroid-sensitive and steroid resistant nephrotic syndrome rituximab was effective in recently published RCTs [50, 51]. In adults, two retrospective analyses described patients with steroid-dependent or frequently relapsing MCD despite immunosuppressive therapy treated with rituximab [52, 53]. Both case series found an increase in remission in about 60% of patients. The first prospective cohort study compared rituximab treatment in 25 patients with steroid-dependent and frequently relapsing MCD to historical controls and confirmed reduction of relapses in adults with MCD [54]. A follow-up study to this prospective cohort study showed 8 relapses in 24 months after complete remission compared to 108 episodes in 24 months before rituximab [55]. A multicenter, longitudinal, intrapatient controlled trial (NEMO study) evaluated the effects of rituximab therapy followed by immunosuppression withdrawal on disease recurrence in children (n = 10) and adults (n = 20) with frequently relapsing or steroid-dependent MCD and FSGS [56]. All patients received rituximab at one (n = 28) or two doses (375 mg/m^2). During the observation period (1 year), rituximab reduced relapses 5-fold compared to the time before rituximab treatment. Treatment with rituximab was safe in this patient cohort. No RCTs in adults have been conducted comparing rituximab treatment in either frequently relapsing or steroid-dependent patients or as a first-line therapy of MCD.

HP Acthar gel (ACTH), as described above, has been used in different underlying causes of nephrotic syndrome

including MCD [57]. In a retrospective case study series, two patients with MCD treated with HP Acthar gel showed complete remission of the nephrotic syndrome. However, no larger trials have evaluated ACTH in MCD in adults yet.

Ongoing RCTs in adult patients with MCD investigate tacrolimus with and without low dose corticosteroids compared with high dose corticosteroids (MinTac study NCT00982072 and T-OPTIMUM trial NCT01763580). In addition, therapy with MMF plus low dose corticosteroids compared to high dose prednisolone will be investigated in a third trial (NCT01185197).

2.2.3. Potential New Targets and Therapeutic Strategies in MCD. Localized upregulation and secretion of hyposialylated glycoprotein angiopoietin-like 4 (Angptl4) from podocytes is one of the key features in rodent models of MCD [58, 59]. Converting hyposialylated Angptl4 to sialylated protein using *N*-acetyl-D-mannosamine, a precursor of sialic acid that can be taken up and stored in podocytes, significantly reduces proteinuria and has the potential for use in small maintenance doses to prevent MCD relapse in rats [60].

Pioglitazone (a PPAR-γ agonist) has shown to slowly progress diabetic nephropathy and to reduce proteinuria. Hence, Agrawal et al. tested whether pioglitazone would enhance the efficacy of glucocorticoids in reducing proteinuria in puromycin aminoglycoside- (PAN-) induced nephrotic syndrome in rats [61]. PAN-induced nephrotic syndrome is a model for FSGS and MCD. In the study by Agrawal et al., glucocorticoids and pioglitazone not only reduced proteinuria in rats but also enhanced efficacy of glucocorticoids in reducing proteinuria by restoring podocyte marker expression, reducing Cox-2 expression, and phosphorylation of the glomerular glucocorticoid receptor [61]. Translation of these findings to a child with refractory nephrotic syndrome showed that pioglitazone reduced proteinuria by 80% and the overall immunosuppression to 64% [61]. However, in this study PAN nephrosis was induced at the same time as beginning the immunosuppressive regimen. Further studies need to investigate the role of pioglitazone as additional therapy for nephrotic syndrome.

Targeting the podocyte cytoskeleton, which is essential in maintaining the glomerular integrity, has gathered more attention lately [62]. Schiffer et al. investigated the role of endocytic protein dynamin regulating the oligomerization of the actin cytoskeleton of podocytes [63]. Targeting dynamin with Bis-T-23 reduced proteinuria in diverse proteinuric models including PAN nephrosis and diverse rodent models [63]. Targeting specifically the actin oligomerization is a promising target for diverse proteinuric kidney diseases.

2.3. Focal Segmental Glomerulosclerosis. Focal segmental glomerulosclerosis (FSGS) is a leading cause of ESRD in the United States and accounts for about 40% of idiopathic nephrotic syndromes in adults [64]. FSGS is diagnosed and classified from renal biopsies [65, 66]. Injury of podocytes initiates the disease process, leading to the classical focal distribution of sclerosis with a segmental pattern within the glomeruli [67]. The etiology is still unknown, but circulating permeability factors have been implicated for a long time [34–36]. As previously mentioned for MCD, patients with FSGS have high relapse rates (up to 40%) [1]. Kidney survival depends on the extent and persistence of proteinuria [1]. Patients with nonnephrotic proteinuria have a good prognosis. A significant minority will not respond to therapy; therefore the benefits and risks of the chosen immunosuppression must be weighed carefully [1].

2.3.1. Guideline Recommendations for FSGS Therapy. Similar to the therapy of MCD, idiopathic FSGS should be treated with corticosteroids according to KDIGO guidelines [1]. Data to support this recommendation is based only on observational studies [1]. The duration of high dose corticosteroid therapy may be extended to 16 weeks if remission is not achieved earlier and can be tapered slowly over an additional period of 6 months [1]. Calcineurin inhibitors can be considered as first-line therapy in patients with contraindications for corticosteroid therapy [1]. Relapsing FSGS should be treated as relapsing MCD (as described above) [1]. There is no agreed definition for steroid resistance in the literature; however KDIGO guidelines suggest that corticosteroids should be given for 16 weeks before steroid resistance is diagnosed. In two RCTs, cyclosporine was tested against no therapy for steroid resistant FSGS. Remission rates were higher in the cyclosporine group [68–70]. Uncontrolled studies have found tacrolimus to be an alternative therapy to cyclosporine in primary FSGS [71, 72].

2.3.2. Clinical Advances and RCTs in FSGS. Laurin et al. analyzed retrospectively a cohort of 458 patients diagnosed with primary FSGS [73]. The study found a significant association between treatment with immunosuppressive therapy (corticosteroids or CNIs) and better renal survival. There was no superiority between the two immunosuppressive modalities used in this study.

A small, single-center study of adults with idiopathic FSGS compared intravenous monthly cyclophosphamide plus steroids to tacrolimus plus steroids for 6 months [74]. Both groups had improved proteinuria and serum albumin with stable GFR but the results were statistically not significant. In an uncontrolled trial of 44 adults with steroid resistant FSGS treated with tacrolimus for 24 weeks, half of the patients achieved complete or partial remission [75].

Case reports and small case series have shown potential for rituximab in steroid-sensitive FSGS, but it appears largely ineffective in steroid resistant disease [76, 77].

A recent small series ($n = 15$) examined adrenocorticotropic hormone gel (HP Acthar gel) in idiopathic FSGS. Sixty percent of patients showed partial remission [57].

Several case reports have shown conflicting results on the treatment of FSGS patients with galactose, which was found to bind potential circulating permeability factors in FSGS [78–80]. Recently, the FONT II trial was published as a phase I/II open label randomized controlled trial comparing standard conservative therapy (SCT) versus SCT plus adalimumab (antibody against tumor necrosis factor-α/TNF-α) versus SCT plus galactose [81]. The primary end point was a 50% reduction of proteinuria with stable GFR. Seven out of 21 patients received SCT plus galactose and three of them

met the primary end point. No improvement was noted in the SCT plus adalimumab group. Further studies are needed to evaluate the role of galactose in the treatment of FSGS.

Abatacept, a costimulatory inhibitor of B7-1 (CD80), induced remission in five patients with idiopathic or recurrent FSGS [82]. One case report confirmed the positive effect of abatacept in a patient with FSGS recurrence in the transplanted organ [83]. However, others could not confirm the overexpression of B7-1 or the effects of abatacept in similar patients [84–87].

Ofatumumab is a fully human monoclonal antibody against CD20. It has recently been shown to be effective in rituximab-resistant nephrotic patients in case reports and small case series (mostly children) with nephrotic syndrome [32, 41, 88, 89]. In children, two RCTs are recruiting patients to test ofatumumab versus placebo in drug resistant nephrotic syndrome (clinicaltrials.gov NCT02394106) and ofatumumab versus rituximab in drug resistant nephrotic syndrome (clinicaltrials.gov NCT02394119). So far, there are no planned or published RCTs or case reports investigating ofatumumab in adults with FSGS.

As described above, HP Acthar gel is currently evaluated in an interventional study for patients with FSGS undergoing renal transplantation (clinicaltrials.gov NCT02683889). In addition, tacrolimus therapy is currently tested in a randomized study compared to therapy with cyclophosphamide in patients with FSGS (clinicaltrials.gov NCT01451489).

Fresolimumab, a human monoclonal antibody neutralizing TGF-beta, was tested in a phase 2 RCT in steroid resistant patients with primary FSGS (clinicaltrials.gov NCT01665391) [90]. Even though the primary and secondary endpoints were not achieved as the study was underpowered, the AE profile was safe [90]. Further studies need to evaluate the role for Fresolimumab in therapy of steroid resistant primary FSGS.

2.3.3. Potential New Targets and Therapeutic Strategies in FSGS. Delville et al. described the identification of an autoantibody panel in recurrent FSGS before transplantation [91]. CD40 is expressed in human cultured podocytes and its expression cannot be induced by challenging in vitro [91]. However, in patients with FSGS, CD40 was detected in glomeruli from recurrent FSGS patients [91]. Purified CD40 autoantibodies from recurrent FSGS sera disrupted the podocyte (human) actin cytoskeleton in vitro [91]. These data suggested that suPAR-β_3-integrin pathway could be involved [91]. Building on these exciting findings, the role of CD40 antibodies in human FSGS disease needs to be further validated. Anti-CD40 blocking antibodies (ASKP1240 or lucatumumab) are already commercially available and could become potential treatment options tested in clinical trials [36].

Urokinase plasminogen activator receptor (uPAR) is a cell membrane glycosylphosphatidylinositol- (GPI-) anchored protein expressed in many cell types, for example, immune cells [92–94], endothelial cells [95], tumor cells [96], tubular epithelial cells [97], and podocytes [98]. Through cleavage of uPAR from its GPI-anchor, the soluble urokinase plasminogen activator receptor (suPAR) is released. Recently, suPAR has emerged as a biomarker in different disease conditions. For example, suPAR concentrations were associated with increased risk of cardiovascular events in the general population [99, 100]. Several studies have described an inverse correlation of suPAR levels with the estimated glomerular filtration rate (eGFR) [101–104]. Since its first description as a permeability factor in FSGS by Wei et al. [105], many researchers have published conflicting results concerning the causative role of suPAR for FSGS [101, 106]. Amiloride, a potassium sparing diuretic, has recently been shown to reduce uPAR expression in podocytes in vitro and in proteinuric rodent models in vivo [107]. Amiloride has been used in case reports for patients with Alport and Fabry's disease to reduce proteinuria in addition to standard therapy [108, 109]. There have been no reports on amiloride therapy in humans with FSGS.

Saquinavir, an HIV protease inhibitor with proteasome inhibiting activity, has been tested in a pilot study and in case reports in patients with a long history of nephrotic syndrome despite immunosuppressive therapy [110, 111]. In this study, saquinavir was used as an add-on therapy to other immunosuppressive agents. One out of four primary-steroid resistant nephrotic syndrome patients and five out of six steroid-dependent nephrotic syndrome patients became infrequent (5) or frequent (1) relapsers [110]. Immunosuppressive dosages, especially corticosteroids, were significantly reduced [110]. In podocytes and peripheral blood mononuclear monocytes, saquinavir blunted NF-κB activation [110]. The observation that the proteasome inhibitor was beneficial in FSGS warrants further investigation and larger studies.

As described above the podocyte cytoskeleton is also an interesting new target in FSGS (see section potential new targets and therapeutic strategies in MCD).

3. Conclusion

Major advances in the pathophysiological understanding, especially of iMN, have led to new treatment strategies in the past years. However, even though the incidence of primary causes of nephrotic syndrome is low, the lack of larger RCTs in this field is striking [112]. Therefore, adult patients with nephrotic syndrome should be treated within clinical trials in the future. Many interesting new treatment targets identified by basic science have been proposed. Further investigation of these new targets and those identified in the future will potentially lead to novel advances in the treatment of nephrotic syndrome, with higher effectiveness in reducing proteinuria at an acceptable level of AEs.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Cytoplasmic Localization of WT1 and Decrease of miRNA-16-1 in Nephrotic Syndrome

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Nephrotic syndrome (NS) is a glomerular disease that is defined by the leakage of protein into the urine and is associated with hypoalbuminemia, hyperlipidemia, and edema. Steroid-resistant NS (SRNS) patients do not respond to treatment with corticosteroids and show decreased Wilms tumor 1 (WT1) expression in podocytes. Downregulation of WT1 has been shown to be affected by certain microRNAs (miRNAs). Twenty-one patients with idiopathic NS (68.75% were SSNS and 31.25% SRNS) and 10 healthy controls were enrolled in the study. Podocyte number and WT1 location were determined by immunofluorescence, and the serum levels of miR-15a, miR-16-1, and miR-193a were quantified by RT-qPCR. Low expression and delocalization of WT1 protein from the nucleus to the cytoplasm were found in kidney biopsies of patients with SRNS and both nuclear and cytoplasmic localization were found in steroid-sensitive NS (SSNS) patients. In sera from NS patients, low expression levels of miR-15a and miR-16-1 were found compared with healthy controls, but only the miR-16-1 expression levels showed statistically significant decrease ($p = 0.019$). The miR-193a expression levels only slightly increased in NS patients. We concluded that low expression and delocalization from the WT1 protein in NS patients contribute to loss of podocytes while modulation from WT1 protein is not associated with the miRNAs analyzed in sera from the patients.

1. Introduction

Nephrotic syndrome (NS) is one of the most common diseases in children and is characterized by the leakage of large amounts of protein into the urine, lipidemia, hypoalbuminemia, and dysfunction of the glomerular filtration barrier [1]; NS is also a major cause of podocyte injury [2]. Podocytes, highly specialized cells of the visceral epithelium, are found in the glomerular membrane of the kidney [3]; they constitute the terminal part of the ultrafiltration barrier that prevents protein loss, which can result in damage and detachment of podocytes. The reduction of glomerular podocytes in lupus nephritis (LN) patients is correlated with the cumulative loss of urinary podocytes and proteinuria during the progression of kidney disease [2, 4].

Some urinary biomarkers have been found to be associated with injured podocytes in urine sediment, such as podocalyxin (PODXL), nephrin (NPHS1), podocin, and Wilms tumor protein (WT1) [5]. WT1 itself has been found in exosomes in urine sediment, indicating podocyte injury [6].

WT1 is essential for urogenital development, and its expression is limited to mature podocytes [7]. The *WT1* gene encodes for a transcription factor that is critical for the development of podocytes and viability; the regulation of podocyte homeostasis occurs via PODXL and NPHS1 [7].

Recent studies have demonstrated that mutations in *WT1* can lead to syndromic forms of steroid-resistant NS (SRNS), such as Denys-Drash or Frasier syndrome, and can cause isolated SRNS [8, 9]. Between 10% and 20% of patients fail to respond to steroid therapy; thus, the prognosis for SRNS is usually poor, due to the increased risk of developing end-stage renal disease [10, 11]. Beltcheva et al. reported a case of a pediatric patient with SRNS that was caused by a novel dominant *WT1* mutation, C11184A, identified in exon 9 [10]. Mutations within *WT1* are frequent causes of sporadic, isolated cases of SRNS in girls [8]. *WT1* expression is modulated by microRNAs (miRNAs) [12], which are small (20–25 nucleotides), noncoding RNA molecules that act as posttranscriptional regulators of gene expression in animals and plants, participating in many key biological processes [13]. In the kidney, miRNAs play critical roles in renal development, homeostasis, and physiological function. Several studies have shown that miRNAs are also key mediators of the pathogenesis of various renal diseases [14–16]. Furthermore, miRNAs can be secreted into the extracellular environment and detected in fluids, including urine and serum. They have been proposed as biomarkers for a wide range of diseases [17, 18].

Gao et al. discovered that miRNAs 15a and 16-1 may function as tumor suppressors to regulate leukemic cell proliferation, potentially by downregulating the *WT1* oncogene [12]; pure curcumin was also recently shown to modulate the expression of WT1, partly by upregulating the expression of miR-15a and miR-16-1 in leukemic cells [19].

In the podocytes of patients with focal segmental glomerulosclerosis (FSGS), the expression of miR-193a was induced, which in turn inhibited the expression of *WT1*, modulating the expression of genes critical for podocyte architecture [20]. The presence of miRNAs in plasma, serum, and urine was also shown to be a possible biomarker of diabetic kidney disease [21]. Another mechanism of modulation of *WT1* that has been observed in experimental mice during the sepsis is the decrease of nuclear WT1 in podocytes and was associated with transcriptional suppression of nephrin and cause albuminuria [22].

The aim of this study was to determine whether there are different expression levels and protein localization of WT1 in SRNS and SSNS patients and its relationship with the miR-15a, miR-16-1, and miR-193a, which modulate the *WT1* expression in other models.

2. Methods

Twenty-one biopsies, serum and urine samples from 11 males and 10 females, from patients diagnosed with NS who met the criteria of International Study of Kidney Disease in Children with proteinuria levels greater than 3.5 g/24 h were included, in the control group serum and urine samples were obtained from 10 subjects without kidney disease, renal biopsies were obtained of autopsy subjects without renal disease who died in accidents as a result of a head injury. Patients who agreed to participate in the study signed a letter of informed consent. This study was performed with patients living in the north-central region from Mexico, according to the principles of the Declaration of Helsinki, and was approved by the ethics committees of our institutions.

2.1. Sample Processing. Freshly urine samples were processed in less than 30 min, and 10 mL of urine was centrifuged for 5 min at 1,500 rpm. After decanting the supernatant, the pellet was resuspended in 1 mL PBS-TS (0.1% Tween-20 and 0.02% SDS in phosphate-buffered saline [PBS]); 25 μL of which was fixed at 55°C for 10 min for continued permeabilization as described below.

2.2. Immunofluorescence. As the localization of WT1 is essential for its biological action, WT1 localization was assessed for all samples. For the analysis of the number of podocytes per glomerulus in both NS patients and healthy subjects, WT1 and nephrin were used as biomarkers for immunofluorescence of 4 μm thick sections of renal tissue mounted on microscope slides. The specimens were dewaxed, permeabilized with Triton X-100 (Triton 0.1% with 1% sodium citrate in PBS), and washed three times with PBS. The tissues were blocked with 20% fetal bovine serum (FBS) in PBS for 30 min and incubated for 1 h with monoclonal anti-WT1 antibody (WT1 [F-6] sc-7585, monoclonal mouse IgG1, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-nephrin monoclonal antibody (H-300 sc-28192, polyclonal rabbit IgG, Santa Cruz Biotechnology) diluted 1 : 100 in 10% FBS in PBS. After washes with PBS, the presence of bound antibody was identified by goat anti-mouse IgG1 Texas Red (sc-2979, Santa Cruz Biotechnology) and goat anti-rabbit IgG-FITC (sc-2012, Santa Cruz Biotechnology) staining. Some slides were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Finally, the slides were mounted and examined by confocal scanning microscopy. The intensity of the signal was expressed in pixels and analyzed using Image-Pro Plus software, version 7.0 (Media Cybernetics, Rockville, MD, USA). The WT1 localization was determined by Pearson correlation coefficient (PCC) [23].

2.3. Blood Collection. Five mL whole blood samples from NS patients and healthy controls were collected in tubes (BD Vacutainer; Becton Dickinson Vacutainer Systems, Franklin Lakes, New Jersey, USA) and then processed for obtain the serum by centrifugation at 1,500 rpm for 10 min. The sera were stored at −70°C until analysis.

2.4. RNA Isolation and Quantification. Total RNA was extracted from patient sera using the miRNeasy Serum/Plasma Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. Two aliquots of 200 μL each were used to obtain sufficient RNA to perform all quality tests. RNA was dissolved in diethylpyrocarbonate-treated water, and RNA quality and quantity were determined by spectrophotometry using the NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA).

2.5. cDNA Synthesis and Real Time RT-PCR (qPCR) Assay for miRNAs Expression. To determine whether miR-15a, miR-16-1, and miR-193a are involved in regulating the expression of *WT1*, their expression was quantified in sera from NS patients

Table 1: Description of NS patients and controls.

	Age	Gender	%	Treatment	%	Proteinuria
Nephrotic syndrome	5.58 (range 2–12 years)	Males	52	SSNS	68.75	4.01 g/24 h
		Females	48	SRNS	31.25	
Healthy subjects	9 (range 3–12 years)	Males	30.77	No treatment		<0.3 g/L
		Females	69.23			

SSNS: steroid-sensitive nephrotic syndrome; SRNS: steroid-resistant nephrotic syndrome.

and healthy controls. Total RNA was polyadenylated and subjected to reverse transcription using an NCode miRNA First-Strand cDNA Synthesis Kit (Invitrogen Carlsbad, CA) according to the manufacturer's instructions.

Real-time qPCR analysis was carried out using SYBR® Green PCR Master Mix (Applied Biosystems; Foster City, CA, USA) on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems). The primers reported by Gao et al. [12] were used to quantify the relative expression levels of miR-15a, miR-16-1, and miR-193a in the samples. Experiments were performed in triplicate. U6 was used as an endogenous control. The expression of each miRNA relative to U6 RNA was calculated using the equation $2^{-\Delta\Delta CT}$ [24–26].

2.6. RNA Isolation and qPCR for WT1 mRNA Expression. Total RNA was isolated from renal biopsies using the High Pure RNA Isolation Kit and High Pure RNA Paraffin Kit (Roche Life Science, Mexico) according to the manufacturer's instructions. Single-stranded cDNA was synthesized by reverse transcription using the miScript Reverse Transcription Kit (Qiagen, United States) according to the manufacturer's instructions. Real-time PCR was performed using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with TaqMan gene expression assays. Comparative real-time PCR assays were performed for each sample in triplicate. The primers for WT1-F: 5′-TCTGCGGAGCCCAATACAG-3′; WT1-R: 5′-CACATCCTGAATGCCTCTGAAGA-3′; and WT1-P FAM: 5′-CACCGTGCGTGTGTATT-TAMRA-3′ were used. The comparative quantification cycle threshold (C_q) method was used to determine the relative expression levels of the *WT1* gene. The *18S rRNA* (Applied Biosystems, Foster City, CA, USA) gene was used for normalization.

2.7. Statistical Analyses. Statistical analyses were performed using SPSS 17.0 software. The results are presented as the mean ± SD. Comparisons were made using an analysis of variance and Student's *t*-test, and the association between miRNA levels and expression of *WT1* was assessed by Pearson's correlation statistical program and considered significant at *p* value less than or equal to 0.05.

3. Results

3.1. Sample Description. Twenty-one samples from patients with NS and 10 healthy subjects without renal disease as control group (Table 1) were analyzed for levels of creatinine (0.39 ± 0.25 mg/dL), proteinuria (4.01 ± 2.7 g/24 h), cholesterol (207.06 ± 91.72 mg/dL), triglycerides (141.56 ± 93.55 mg/dL), and albumin (3.75 ± 0.83 g/dL). Control renal biopsies were obtained from autopsy subjects without renal disease who died in accidents as a result of a head injury.

3.2. WT1 Localization in Podocytes in NS Patients and Healthy Subjects. Immunofluorescence of renal biopsy tissue using WT1 as a biomarker showed a decrease of 27% in NS patients compared to healthy subjects (Figure 1(a)). The localization of WT1 in SRNS patients was primarily cytoplasmic, whereas WT1 in SSNS patients showed both a nuclear and a cytoplasmic localization while in healthy controls it was mainly nuclear. The localization assay was performed by the colocalization of DAPI (nuclear localization) and Texas Red (WT1), showing a statistically significant difference between SSNS (PPC = 0.51 ± 0.03) and SRNS (PPC = 0.29 ± 0.01) patients compared with healthy subjects (PPC = 0.73 ± 0.02) ($p = 0.00001$) (Figure 1(c)). In urine sediments of SRNS patients, detached podocytes showed a cytoplasmic localization of WT1 (PPC = 0.35 ± 0.08) (Figure 1(b)). These results were significantly different from those in healthy subjects (PPC = 0.64 ± 0.03) ($p \leq 0.0001$; Figure 1(d)).

3.3. Analysis of WT1 Expression in Podocytes of NS Patients and Healthy Controls. *WT1* expression analysis in podocytes was performed by two methods: immunofluorescence and RT-qPCR. *WT1* expression by intensity pixels in renal biopsies of patients with NS was statistically different than healthy controls ($p = 0.0001$) and between patients with SSNS and SRNS ($p = 0.05$; Figure 2(a)), with highest levels in healthy controls and lowest levels in SRNS patients. *WT1* RNA expression levels were analyzed by RT-qPCR in renal biopsies of NS patients and healthy controls, showing significantly lower (66%) WT1 mRNA levels in NS patients ($p = 0.017$), indicating a decrease in basal *WT1* levels in the podocytes of NS patients (Figure 2(b)).

3.4. Expression of miR-15a, miR-16-1, and miR-193a Levels in Sera from NS Patients. The expression levels of miR-15a ($2^{-\Delta\Delta CT}$) did not differ between the different groups (SRNS 0.33 ± 0.12, SSNS 0.91 ± 0.44, and HS 1.54 ± 0.54 of relative expression) ($p = 0.138$; Figure 3(a)) or between patients with SRNS and SSNS compared to the control group ($p = 0.31$; Figure 3(b)). While the expression of miR-16-1 was significantly lower (0.54 ± 0.144 relative expression) in the sera of NS patients compared with healthy controls (1.86 ± 0.82 relative expression) ($p = 0.019$; Figure 3(c)), there was no difference between SSNS and SRNS patients ($p = 0.14$; Figure 3(d)). The relative expression of miR-193 ($2^{-\Delta\Delta CT}$) did not differ between NS patients (2.14 ± 0.36 relative

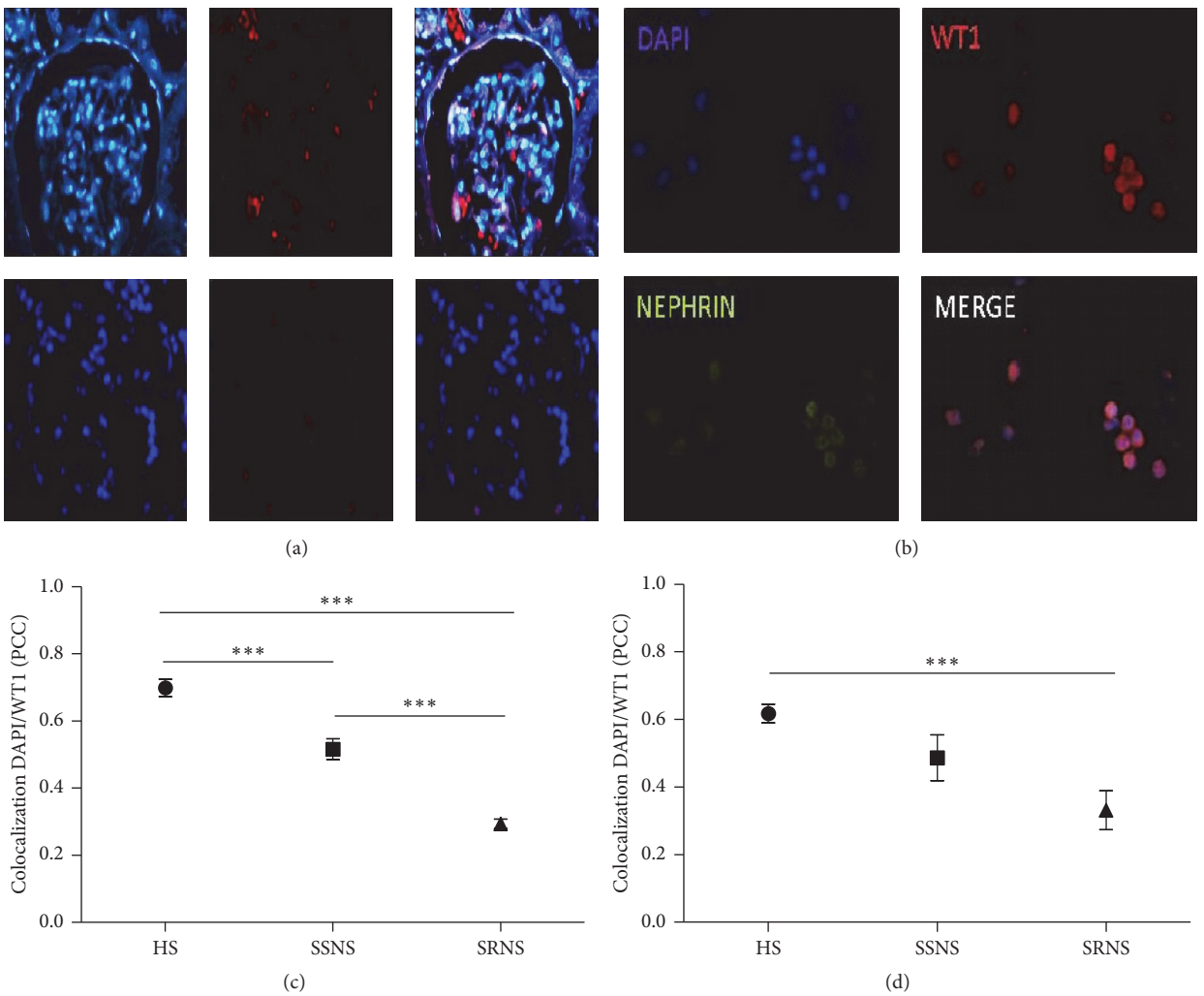

Figure 1: WT1 expression and localization in podocytes from biopsies and urinary sediments of patients with NS. (a) Immunofluorescence of renal biopsies (magnification, ×40) of patients with nephrotic syndrome (NS). (b) Immunofluorescence of podocytes in urine sediments: nephrin detection was used as a second marker of podocytes (magnification, ×40). (c) Colocalization of DAPI and Wilms tumor 1 (WT1) in nucleus/cytoplasm of podocytes in kidney biopsies. (d) Colocalization of DAPI and WT1 in nucleus/cytoplasm of podocytes in urinary sediments. The colocalization was determined by Pearson correlation coefficient (PCC). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

expression) and controls (2.30 ± 0.75 relative expression, $p = 0.59$; Figure 3(e)) or between patients with SRNS and SSNS ($p = 0.91$; Figure 3(f)).

3.5. Relationship between WT1 and miRNAs. We did not find a significant correlation between WT1 expression and serum concentrations of miR-15a, miR-16-1, and miR-193a in NS patients (miR-15a: $r = 0.118$, $p = 0.882$; miR-16-1: $r = 0.301$, $p = 0.969$; and miR-193a: $r = 0.213$, $p = 0.787$). However, a positive correlation was found between miR-15a and miR-16-1 ($r = 0.994$, $p = 0.006$). There was no significant relationship between the particular miRNA levels.

4. Discussion

Epidemiological studies indicate that NS remains the most common manifestation of glomerular disease in childhood, often causing death from untreated infections [27]. The present study reviewed 21 children with NS in the Mexican mestizo population, with an average age of 5.8 years.

Idiopathic NS is one of the most common glomerular diseases in pediatrics. The response to steroids is the best prognostic factor in this disease, and most children with NS respond to corticosteroids. However, 70% of children experience recurrence, with episodes of edema and proteinuria [28]; fewer than 3% of patients with SSNS progress to chronic renal failure compared to 50% patients with SRNS [29].

The WT1 protein has been used as a biomarker of podocyte loss from glomeruli in kidney disease [30, 31] and of the presence of damaged podocytes in urinary sediment [32, 33]. In this work, we clearly observed a reduction of podocytes in renal biopsies of children with NS and the presence of podocytes in the urine, but the reason for such podocyte

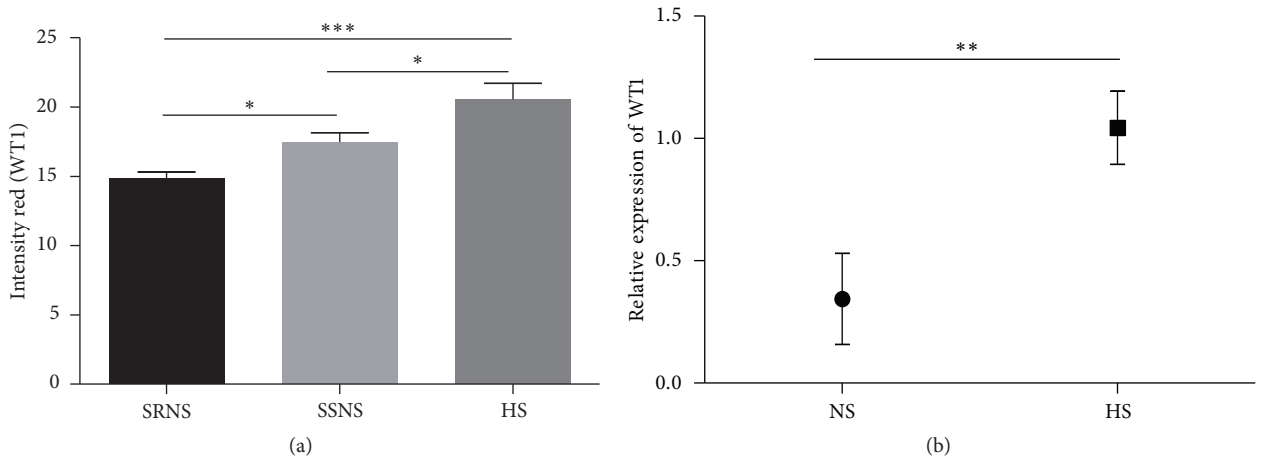

Figure 2: WT1 expression in podocytes from renal biopsies of patients with NS. (a) WT1 expression immunofluorescence. HS: healthy subjects; SSNS: steroid-sensitive NS; and SRNS: steroid-resistant NS. WT1 expression was measured by pixel intensity. (b) Relative expression levels of WT1 by real-time PCR. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

detachment is not clear. The cause may be an imbalance between different isoforms of WT1 or decreased basal protein expression. Different research groups have observed a decrease in WT1 protein expression in children with SRNS, which was often accompanied by *WT1* mutations in exons 8 and 9 [8, 34, 35]. However, another report suggested that reduced expression levels of WT1 cause glomerulonephritis and mesangial sclerosis, depending on the level of the protein [36].

We found normal WT1 expression in the nuclei of podocytes from biopsies of healthy controls without kidney disease; however, in SSNS patients, WT1 expression was localized to both the nucleus and cytoplasm, whereas WT1 expression is primarily cytoplasmic in SRNS patients, suggesting that delocalization of WT1 from the nucleus can lead to a loss of function as a transcription factor. Decreased expression levels of WT1 lead to downregulation of its target genes *PODXL* (podocalyxin) and *NPHS1* (nephrin), as well as several other genes crucial for the architecture of podocytes, initiating a catastrophic collapse of the entire podocyte-stabilizing system [20]. Additionally, Kato et al. [22] observed that in Lipopolysaccharide (LPS) treated mice, a loss of albumin decreased nephrin levels and the nuclear localization of WT1 in podocytes. The nuclear localization of WT1, as well as nephrin mRNA and protein levels, returned to near basal levels 72 hours after LPS with recovery of albumin levels [22].

The loss of *WT1* gene expression could be a fatal consequence as described above [9]; the regulation of *WT1* is provided by certain genes, such as *PAX2* and *PAX8*, or epigenetic modulation mechanisms, such as miRNAs [12, 19]. In addition, *WT1* has the ability to self-regulate [37]. In this study, we assessed whether low WT1 expression in podocytes correlates with the circulating levels of miR-15a, miR-16-1, and miR-193a in the sera of NS patients. Our results indicate that the expression of miR-15a was higher in healthy controls than in NS patients but was not statistically significant. Similar results were observed for miR-16-1, but the difference between the expression of miR-16-1 in NS patients and in healthy controls was significant, indicating an inverse relationship between miRNAs and WT1 expression. Gao et al. reported that miR-15a and miR-16-1 inhibit the proliferation of leukemic cells by decreasing WT1 levels via joining *WT1*-3′UTR. The expression of these miRNAs inhibits cell proliferation, promotes apoptosis of cancer cells, and suppresses tumorigenicity both in vitro and in vivo [12]. Both miR-15a and miR-16-1 have been shown to negatively affect several oncogenes, including *BCL2*, *MCL1*, *CCND1*, and *WNT3A*. Downregulation of these miRNAs has been reported in chronic lymphocytic lymphoma, pituitary adenoma, and prostatic carcinoma [37, 38], while miR-193a was not found to elicit any significant effects on these cancers. However, the data reported by Gebeshuber et al. [20] indicated that miR-193a is involved in FSGS by decreasing WT1 levels. In conclusion, we found low WT1 expression and delocalization in NS patients. We also observed higher expression levels of miR-15a and miR-16-1 in healthy controls compared with NS patients, but only miR-16-1 levels were statistically different. However, we did not find a correlation between miRNA expression and WT1 levels. It is important to extend the search for miRNAs as biomarkers of renal diseases as well as to understand the basic mechanisms of gene regulation of podocytes and the glomerular filtration system.

Disclosure

The authors alone are responsible for the content and writing of the article.

Competing Interests

The authors report no conflict of interests.

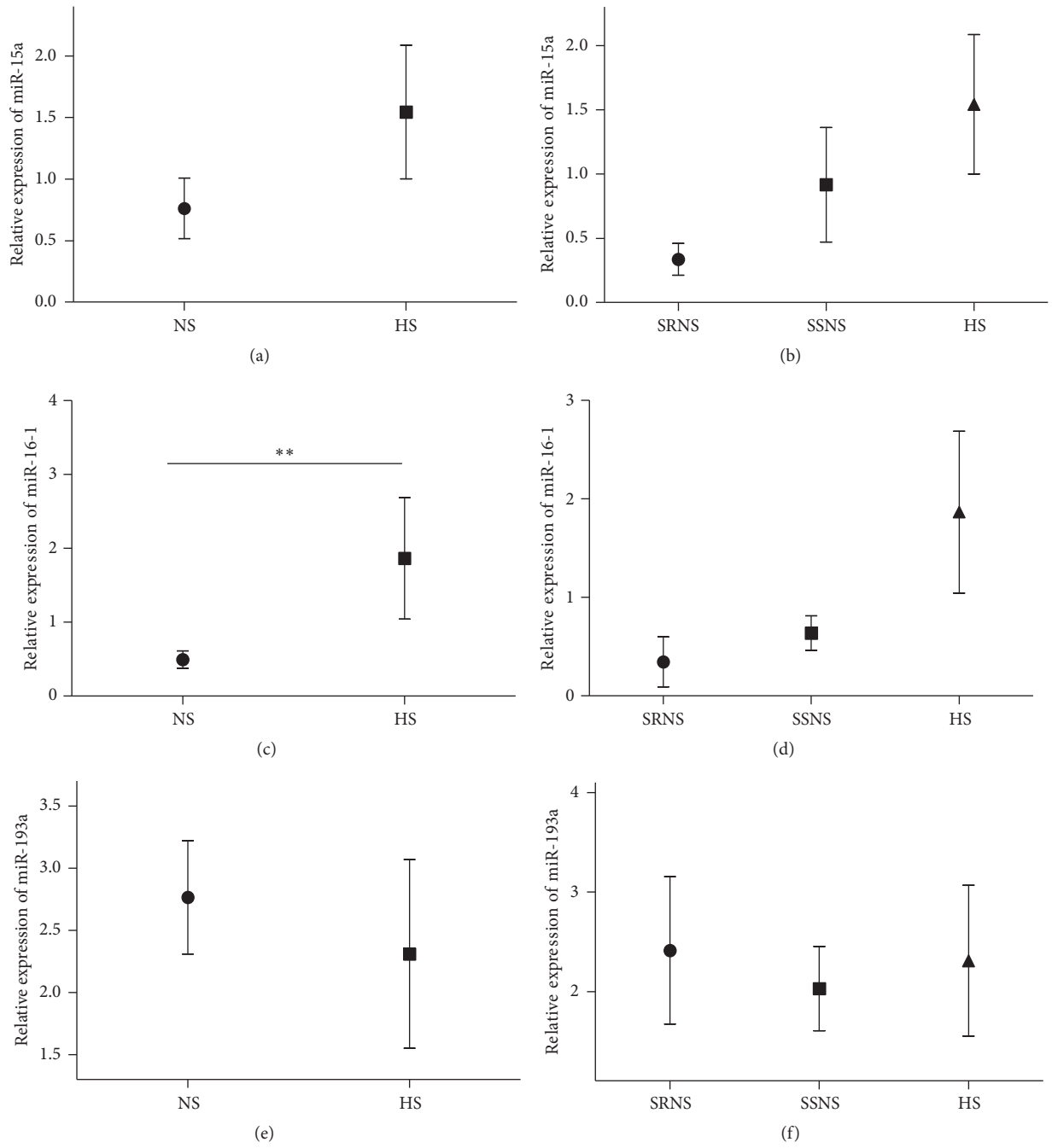

Figure 3: Expression of miR-15a, miR-16-1, and miR-193a in sera from patients with NS. Relative expression levels of miR-15a (a) in patients with NS (nephrotic syndrome) and healthy subjects (HS) and (b) in patients with steroid-sensitive NS (SSNS) and steroid-resistant NS (SRNS). miR-16-1 (c) in patients with NS and HS and (d) in patients with SSNS and SRNS and miR-193a (e) in patients with NS and HS and (f) in patients with SSNS and SRNS.$^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

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