NEPHROTIC SYNDROME IN CHILDREN

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NEPHROTIC SYNDROME IN CHILDREN

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Table of Contents

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Editorial: Nephrotic Syndrome in Children

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Editorial on the Research Topic

Nephrotic Syndrome in Children

Although rare in absolute terms, with an incidence of 2–7/100,000 children/year (1), the nephrotic syndrome is the most common glomerular disease in childhood. As a clinicopathological entity, it is characterized by abnormal glomerular basement membrane permeability to plasma proteins (mainly albumin) resulting in massive proteinuria (albuminuria), and varying degrees of hypoalbuminemia edema and hyperlipidemia. Seventy to 80% of children with the nephrotic syndrome attain complete remission with various corticosteroid regimens and are labeled as steroid sensitive. They have no abnormalities detected on kidney biopsy by light microcopy, hence the term minimal change disease. Unfortunately, two out of three steroid responders have at least one relapse within the first 6 months from stopping treatment (2).

The current issue of Frontiers in Pediatric Nephrology is dedicated to the nephrotic syndrome. Fourteen articles, including 5 case reports, address various aspects of this disease. Of these, four, including a case report, are devoted to different therapeutic approaches to the nephrotic syndrome.

In a multicenter prospective cohort study from Italy, Pasini et al. demonstrated that time to remission in children receiving different steroids regimens had no effect on the relapse rate. They found, however, that younger age and low total serum protein were independent predictors of relapse risk. The controversy over the treatment with corticosteroids and whether the cumulative dose or the duration of therapy may affect the relapse rate is not fully resolved and the jury is still out (1, 3). Two meta-analyses from China, one addressing immunosuppressive therapy with rituximab, Gao et al. the other with mycophenolate mofetil, Xiang et al. for steroid dependent (SDNS) and frequent relapsing nephrotic syndrome (FRNS) attest to the efficacy and safety of these agents in reducing the number of relapses in addition to their steroid sparing effect. The first reports about benefits of rituximab in nephrotic syndrome were published in the early 2000's and consisted of case reports and small case series ranging from 1 to 24 patients with SDNS or FRNS (4). In addition to its immunological role, Rituximab may have direct and non-immunological effects on podocytes associated with inducing remission of proteinuria in children and adults with FSGS (5). Mycophenolate mofetil has been described as a promising drug in SDNS, mainly in maintaining long-term remission. This drug seems to be well-tolerated in most children and its efficacy in maintaining remission in SDNS and FRNS has been demonstrated, especially when compared to other immunosuppressives that might be nephrotoxic and require frequent monitoring of blood levels (6).

Lastly, Ma et al. from China report the case of a 7-year-old girl with SDNS due to C1q nephropathy who went into complete remission with a low dose of rituximab (93 mg/m^2). This is one fourth of the usual dose used in patients with SDNS and FRNS. The authors raise the issue of reconsidering the dose in this disease and we believe it may be worth trying such a dose in a

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controlled manner in those patients as well. This might help avoid both short- and long-term side effects of rituximab.

Five papers address molecular and genetic aspects of the nephrotic syndrome:

In a study by Bai et al. a novel missense mutation on the NPHS2 gene was found in Chinese children with steroid-resistant nephrotic syndrome. This gene plays a significant role in proper podocin protein function and its pathogenesis (7). Thus, we encourage pediatric nephrologists to perform genetic testing on all patients with SRNS, especially those with early-onset SRNS and a positive family history as it helps in avoiding unnecessary immunosuppressive and potentially toxic medications. Looking to other possible genetic mutations that play a role in patients with SRNS. Liu et al. from China, identified a novel, likely pathogenic mutation in the CD2AP gene. This is a crucial protein for slit-diaphragm assembly and function (8). Again, such new mutations ought to lead to multicenter, international collaborative studies that might help identify other pathogenic variants in patients with SRNS. Jacob et al. from the United Arab Emirates report the case of a male with congenital nephrotic syndrome (CNS) associated CMV infection but no syndromic stigmata or systemic manifestations. Kidney biopsy showed crescents, fibrosis and tubulointerstitial changes, but was negative for CMV inclusion by EM. Genetic studies revealed a novel homozygous mutation in the *NPHS1* gene, highlighting the importance of genetic testing all patients with CNS.

Shah et al. report the case of 15-year-old girl who developed acute onset nephrotic syndrome with biopsy proven membranous nephropathy in association with repeated use of the NSAD ibuprofen for dysmenorrhea. While NSAIDs are a common cause of acute interstitial nephritis and AKI with nephrotic range proteinuria (9) the case reported here was that of a pure membranous nephropathy, a rare complication of NSAID therapy indeed.

In the era of B cell depleting agents for the management of steroid dependent or steroid resistant forms of idiopathic nephrotic syndrome (INS) it is obvious that B cells are believed to have major impact in its pathophysiology. However, research also focuses on interleukins and Al Rushood et al. have searched for polymorphisms in the IL4 and IL13 gene. Only the IL13 RQ genotype polymorphisms have been found more often in steroid sensitive patients compared to steroid resistant ones, but no IL polymorphism could be identified as susceptibility factor for INS compared to controls.

The remaining five papers deal with different and interesting aspects of the nephrotic syndrome, including metabolic and infectious complications and the role of T cell cytokines in the pathogenesis of nephrotic syndrome.

Turolo et al. report from Milan, Italy report on the persistent elevation of the omega-6 fatty acids, arachidonic acid and its precursor linoleic acid in nephrotic children during remission, while free of proteinuria. The authors go on to suggest that these fatty acid levels might be regarded as candidate biomarkers for the risk of relapse in children with nephrotic syndrome. A somewhat parallel observation was reported many years ago by Zilleruelo et al. (10) in children with nephrotic syndrome who showed elevated total cholesterol, triglycerides, LDL and VLDL during remission.

Another interesting aspect concerning T cell function has been examined by Ni et al. T Helper type 2 (Th2) cells secrete various cytokines such as IL4 and IL13 which trigger IgE secretion. High levels of IgE in patients with active INS are believed to be due to these changes in Th2 cell function. The authors showed, that micro-RNAs seem to play a role for Th2 expression and in children with active non-atopic INS, the levels of miRNA-24 and-27 are decreased, allowing a higher number of Th2 cells and a Th1/Th2 imbalance.

Infections are a common cause of morbidity, and before the era of antibiotics, the highest cause of mortality in children with nephrotic syndrome (11). Zhang et al. from China investigated the risk factors in a large number of children with nephrotic syndrome. As expected, steroid resistance combined with the use of immunosuppressive drugs were significant risk factors for the development of severe infection. Gram positive bacteria, elevated CRP above 8 mg/L and low C3 complement level ($<$0.55 gm/L) and a low absolute lymphocyte count ($<1.5 \times 10^9$/L) were major risk factors for severe infection and mortality.

As pediatric patients with INS are at risk for infections which may be preventable by specific vaccinations, there is a strong need for clear information from the caregivers to the patients' families. Vaccination strategies (in particular anti-pneumococcal vaccination) have improved tremendously and the number of invasive pneumococcal infections in patients with active INS has decreased. Tran et al. assessed parental and pediatric nephrologist knowledge about immunization practices in children with nephrotic syndrome. Surprisingly only 44% of pediatric nephrologists adhered to the Advisory Committee on Immunization Practices (ACIP) guidelines for inactive vaccines and only 22% for live vaccines.

Congenital nephrotic syndrome is a severe clinical entity with several consequences for the patients and their families. As profound hypoalbuminemia increased the risk for developmental problems, in particular under the age of 3 years, strategies to correct at least partially the serum albumin level have to be established, but these often require long term hospitalization. The work of Eugenia Serramontmany et al. showed that in those patients who require only one daily albumin infusion, home albumin infusion therapy is a reasonable option and does not increase the risk of complications such as central venous catheter infections.

Idiopathic nephrotic syndrome remains enigmatic in many ways, whether from the etiologic, pathophysiologic or therapeutic standpoints. Prevention of related complications is still a matter of debate and many strategies deserve to be explored. The decision, when to use which immunosuppression and for what patient will remain debatable until the precise pathophysiology of INS is elucidated.

AUTHOR CONTRIBUTIONS

All authors contributed in a significant capacity to this editorial on Nephrotic syndrome in children.

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ORIGINAL RESEARCH
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Interleukin-4 and Interleukin-13 Gene Polymorphisms in Children With Idiopathic Nephrotic Syndrome

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Background: Idiopathic Nephrotic syndrome (INS) is an immune-mediated disease in which a number of cytokines, including IL-4 and IL-13, have been implicated in the pathogenesis. Cytokine gene polymorphisms might affect their levels and activity. Therefore, may affect INS susceptibility and response to treatment. The aim of the study was to determine the association of IL-4 and IL-13 gene polymorphisms and INS susceptibility and their effects on steroid responsiveness in children.

Methods: The polymorphisms in IL-4 and IL-13 genes were detected by PCR-RFLP in 155 INS patients and 64 controls.

Results: A total of 132 steroid-sensitive (SS) and 23 steroid resistance (SR) INS patients; mean age 7.3 ± 4.0 years, were included. Male: Female ratio was 2:1. No significant statistical differences were detected in the frequency of CC, CT, and TT genotypes of IL-4 gene compared to controls ($P = 0.57$, 0.61, and 1.00, respectively). There was no significant difference in the T and C-allele frequencies, in SS and SR subgroups. Analysis of IL-13 gene polymorphism also did not show significant statistical differences in the frequency of QQ, RQ, and RR genotypes compared to controls ($P = 0.74$, 1.00, and 0.68, respectively). No significant difference was found in the Q and R-allele frequency. However, the heterozygous RQ genotype of the IL13 gene was significantly higher in SS INS patients compared to the SR INS cases ($P = 0.04$).

Conclusion: Our findings did not show an association between IL-4 and IL-13 gene polymorphisms and INS susceptibility. However, IL-13 RQ genotype was expressed more in children with INS who are steroid sensitive.

Keywords: interleukin-4, interleukin-13, nephrotic syndrome, gene polymorphisms, cytokine, steroids

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INTRODUCTION

Idiopathic nephrotic syndrome (INS) is the most frequent form of nephrotic syndrome in children, representing more than 90% of cases between the age of 1 to 10 years (1). Immunological disturbances, especially T cell imbalance, have been implicated in the pathogenesis of the disease (1, 2). This association was suggested by the response to immunosuppressive treatment and the association between the disease with atopy and elevated serum IgE levels (1, 2).

T helper cells (Th2) with its signature cytokines such as IL-4 and IL-13 have been studied as potential important role players in the pathogenesis of the INS (3, 4). IL-4 is a Th2 cytokine produced by basophils, mast cells and activated TH2 cells (5), and plays an essential role in IgE

regulation, Th2 differentiation, Th1 inhibition and induction and maintenance of allergy (6, 7). It might also act as an anti-tumor and an anti-inflammatory agent (8, 9).

On the other hand, IL-13 is an immunoregulatory protein produced by T-cells subsets, basophils, eosinophils and mast cells. It is believed to be an important mediator in allergic disease as its levels and genetic polymorphism variants have been shown to be associated with elevated serum IgE levels in atopic and asthmatic patients of different racial backgrounds (10–13).

Both IL-4 and IL-13 genes are located in a region of 140 kb on chromosome 5q31-33 that codes for a cluster of Th2 cytokines (10, 14). They share a common IL-4 receptor α chain (IL4 RA) in the multimeric IL-4 and IL-13 receptor complexes (15). Therefore, they share many biological activities that affect clinical conditions.

Gene Polymorphisms in the regulatory regions of these cytokines can influence the amount of cytokine produced as well as their biological activity and potency at their receptor sites (14). Cytokine gene polymorphism including that of IL-4 and IL-13 in nephrotic syndrome of children with an Arab race has not been explored previously. The aim of this study is to determine any association between IL-4 and cytokine IL-13 gene polymorphisms with the susceptibility to INS in Kuwaiti children and their effects on response to steroid treatment.

METHODS

Children less than 12 years of age with a confirmed diagnosis of Idiopathic nephrotic syndrome (INS) were included in this study. All had INS with a clinical and/or histopathological diagnosis of MCNS. Subjects were evaluated at a specialized pediatric nephrology clinic in Mubarak Al-Kabir University Hospital during the period 2012–2018. INS was defined as the presence of generalized edema, nephrotic- range proteinuria, hypoalbuminemia with or without hyperlipidemia (16). A total of 155 children were included. Patients were subdivided according to their response to steroids into steroid sensitive (SS) and steroid resistant (SR). Steroid responsiveness was defined as the disappearance of proteinuria (negative to trace in a urine dipstick for 3 consecutive days, or a urine protein/creatinine level of <0.2) within the first 4-week course of full dose prednisolone therapy (60 mg/M^2/day). Steroid resistance was defined as the persistence of proteinuria after a 4-week course of full dose prednisolone. Patients with poor compliance and not on regular follow-up were excluded as well as those with infrequent relapses. All SSNS patients were in full remission at the time of the study.

All patients underwent a thorough physical examination by an experienced pediatrician. A total of 64 age and gender- matched healthy children of the same ethnic background, were included as controls. All were examined by a senior pediatrician to exclude any renal disease. Controls were selected from patients visiting Hospital's Emergency Department for minor illnesses such as upper respiratory tract infection or acute gastroenteritis.

In all the study subjects (patients and controls), tests for blood urea, serum creatinine, total protein, albumin level and a complete lipid profile were carried out. Urine protein: creatinine ratio was also determined for all the study subjects.

At the end of the study, all SSNS patients had normal serum creatinine. Sixty-five percent of the patients were in remission for more than 6 months, while 35% continued to relapse, but less frequently, despite receiving second-line immunosuppressive treatment. For SSNS patients, renal biopsy was performed for steroid dependent patients and for those with frequent relapses (> 6 relapses/year) before initiating cytotoxic therapy.

Genetic study was done in 12 SRNS patients and they were NPHS1 negative. The genetic study for the rest of the SRNS patients was not available as it should be sent outside the country.

Genotyping of IL4 and IL13 Gene Polymorphisms

DNA was isolated from the peripheral leukocytes using a standard method (17). The genotypes were determined by PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism) method using the primers listed below (18, 19):

IL-4 Gene C590T Polymorphism

Forward primer: 5′-ACTAGGCTCACCTGATACG-3′
Reverse primer: 5′-GTTGTAATGCAGTCCTCCTG-3′

The PCR reactions were carried out in a total volume of 25 μl containing 100 ng of genomic DNA, 10 pmoles of each primer, 2 mM $MgCl_2$, 0.2 mM deoxynucleotides (dNTPs), 1 × buffer, and 2U of Taq DNA polymerase. The amplification was performed for 35 cycles with an annealing temperature of 58°C for 1 min for the IL-4 gene C590T polymorphism. The C—>T transition at codon 590 of the IL-4 gene abolished a restriction site for *Bsm*F1 in the T-allele. The polymorphism was identified by *Bsm*F1 restriction endonuclease digestion of the PCR-amplified fragment. The cleavage products were analyzed by 2% agarose gel electrophoresis and visualized under UV light after staining with ethidium bromide. The product sizes were 192 and 60 bp for the C-allele and 252 bp for the T-allele, respectively.

IL-13 Gene Polymorphism

The IL-13 gene polymorphisms R130Q (rs20541) and IL-13 gene promoter polymorphism (C- 1112T) was determined by PCR-RFLP methods described earlier (18, 19). The PCR primer sequences used were:

Sense primer: 5′-CTTCCGTGAGGACTGAATGAGAC GGTC-3′
Antisense primer: 5′-GCAAATAATGATGCTTTCGAAGTT TCAGTGGA-3′

For the R130Q polymorphism, amplification was carried out after denaturation at 94^0C for 5 min followed by 35 cycles of 94^0C for 45 s, 67^0C for 45 s and 72^0C for 30 s followed by an extension at 72^0C for 5 min. The PCR products were cleaved with restriction enzyme *Nla*IV (0.5 U) at 37^0C for 3 h and analyzed by agarose gel electrophoresis as described earlier. The expected cleavage products were 210 and 26 bp when a normal R130-genotype is present and in the case of mutant −130Q genotype the expected product size 178 bp, 32 and 26 bp, respectively.

Statistical Analysis

The genotypes were determined by gene counting and the data was analyzed using the Chi-squared test and the Fisher's exact test. Odds Ratio (OR) was calculated with 95% Confidence Interval (CI). *P*-values of 0.05 or less were considered as statistically significant.

Ethical approval was obtained from the Health Sciences Center Committee for the Protection of Human Subjects in Research, Kuwait University, as well as, Ministry of Health Ethics Committee. An informed consent was obtained from care givers of both patients and controls as per guidelines of the Committees.

RESULTS

The study included a total of 155 Kuwaiti children with INS. Of these, 132 were steroid-sensitive (SS) and 23 were steroid resistant (SR). The mean age in the INS patient group was 7.6 ± 4.3 years and Male: Female ratio was 2:1. The clinical and biochemical characteristics of INS patients and controls have been presented in **Table 1**. A total of 83 patients had history of atopy (53.5%), with some having more than one form of atopy. Asthma was reported in 53 patients (34%), eczema in 46 patients (27%), allergic conjunctivitis in 11 (7%), and allergic rhinosinusitis in 7 patients (4.5%). Food allergy was not reported in any patient. Atopy was not reported in any of the controls.

Genotype analysis of IL-4 gene polymorphism was inconclusive in 2 INS patients of the SS subgroup which were excluded from the statistical analysis for IL-4 gene polymorphism. From a total of 153 INS subjects and 64 controls, the CC genotype of IL-4 gene polymorphism was detected in 64% of the INS patients compared to 69.5% in the controls ($P = 0.57$). The heterozygous CT genotype was detected in 30% of INS patients compared to 25.5% in the controls ($P = 0.61$). The TT-genotype was detected in 6% of INS patients and in 5% of the controls ($P = 1.00$). The C-allele frequency in homozygous and heterozygous forms was found in 94% of INS patients compared to 95% of the controls ($P = 1.00$). The T-allele frequency in homozygous and heterozygous forms was found in 35.7% of INS patients compared to 30.5% of the controls ($P = 0.57$). **Table 2** summarized the gene and allele frequency of IL-4 gene polymorphism in both patients and controls. No significant difference was detected in any of the genotype frequencies between the SS and SR sub-groups when compared with each other or when compared to the controls (**Table 3**).

In the case of IL-13 gene polymorphism, genotyping was conclusive for all the 155 INS patients and 64 controls and the results have been presented in **Table 4**. The QQ genotype of IL-13 gene polymorphism was detected in 70.3% of the INS patients compared to 73.4% of the controls ($P = 0.74$). The heterozygous RQ genotype was detected in 25.8% of INS patients compared 28% of the controls ($P = 1.0$). The RR-genotype was detected in 3.9% of INS patients and 1.6% of the controls ($P = 0.68$). The Q-allele frequency in homozygous and heterozygous forms was found in 83.2% of INS patients compared to 86% of the controls ($P = 0.57$). The R-allele frequency in homozygous and heterozygous forms was found in 16.7% of INS patients compared to 14% of the controls ($P = 0.57$). When the genotype frequencies were compared between the SS and SR sub-groups, only the RQ genotype showed a statistically significant association with steroid sensitivity ($P = 0.04$; **Table 5**).

The data of SS patients and SR patients were studied separately and compared to controls. When IL-4 gene polymorphisms data of SR and SS patients were compared to controls separately, no significant differences in the genotype or allele frequencies were detected, as shown in **Tables 6**, **7**.

Similarly, IL-13 gene polymorphisms data of SR and SS patients were compared to controls separately. No significant differences in the genotype or allele frequencies were detected, when each sub-group compared to controls, as shown in **Tables 8**, **9**.

TABLE 1 | Clinical and laboratory data of Kuwaiti children with Idiopathic Nephrotic Syndrome (INS) and the controls.

Range		INS patients (*n* = 155)	Controls (*n* = 64)	Normal
Mean age (years)		7.6 ± 4.3	7.1 ± 3.7	
Gender	Male	104	42	
	Female	51	22	
Atopy (*n*)		83 (53.5%)	0 (0%)	
Mean serum creatinine (μmol/L)		47 ± 7	56 ± *8*	(15–88)
Mean serum protein (g/L)		50 ± 2	73 ± 2	(68–80)
Mean serum albumin (g/L)		21 ± 3	34 ± 3	(30–40)
Mean serum cholesterol (mmol/L)		6.5 ± 0.4	3.2 ± 0.6	(3.1–5.2)
Mean UP: Cr ratio* (mg/mg)		2.8±0.7	0.02±0.05	(≤ 0.2)

**UP, Urine Protein; Cr, Creatinine.*

TABLE 2 | Interlukin-4 gene polymorphism in Kuwaiti children with INS and the controls.

Genotype	INS (%) *n* = 153	Controls (%) (*n* = 64)	Odds ratio (OR)	95% confidence Interval (CI)	*P*-values
CC	99 (64.71)	44 (68.75)	0.83	0.45–1.56	0.68
CT	45 (29.41)	17 (26.56)	1.15	0.59–2.22	0.79
TT	9 (5.88)	3 (4.69)	1.27	0.33–4.86	1.00
C allele	243/306	105/128	0.84	0.49–1.44	0.62
T allele	63/306	23/128	1.18	0.69–2.01	0.62

TABLE 3 | Comparison of IL-4 gene polymorphism genotype and allele frequencies between steroid sensitive (SS) and steroid resistant (SR) Kuwaiti INS patients.

Genotype	SS (%) *n* = 130	SR (%) (*n* = 23)	Odds ratio (OR)	95% confidence interval (CI)	*P*-values
CC	83 (63.85)	16 (69.57)	0.77	0.29–2.01	0.64
CT	40 (30.77)	5 (21.74)	1.60	0.55–4.61	0.46
TT	7 (5.38)	2 (8.69)	0.59	0.12–3.08	0.63
C allele	226/260	37/46	1.62	0.72–3.65	0.25
T allele	54/260	9/46	1.08	0.49–2.37	1.00

TABLE 4 | Genotype and allele frequencies of Interleukin-13 gene polymorphism in Kuwaiti children with INS and controls.

Genotype	INS (%) $n = 155$	Controls (%) ($n = 64$)	Odds ratio (OR)	95% confidence interval (CI)	*P*-values
QQ	109 (70.32)	47 (73.44)	0.86	0.45–1.65	0.74
RQ	40 (25.81)	16 (25.0)	1.04	0.53–2.04	1.00
RR	6 (3.87)	1 (1.56)	2.54	0.29–21.52	0.68
Q allele	258/310	110/128	0.81	0.45–1.45	0.57
R allele	52/310	18/128	1.23	0.69–2.20	0.57

TABLE 5 | Comparison of IL-13 gene polymorphism genotype and allele frequencies between steroid sensitive (SS) and steroid resistant (SR) Kuwaiti INS patients.

Genotype	SS (%) $n = 132$	SR (%) ($n = 23$)	Odds ratio (OR)	95% confidence interval (CI)	*P*-values
QQ	90 (68.18)	19 (82.60)	0.45	0.14–1.41	0.22
RQ	38 (28.79)	2 (8.70)	4.25	0.95–19.00	0.04
RR	4 (3.03)	2 (8.70)	0.33	0.05–1.90	0.22
Q allele	218/264	40/46	0.71	0.28–1.78	0.53
R allele	46/264	6/46	1.41	0.56–3.51	0.53

TABLE 6 | Comparison of IL-4 gene polymorphism genotype and allele frequencies between Steroid Sensitive (SS) patients and controls.

Genotype	SS (%) $n = 130$	Controls (%) ($n = 64$)	Odds ratio (OR)	95% confidence interval (CI)	*P*-values
CC	83 (63.85)	44 (68.75)	0.80	0.42–1.52	0.61
CT	40 (30.77)	17 (26.56)	1.23	0.63–2.40	0.66
TT	7 (5.38)	3 (4.69)	1.16	0.29–4.63	1.00
C allele	226/260	105/128	1.46	0.82–2.60	0.26
T allele	54/260	23/128	1.20	0.70–2.06	0.61

TABLE 7 | Comparison of IL-4 gene polymorphism genotype and allele frequencies between Steroid Resistant (SR) patients and controls.

Genotype	SR (%) ($n = 23$)	Controls (%) ($n = 64$)	Odds ratio (OR)	95% confidence interval (CI)	*P*-values
CC	16 (69.57)	44 (68.75)	1.04	0.37–2.92	1.00
CT	5 (21.74)	17 (26.56)	0.77	0.25–2.39	0.78
TT	2 (8.69)	3 (4.69)	1.94	0.30–12.40	0.60
C allele	37/46	105/128	0.90	0.38–2.12	0.83
T allele	9/46	23/128	1.11	0.47–2.62	0.83

TABLE 8 | Comparison of IL-13 gene polymorphism genotype and allele frequencies between Steroid Sensitive (SS) patients and controls.

Genotype	SS (%) $n = 132$	Controls (%) ($n = 64$)	Odds ratio (OR)	95% confidence interval (CI)	*P*-values
QQ	90 (68.18)	47 (73.44)	0.78	0.40–1.51	0.56
RQ	38 (28.79)	16 (25.0)	1.21	0.61–2.39	0.70
RR	4 (3.03)	1 (1.56)	1.97	0.22–17.99	1.00
Q allele	218/264	110/128	0.78	0.43–1.40	0.48
R allele	46/264	18/128	1.29	0.71–2.33	0.48

TABLE 9 | Comparison of IL-13 gene polymorphism genotype and allele frequencies between Steroid Resistant (SR) patients and controls.

Genotype	SR (%) ($n = 23$)	Controls (%) ($n = 64$)	Odds ratio (OR)	95% confidence interval (CI)	*P*-values
QQ	19 (82.60)	47 (73.44)	1.72	0.51–5.78	0.57
RQ	2 (8.70)	16 (25.0)	0.29	0.06–1.36	0.14
RR	2 (8.70)	1 (1.56)	6.00	0.52–69.62	0.17
Q allele	40/46	110/128	1.09	0.40–2.94	1.00
R allele	6/46	18/128	0.92	0.34–2.47	1.00

DISCUSSION

The immunopathogenesis of INS has been studied extensively during the recent years (1, 5, 10). The effects of various cytokines on the glomerular basement membrane in the kidneys is widely supported by many studies on different populations (1, 5, 10). IL-4 and IL-13 are two important mediators which have recently attracted attention in studies exploring the pathogenesis of INS. As many INS patients commonly develop atopy, and IL-4 and IL-13 are the major mediators involved in that process, it was not surprising to incriminate these two cytokines in the immune-pathogenesis of the INS. Several previous reports have demonstrated that both these cytokines share a number of biological activities including IgE isotope switching, CD23 induction and stimulation of eosinophils activity as they both share a common IL-4 receptor alpha chain (IL-4RA) in the multicentric IL-4 and IL-13 receptor complexes (5, 6, 8, 15).

In the present study, we did not find an association between IL-4 or IL-13 gene polymorphism and predisposition to INS in Kuwaiti Arab children.

Many previous studies reported conflicting results (2, 10, 20). When our findings were compared with other populations worldwide, our results were consistent with data obtained from Caucasians in the UK where IL-4 gene polymorphism was studied in 100 patients and 63 controls with no significant difference between allele distribution in INS patients and the controls (2). However, Kobayashi et al. (20) studied IL-4 gene polymorphism in 58 Japanese children and found that the frequency of the T allele was significantly lower in INS group than the controls. It has been previously reported that T allele of the 590C/T polymorphism was associated with increased IL-4 gene promoter activity (21, 22). Another study carried out in India on 150 children with INS, demonstrated that IL-4-C590T polymorphism may influence the prognosis and the clinical course of the disease (23).

Our study did not show any association between IL-13 gene polymorphism and susceptibility of children with an Arab racial background to INS. However, the RQ genotype was associated with steroid sensitivity.

These findings were consistent with studies reported in different other populations worldwide. Data in both Asian and Caucasian populations suggest that there is no association of IL-13 gene polymorphism with susceptibility to minimal change nephrotic syndrome (10, 24, 25). Similar results have been reported from British INS patients (24) as well as on German INS patients (25). In Singapore Asian children also, no significant association of IL-13 gene polymorphism with INS was detected (10).

The lack of association of both 1L-4 as well as IL-13 gene polymorphisms and INS reported in our study as well as other studies seems to be unexpectedly surprising knowing the previously documented effects of both these cytokines in kidneys. Many previous *in vivo* as well as *in vitro* studies had supported the important roles of both cytokines and their receptors in the pathogenesis of INS. Podocytes in the glomerular basement membranes in kidneys of express receptors for both interleukins IL-4 and IL-13 (26, 27). These cytokines have been shown *in vitro* to have direct effects on podocyte mediator production and barrier function (26–28). A mechanism whereby excess IL-13 might induce nephrotic syndrome is illustrated by changes in podocyte protein trafficking and proteolytic enzyme secretion seen when these cells are incubated with IL-4 or IL-13 *in vitro* (29).

Another intriguing animal study directly addressed the role of IL-13 by overexpressing it in the rats and reported that an MCN-like nephropathy was induced, with changes in podocyte structure and gene expression similar to those seen in human diseases (30).

Moreover, Van den Berg showed the presence of IL-13 receptors on glomerular epithelial cells, the stimulation of which, resulted in a decrease in the transepithelial electrical resistance suggesting a possible direct effect of this cytokine on the podocytes and its function in preserving circulatory albumin and preventing albuminuria, which is the hallmark of INS (27). It has been reported that children with nephrotic syndrome who had higher initial serum IgE levels, had higher relapsing rates, longer duration of steroid therapy before remission and higher serum IL-4 and IL-5 levels (31).

A dysfunctional glomerular filtration barrier characterizes nephrotic syndrome, the pathophysiology of which is highly complex. T cells, mainly Th2 and Th17, play an important role. The upregulation of specific cytokines (IL-4, IL-5, IL-9, and IL-13) was previously shown to promote the development of NS (32). Therefore, studying the gene polymorphisms of these cytokines is essential.

Our study might be limited by a relatively small sample size. It would be prudent to have the serum cytokine levels for correlation. Despite being a single center study, ours is the focal center for nephrotic syndrome in the entire country where all the complex cases are referred so it covers the whole country. Although we studied patients with the same ethnic background, it is actually a strength of our study because this type of genetic association has not been performed in any population in the Gulf Region. We studied the homozygous and the heterozygous genotypes. The allele frequencies in the homozygous and heterozygous forms were included.

In conclusion, we report no association between IL-4 and IL-13 gene polymorphisms and the predisposition of INS in Kuwaiti Arab children. Of note, IL-13 RQ genotype was expressed more in children with INS who are steroid sensitive. Further larger studies, involving samples of different ethnic backgrounds, are warranted to determine the genetic predisposition of INS, as well as, steroid responsiveness in children.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, upon request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Health Sciences Center committee for the protection of human subjects in research, Kuwait University, as well as, Ministry of Health ethics committee, Kuwait. Written informed consent to participate in this study was provided by the participants' legal guardian, patients and controls, as per the committees recommendations.

AUTHOR CONTRIBUTIONS

MA and AA-E: substantial contributions to conception and design. AA-E: acquisition of data. MH: gene studies and data analysis. MA and AA-E: interpretation of the results. MA and AA-E: drafting the article. AA-E and MH: revising it critically for important intellectual content. All authors: final approval of the version to be published.

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CASE REPORT
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Case Report: CMV-Associated Congenital Nephrotic Syndrome

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Background: Congenital nephrotic syndrome, historically defined by the onset of large proteinuria during the first 3 months of life, is a rare clinical disorder, generally with poor outcome. It is caused by pathogenic variants in genes associated with this syndrome or by fetal infections disrupting podocyte and/or glomerular basement membrane integrity. Here we describe an infant with congenital CMV infection and nephrotic syndrome that failed to respond to targeted antiviral therapy. Case and literature survey highlight the importance of the "tetrad" of clinical, virologic, histologic, and genetic workup to better understand the pathogenesis of CMV-associated congenital and infantile nephrotic syndromes.

Case Presentation: A male infant was referred at 9 weeks of life with progressive abdominal distention, scrotal edema, and vomiting. Pregnancy was complicated by oligohydramnios and pre-maturity (34 weeks). He was found to have nephrotic syndrome and anemia, normal platelet and white blood cell count, no splenomegaly, and no syndromic features. Diagnostic workup revealed active CMV infection (positive CMV IgM/PCR in plasma) and decreased C3 and C4. Maternal anti-CMV IgG was positive, IgM negative. Kidney biopsy demonstrated focal mesangial proliferative and sclerosing glomerulonephritis with few fibrocellular crescents, interstitial T- and B-lymphocyte infiltrates, and fibrosis/tubular atrophy. Immunofluorescence was negative. Electron microscopy showed diffuse podocyte effacement, but no cytomegalic inclusions or endothelial tubuloreticular arrays. After 4 weeks of treatment with valganciclovir, plasma and urine CMV PCR were negative, without improvement of the proteinuria. Unfortunately, the patient succumbed to fulminant pneumococcal infection at 7 months of age. Whole exome sequencing and targeted gene analysis identified a novel homozygous, pathogenic variant (2071+1G>T) in *NPHS1*.

Literature Review and Discussion: The role of CMV infection in isolated congenital nephrotic syndrome and the corresponding pathological changes are still debated. A search of the literature identified only three previous reports of infants with congenital nephrotic syndrome and evidence of CMV infection, who also underwent kidney biopsy and genetic studies.

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Conclusion: Complete workup of congenital infections associated with nephrotic syndrome is warranted for a better understanding of their pathogenesis ("diagnostic triad" of viral, biopsy, and genetic studies). Molecular testing is essential for acute and long-term prognosis and treatment plan.

Keywords: Finnish-type nephrotic syndrome, *NPHS1*, cytomegalovirus, *Streptococcus pneumoniae*, case report, glomerulonephritis, infantile nephrotic syndrome

INTRODUCTION

Congenital nephrotic syndrome (CNS) is a rare disease with poor renal and overall outcome. It is defined by the occurrence of large proteinuria and hypoproteinemia, resulting in generalized edema during the first 3 months of life (1). The estimated incidence is 1–3 per 100,000 children worldwide (2–4). The etiology of the CNS is heterogeneous and may present as part of a genetic syndrome.

Congenital infections, particularly CMV and *Treponema pallidum* (syphilis), and occasionally *Toxoplasma gondii*, and other pathogens, have long been associated with rare instances of CNS (5–12). Although CMV and cytomegalic inclusions have been demonstrated (predominantly) in renal tubular epithelial cells of patients with various CMV-associated glomerulopathies (7, 13), the causative role and the pathomechanism of the virus in cases of glomerulonephritis and nephrotic syndrome are still debated (4, 10, 14).

Recent surveys revealed the presence of disease causing genetic variants in up to 80% of CNS cases (4, 10, 15, 16). *Bona fide* pathogenic variants commonly lead to profound structural and functional abnormalities of the podocyte and/or glomerular basement membrane that compromise the glomerular filtration barrier. The prototypic, "Finnish type" congenital nephrotic syndrome is due to biallelic pathogenic variants in *NPHS1* (17, 18). Genes occasionally involved in congenital or infantile nephrotic syndrome with overlapping clinical and histological phenotypes include *NPHS2* (podocin), *LAMB2 (beta2-laminin)*, and *WT1* (Wilms Tumor 1 transcription factor), among others (4).

Early subtle histological alterations, such as microcystic proximal tubular dilatation, eventually lead to nephron loss and end stage kidney disease (ESKD) by the age of 2–3 years (1, 15, 19, 20). The spectrum of histological changes encompasses diffuse mesangial sclerosis (DMS), focal segmental glomerulosclerosis (FSGS), membranous glomerulopathy, and minimal change disease that are largely determined by the type of mutation and age at biopsy (1, 4, 15, 20, 21). The long-term management of patients with CNS remains challenging and may require early nephrectomy to minimize the pervasive effects of massive proteinuria, and subsequent kidney transplantation (4, 18, 22, 23).

We hypothesized that previously postulated infectious etiologies of CNS cases are biased due to the lack of biopsies and—historically not feasible—comprehensive genetic studies.

Here, we report an infant with CNS and CMV infection and demonstrate the importance of timely and complete diagnostic workup, i.e., the tetrad of clinical findings and virological/infectious disease, histopathologic, and molecular genetic studies.

CASE DESCRIPTION

A 9-week-old male (ex 34 weeks gestational age, corrected age 3 weeks) had been referred to our Emergency Department with suspected surgical abdomen. He presented a 3-day history of non-bilious, non-bloody vomiting and a 2-week-history of increasing abdominal distention and scrotal swelling. The patient was the second child of his parents who are from Kerala, India, and distantly related (3rd degree cousins). The couple's firstborn son is healthy.

Pregnancy was complicated by moderate oligohydramnios and decreased fetal movements which led to urgent C-section. Mother denied fever or rash during pregnancy, and her urinalysis was normal.

Birth weight was 1970 g (20th weight percentile). The weight of the placenta is not known. APGAR was 8 and 8 after 1 and 5 min, respectively. He received CPAP and then oxygen via nasal cannula for a total of 3 days. A post-natal brain ultrasound study was reportedly normal, TSH was 12 mU/L, and he was discharged home after 6 days. Immunizations were up-to-date, including PCV13.

Clinical Exam

At presentation, the infant was alert, irritable, pale and grunting. He was normocephalic without dysmorphic features. There was no rash, no jaundice, no petechiae or ecchymoses. He was tachycardic at 175 beats per minute and tachypneic. The remainder of the cardiovascular and pulmonary findings was unremarkable. The abdomen was distended, tense and shiny with large ascites and no palpably enlarged liver or spleen. Substantial scrotal edema was noted. Weight (with edema) was 3.5 kg (0.01%, z score −3.83), length 52.5 cm (0.00%, z score −4.31), and head circumference 35.5 cm (0.04%, z score −3.35).

Investigations

At the time of admission, there was large proteinuria, hypoalbuminemia, and hypercholesterolemia, consistent with nephrotic syndrome. Nephrotic range proteinuria was defined as a spot urine protein-to-creatinine ratio of >0.23 g/mmol (corresponding to >2.0 g protein/g creatinine). For details and definitions, see **Table 1**). The hemoglobin (Hb) level dropped from 100 to 69 g/L over the first 10 days of admission, with inadequate reticulocyte response and normal serum ferritin and iron levels. Serum C3 and C4 concentrations, measured on

TABLE 1 | Laboratory results during the disease course[a].

Parameter	Reference ranges	Presentation (age 9 weeks)	Start VGC (age 10 weeks)	One month VGC (age 14 weeks)	Last results (age 7.4 months)
Urine protein (g/L)	g/L	>2.0 (4+)	>2.0 (4+)	17.2	12.2
U protein creatinine ratio, nephrotic range	>0.23 g/mmol (>2.0 g/g)	>0.4 [b] (>3.52 g/g)	>0.7 [b] (>6.92 g/g)	6.44 (56.94 g/g)	3.71 (32.75 g/g)
Hematuria (dipstick/microscopy)	Dipstick negative 0–3/HPF	Small -	Small -	3+ >35	3+ >35
Serum albumin	33–54 g/L	9	17 [c]	9	12
Total serum protein	51–73 g/L	24.5	33.2	25.5	-
Toxoplasma IgM/IgG		neg/neg	-	-	-
Rubella IgM/IgG		neg/neg	-	-	-
CMV IgM	<0.69 COI	4.56	-	-	-
CMV IgG	<0.49 U/mL	5.54	-	-	-
CMV PCR blood		positive	-	negative	-
CMV PCR urine		positive	-	negative	-
HSV 1 & 2 PCR blood		neg/neg	-	-	-
Syphilis (*T. pallidum*)		Neg	-	-	-
Hepatitis B (HBs) Ag	<1.0 COI	-	-	0.394	-
Anti-HBs Ab	<10 mIU/mL	<2.00	-	4.2 [d]	-
Anti-HBc Ab		Non-reactive	-	-	-
Hepatitis C (anti-HCV Ab)		Non-reactive	-	-	-
EBV PCR (blood)		negative	-	-	-
Hemoglobin	111–131 g/L	100 [e]	69 [f]	93	106
WBC	6.0–16.0 × 10^9/L	11.7	12.6	5.6	16.8
Absolute neutrophil count	1.00–6.00 × 10^9/L	1.62	0.79	0.79 [g]	4.8
Platelets	200–550 × 10^9/L	328	357	584 [h]	827
Serum IgG	3.09–15.73 g/L	<0.4	-	-	-
Serum IgA	0.08–0.58 g/L	0.11	-	-	-
Serum IgM	0.04–0.89 g/L	0.49	-	-	-
Serum C3	0.82–1.67 g/L	-	0.31	-	-
Serum C4	0.14–0.44 g/L	-	0.08	-	-
C-reactive protein (CRP)	0–2.80 mg/L	0.42	-	0.63	-
Cholesterol	2.1–3.8 mmol/L	10.4	-	-	10.4 [i]
ALT	0–57 U/L	17	11	12	10 [i]
AST	0–110 U/L	28	40	27	30 [i]
GGT	0–203 U/L	1036/519 [j]	226	161	61 [i]
Total bilirubin	0–20.52 μmol/L	9.75/41.55 [j]	13.68	< 2.39	< 2.39 [i]
25 OH D3	75–250 nmol/L	32.5	-	-	27.5 [l]
1,25 $(OH)_2$ D3	48–190 pmol/L	-	-	-	221 [l]
Intact PTH	1.6–6.7 pmol/L	10.8	-	-	11.3 [l]
TSH	0.73–8.35 mU/L	23.23	4.97 [k]	-	4.11 [m]
Free T4	11.9–25.6 pmol/L	7.1	22.2 [k]	-	12.4 [m]
Body weight	kg	3.50 [n]	3.20	4.05	5.22
WHO growth chart	Centile (z score)	0.01 (−3.83)	0.00 (−4.89)	0.00 (−4.01)	0.00 (−4.21)
Head circumference	cm	35.5	-	-	-
WHO growth chart	Centile (z score)	0.04 (−3.35)	-	-	-

[a] *COI cut-off index, HPF high power field, VGC valganciclovir, - not obtained at that indicated interval.*

[b] *Protein titration in urine was not available initially. See Kidney Disease Improving Global Outcomes (KDIGO) Clinical Practice Guideline for Glomerulonephritis (2012) for the definition of nephrotic-range proteinuria.*

[c] *Following albumin infusions.*

[d] *Measured 2 weeks after commencing after HBV vaccination.*

[e] *Normocytic anemia with inadequately low reticulocyte count (2.38%).*

[f] *Patient received RBC transfusion following this result.*

[g] *Subsequent neutrophil counts remained >2 × 10^9/L.*

[h] *Persistently elevated platelet counts following treatment with VGC and the suppression of detectable CMV DNA.*

[i] *Last measurement at age 5.1 months.*

[j] *Change from day 1 to day 3 of admission; direct bilirubin was about 20% of total bilirubin.*

[k] *Rise of serum bilirubin concentration prior to commencement of VGC treatment.*

[l] *Supplementation with 2,000 IU cholecalciferol daily.*

[m] *Measurements during L-thyroxin supplementation.*

[n] *edematous.*

day 11 of admission, were decreased. The concomitant direct agglutination (Coombs) test was negative. Secondary findings were hypogammaglobulinemia, hypothyroidism with elevated TSH and low free T4 levels, hypovitaminosis D, low-normal serum ionized calcium (1.17 mmol/L) and moderately elevated intact PTH. ALT and AST were normal. GGT and bilirubin concentrations peaked during the first few days after admission (see **Table 1**).

The ultrasound (US) scan showed enlarged kindeys with mildly increased cortical echogenicity. Liver and spleen were of normal size and echotexture. The brain US study was normal, except the presence of thalamostriate mineralizing vasculopathy.

Cardiac echography demonstrated a small perimembranous ventricular septal defect of 2–3 mm with left-to-right shunt and patent foramen ovale, none requiring specific interventions.

Infectious disease workup revealed anti-CMV IgM antibodies and CMV DNA (by PCR) in blood and urine. Maternal screening during pregnancy for toxoplasma (IgG and IgM), rubella (IgM), HIV 1 & 2 antibodies/P24 antigen, hepatitis B (HBsAg) and hepatitis C (antibodies), and syphilis was negative. Subsequent CMV testing showed high maternal serum concentrations of CMV IgG, but no anti-CMV IgM.

Treatment of the Patient

Treatment consisted of frequent albumin infusions, diuretics and ACE inhibition, with improvement of ascites and peripheral edema. He also received L-thyroxin, vitamin D and oral penicillin prophylaxis, low-dose acetyl salicylic acid, iron, and indomethacin. In addition, he was vaccinated against *Streptococcus pneumoniae* (PCV13, twice) and hepatitis B.

Valganciclovir treatment was initiated 9 days after presentation at a dose of 22 mg/kg/day. CMV DNA became undetectable in plasma and urine after 1 month of antiviral therapy, however proteinuria failed to improve (**Table 1**) strengthening the presumptive clinical diagnosis of a genetic form of congenital nephrotic syndrome.

The patient's energy and protein intake remained precariously inadequate. However, parents were reluctant to agree to g-tube insertion or (unilateral) nephrectomy. Tragically, at the age of 7.9 months, following a few days of lapsed penicillin administration due to vomiting and diarrhea, the patient succumbed to fulminant sepsis, within hours after arrival in the Emergency Department, caused by pan-sensitive *S. pneumoniae*.

Kidney Biopsy

A kidney biopsy was performed 15 days after hospitalization (6 days after initiation of VGC treatment) to differentiate the pathohistological changes underlying the nephrotic presentation (1, 2, 4, 12). The majority of the >100 sampled glomeruli showed mild mesangial hypercellularity with patent capillaries and normal glomerular basement membrane (GBM) contours. Ten percent of the glomeruli were globally sclerosed or segmentally scarred. Three glomeruli showed active cellular or fibrocellular crescents with proliferation in the Bowman space and focal ruptures of GBM. There was a patchy, mononuclear tubulointerstitial inflammatory infiltrate. Infiltrating interstitial lymphocytes stained positive for CD3 and CD20, respectively, indicating the presence of T- and B-cells. Trichrome staining showed fibrosis in <5% of the cortex. Arteries and arterioles were histologically normal. Immunohistochemical staining for CMV was negative. Routine immunofluorescence showed faint, likely non-specific staining for IgM and C3. Electron microscopy revealed diffuse podocyte foot process effacement with prominent microvillous transformation, but no immune-type deposits. Endothelial fenestrations were intact, and no viral or endothelial tubuloreticular inclusions were noted (**Figure 1**).

Genetic Studies

Genomic DNA, extracted from peripheral blood cells, underwent a series of ultra-sonication, chemical, and enzymatic steps to generate a sequencing-ready library of short fragments (300–400 bp) using the SureSelect^{XT} kit (Agilent, USA). RNA capture probes targeting all coding regions were used to enrich for whole exome regions using the SureSelect Clinical Research Exome V2 kit (Agilent, USA). The enriched library was then subjected to next generation sequencing (2 × 150 bp) using the SP flow cell and the NovaSeq platform (Illumina, USA). Sequencing data were then processed using an in-house custom made bioinformatics pipeline to retain high quality sequencing reads with at least 100X coverage across all coding regions. Rare variants in 99 genes associated with nephrotic syndrome (**Supplementary Table 1**) were filtered for analysis and interpretation using the American College of Medical Genetics and Genomics (ACMGG) Sequence variant interpretation guideline (24). Two variants in the *NPHS1* and the *ITSN2* genes met the ACMGG criteria for reporting. *NPHS1* encodes nephrin which is central for the integrity of the podocyte slit diaphragm. Pathogenic variants in *NPHS1* cause classical Finnish-type congenital nephrotic syndrome (17). The apparently homozygous 2071+1G>T variant in *NPHS1* (NM_004646.3) has not been previously reported in individuals with disease and is absent from large population studies such as the Genome Aggregation Database (genomAD) and the Greater Middle East (GME) Variome database. This variant occurs in the conserved region (±1,2) of the splice consensus sequence of the only known *NPHS1* transcript, and is predicted to cause altered splicing leading to an abnormal or absent protein (25). The mutation is apparently homozygous, although a large deletion of the second allele could not be ruled out since parental testing was not conducted.

A heterozygous c.2713G>A p. (Ala905Thr) missense variant was also reported in *ITSN2* (NM_006277.2), the gene encoding intersectin 2, a member of the guanine exchange factor (GEF) family of proteins that activate Cdc42. This variant was classified as uncertain due to lack of sufficient evidence supporting its clinical significance. Bi-allelic pathogenic variants in *ITSN2* have been reported as a novel cause of nephrotic syndrome (26). The identified variant is absent from large population studies such as the Genome Aggregation Database (gnomAD) and the Greater Middle East (GME) Variome database. Computational prediction tools did not provide evidence for or against pathogenicity. No variants were discovered in complement-related genes or genes associated with (other) immunodeficiencies.

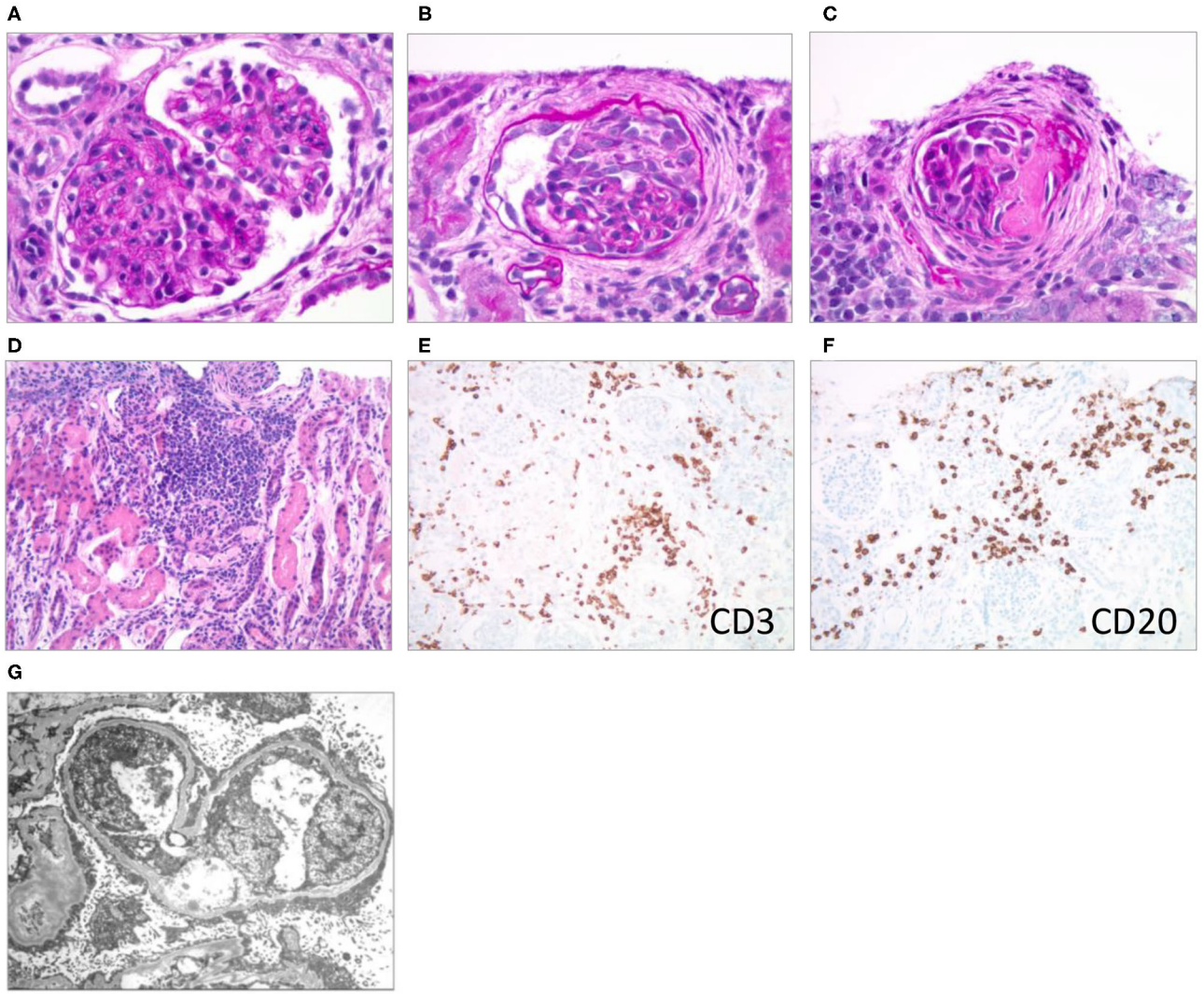

FIGURE 1 | Renal pathological presentation (kidney biopsy of the proband 15 days after admission). **(A–C)** Brightfield microscopy (PAS stain x600); **(D)** brightfield (H & E stain x200); **(E,F)** immunohistochemistry (x200); **(G)** electron microscopy. **(A)** Mesangial and endocapillary hypercellularity, **(B)** cellular crescent, **(C)** segmental scar/fibrous crescent, **(D–F)** interstitial infiltrates, **(G)** diffuse podocyte foot process effacement with prominent microvillous transformation.

SURVEY OF THE LITERATURE

CMV-Associated Congenital and Infantile Nephrotic Syndrome

Following the identification of a pathogenic *NPHS1* variant as the genetic cause of the patient's nephrotic syndrome, we wondered whether recognizable histopathological features would allow differentiating CMV-associated lesions (and CMV CNS) from CNS due to defined genetic variants. We therefore searched the available literature (PubMed and Google Scholar, without language restriction) for CMV-associated/infantile nephrotic syndrome with histological and genetic findings ("tetrad" of documented CMV infection and clinical features, kidney biopsy, and mutation screen).

Our survey identified only three cases with a complete tetrad: one patient with a homozygous *NPHS2* pathogenic variant, one patient with reported pathogenic variants in both *NPHS1* and *COL4A5* (however the number of variants and the phase of the variants in each gene was not detailed), and one with no detectable variants in *NPH1*, *NPH2,* and *WT1* (**Table 2**; patients #3, 7, and 6, respectively) (12, 14, 31). All three patients were treated with ganciclovir (GCV). Only patient #6 achieved sustained remission of proteinuria (31).

We then searched for reports of patients with CMV-associated congenital or infantile nephrotic syndrome, who also had a kidney biopsy. All identified cases (#1, 2, 4, 5) were treated with GCV (or GCV, followed by VGC), and all entered stable, proteinuria-free remission (**Table 2**). Mean antiviral treatment duration was 38 ± 27 (median 30) days; proteinuria improved substantially or disappeared after a mean of 20 ± 5 (median 19) days. The nephrotic syndrome was GCV-resistant in three cases (#3, 7, and 8). These patients were significantly younger at

TABLE 2 | CMV-associated congenital and infantile nephrotic syndrome (Literature review and proband).

#	Age onset Sex (gestation)	Pregnancy/ perinatal	Clinical presentation	Urinalysis Serum albumin	Infectious diagnostic	Treatment	Proteinuria after antiviral	Outcome	Genetic variants	References
1	5 mo F (39 w)	PBWR 0.2 (Maternal NS 8 mo in pregnancy (FSGS), resistant to Pred & POCY)	Normal at birth At 5 mo generalized edema At 6.5 mo fever, purpuric maculo-papular rash, hepatomegaly elevated ALT, LDH	Large proteinuria (>40 mg/m^2/h) Hypoalbuminemia (23 g/L)	At 5 mo CMV IgM+ (mat CMV IgM-, IgG+) (both Toxo, HSV, VZV, RV, Syph, HBV, HCV neg) (C3 &C4 N) At 6.5 mo CMV B-, U+, IgM+	At 5mo: High-dose Pred (ineffective) At 6.5mo GCV x 15 d	**Resolved** (proteinuria improved at 15d)	At 12 mo Clinically well, all symptoms resolved Sustained proteinuria remission, N renal function	ND	(27)
2	5 mo M (Term)	PBWR 0.19	Acute, generalized edema after URTI/vomiting US KUB normal	Large proteinuria (>40 mg/m^2/h) Hypoalbuminemia (6.9 g/L)	CMV IgM+, IgG+ (EBV, HBV/HBC, HIV, HSV, Syph, VZV neg) (mat CMV IgM-, IgG+)	Pred 60 x4w (ineffective), then oral GCV x12w	**Resolved** (remission at 4 w GCV)	Sustained proteinuria remission Stable at 17 mo FU	ND	(28)
3	45 d F (36 w)	Mild dysmorphic features Antenatal US large, echogenic kidneys) Ebstein anomaly	Generalized edema/ascites/pleural effusion Bilateral vitreitis Bilateral SNHL Increased renal echogenicity	Large proteinuria (170 mg/m^2/h) Hypoalbuminemia (22 g/L)	CMV U+, IgM+ (Toxo, RV, Syph, HBV, HCV, HIV neg) C normal	Daily IV Alb/ diuretic GCV (duration NR)	Persistent	Resolution SNHL & vitreitis 6 mo post-GCV ESRD at 21 mo	*NPHS2* [a]	(14)
4	57 d F (33 w)	Pregnancy uncomplicated Not dysmorphic	Generalized edema, HTN Respiratory symptoms Anemia 58 g/L, platelets 75/nL Enlarged, echogenic kidneys Brain cortical atrophy (no calcifications, no retinitis)	Large proteinuria (454 mg/m^2/h) Hypoalbuminemia (20 g/L) UA: WBC, RBC, gran casts	CMV IgG+, IgM- CMV PCR serum high (neg HBV/HCV, HSV1/2, RV, toxo Ab) (Mat CMV IgM-, IgG+)	Daily IV Alb & diuretics Captopril IV GCV x 3 w	**Resolved** Upc 0.079 g/mmol at 3w	Hematologic response, HTN resolved Remission >14 mo FU	ND	(29)
5	6 mo	NR	Persistent watery diarrhea Generalized edema & ascites Hb 107 g/L Plt 406/nL	Large proteinuria (28 g/L) Hypoalbuminemia (10 g/L)	CMV PCR (blood/urine?)	IV alb x 19d Captopril IV GCV x 2w, then VGC x 4w	**Resolved** Up low (CMV PCR neg) at 19d GCV/VGC	Resolution of colitis CMV PCR neg 19d Stable remission 1y	ND	(30)

(Continued)

TABLE 2 | Continued

#	Age onset Sex (gestation)	Pregnancy/ perinatal	Clinical presentation	Urinalysis Serum albumin	Infectious diagnostic	Treatment	Proteinuria after antiviral	Outcome	Genetic variants	References
6	5 mo M	Diamniotic twin pregnancy	Gastroenteritis Mild edema Large kidneys No dysmorphism/brain lesions Normal ophthalmological exam	Large proteinuria (Upc 7.83 g/mmol) Hypoalbuminemia (10.3 g/L) Normal GFR	CMV B-, PCR+ (EBV (PCR), HBV, HCV, HIV, Syph neg)	IV Alb x 16d Captopril x 16d IV GCV x 15d (from D15), then VGC x 15d	**Resolved** Up 0.15 g/L at 15d, Salb N	Sustained remission over 30 mo FU	Negative (*NPHS1*, *NPHS2, WT1*) [b]	(31)
7	15 d	NR	"asymptomatic"	Large proteinuria (3+, 2.82 g/mmol) Hypoalbuminemia (21.7 –>11.0 g/L) Normal cholesterol	CMV IgM+	GCV 10 x 4w	Partial remission (Upc 0.189, S-Alb 30.4 g/L)	CKD at 13mo (eGFR 55 mL/min/1.73m^2) Proteinuria 3+	*NPHS1*, *COL4A5* [c]	(12)
8	51 d M (34 w)	Oligohydramnios asymmetric IUGR	Ascites, scrotal edema FFT, developmental delay Anemia (100 69 g/L) GGT/mild hyperbilirubinemia Thalamostriate mineralizing vasculopathy	Large proteinuria (4+, 3.4 g/mmol) Microhematuria (15-20 RBC/HPF) Hypoalbuminemia (9 g/L) eGFR normal	CMV IgM+ CMV B-PCR+ CMV U-PCR+ (Toxo, RV IgM, Syph neg) Mat CMV IgG+, IgM- Low C3 &C4	Captopril VGC IV Alb/diuretic Pen-VK prophylaxis	persistent	CMV PCR neg 4 w post VGC, FFT Death pneumococcal sepsis at 7.9 mo	*NPHS1 (INTS2 – het)* [d]	This report

[a] *Homozygous for nonsense mutation c.412C<T (p.Arg138X), carrier status confirmed for both parents (14, 32).*

[b] *Testing was limited to the indicated genes. Methodological details are not reported.*

[c] *NPHS1 variant reported as c.2396G>T (p. Gly799Val) and c.1339G>A (p. Glu477Lys), COL4A5 variant not specified (12).*

[d] *Detail see Results section.*

CKD, chronic kidney disease; d, day(s); eGFR, estimated glomerular filtration rate (33); ESRD, end stage renal disease; FFT, failure to thrive; GGT, gamma glutamyl transferase; GCV, ganciclovir (IV); Hb, hemoglobin; HPF, high power field; HTN, hypertension, mo month(s); N, normal; NR, not reported; PBWR, placenta birthweight ratio; Plt, platelet(s); Upc, (urine protein-to-creatinine ratio), VGC, valganciclovir (oral); w, week(s).

SI unit conversion Upc (g/mmol) × 8.84 = g/g (reference range for nephrotic range proteinuria >0.23 g/mmol, corresponding to 2.0 g/g).

nephrotic syndrome onset (median 1.5, range 0.5–1.7 months) than GCV "sensitive" patients (median 5, range 1.9–6 months) ($P < 0.05$, unpaired t-test). There was no apparent difference in the presenting nephrotic features (urine protein excretion and serum albumin concentrations) between GCV/VGC responsive and refractory patients.

Kidney Biopsy Results in the Literature

Results of the biopsy survey are detailed in **Table 3**. In this analysis we included also patients without genetic results under the assumption that stable remission from CMV-associated nephrotic syndrome indicates the absence of podocyte gene mutations. We identified and evaluated eight patients (including our case) with a total of 9 biopsies. The biopsies of four of the five GCV "sensitive" (mutation "negative") patients (#1, 2, 4, and 6) (27–29, 31) revealed mild-moderate mesangial cell proliferation with or without (mesangial) matrix increase. One of these biopsies also demonstrated mesangial sclerosis (#4). This contrasts with the findings in patients with GCV "resistant" (mutation "positive") CNS (#3, 7, and 8) (12, 14), where light microscopic findings demonstrate a spectrum of normal-appearing glomeruli (#3a), glomerular sclerosis (#3b and 8) and occasional cellular or fibrocellular crescents, as well as focal mesangial proliferation (#7 and 8).

DISCUSSION

The presented case demonstrates features of classical (Finnish-type) congenital nephrotic syndrome (CNS) due to a homozygous *NPHS1* pathogenic variant, complicated by congenital CMV infection. There was no retinopathy or microcephaly, and apart from (non-specific) thalamostriate mineralizing vasculopathy (34, 35), the infant had no detectable cerebral abnormalities by ultrasound. However, he had substantial anemia (resulting in RBC transfusion), neutropenia (without thrombocytopenia) and temporary GGT and bilirubin elevation (without hepato- or splenomegaly). The C3 and C4 hypocomplementemia was surprising and deserves further inquiry in comparable cases of congenital/Finnish type nephrotic syndrome and of congenital CMV infections, whereas the extreme hypogammaglobulinemia resulted in all likelihood from massive protein loss via the disrupted glomerular filtration barrier.

Intrauterine or perinatal CMV exposure may have been responsible, at least in part, for the presenting signs and symptoms in our patient. However, there are no criteria to predict the role of CMV in this or similar patients' nephrotic syndromes. Due to health insurance reasons, we were unable to pursue early genetic testing. However, we proceeded with a kidney biopsy for diagnostic and therapeutic guidance (1, 4).

The histological findings were consistent with what has been described in CMV-related congenital nephrotic syndrome, including proliferative lesions and diffuse podocyte effacement. Lack of CMV detection (or inclusion bodies) in renal tissue makes it less likely that the virus was the direct cause of the observed morphological changes. Some of the demonstrated lesions may have arisen as part of the inflammatory and cytokine response of the host, albeit without virus or interferon-gamma-induced (endothelial) tubuloreticular arrays (36, 37). Interestingly, serum C3 and C4 protein levels were decreased during the early course, yet we failed to observe unequivocal immune deposits in the kidney biopsy.

Antiviral treatment was combined with supportive measures, mainly diuretic-assisted albumin infusions, thyroid hormone and vitamin D which controlled the profound edema and hormonal deficiencies. Persistence of large proteinuria and dependency on albumin infusions after the CMV PCR had become negative, favored a genetic cause of the nephrotic syndrome. Attempts to convince the parents to optimize nutrition (g-tube feeding) and (unilateral) nephrectomy to reduce protein losses (2, 4, 22) remained unsuccessful. Unfortunately, he succumbed to foudroyant pneumococcal sepsis at almost 8 months of age, despite repeat vaccination against *S. pneumoniae* and the prescription of antibiotic prophylaxis, which highlights the highly immunocompromised status of infants with severe nephrotic syndrome. We were eventually able to perform whole exome sequencing and can at least offer genetic counseling for the family.

The occurrence of NS in newborns with intrauterine exposure to CMV has been known since more than 50 years (7, 13). While infants with overwhelming congenital CMV disease demonstrate CMV invasion and proliferation in the kidney along with many other tissues (7), the mechanism of CMV-induced glomerular injury and podocytopathy is not well-defined. Previous studies have addressed direct, virus-mediated tissue injury and injury induced by the host immune response, such as T cell infiltration or immune complex formation (38, 39). However, the natural history and outcome of theses lesions has not been adequately documented, and there is a paucity of cases correlating virological and serological results with renal tissue and genetic studies.

The historical attribution of (severe) CNS to concurrent (congenital) CMV infection, including some of the histological changes, needs be revisited (4, 14). This notion does not negate the possible occurrence of (large) proteinuria and edema in newborns with severe, CMV-induced injuries, such as microcephaly or sensorineural hearing loss, with or without hepato- and splenomegaly and cytopenia.

Although the number of reported cases of CMV-associated CNS with a complete diagnostic "tetrad" is surprisingly small (**Table 2**), our analysis seems to confirm that an onset of nephrotic syndrome within 3 months of life predicts an underlying variant within podocyte or GBM-related genes, regardless of a coincidental CMV infection. In contrast, onset of nephrotic syndrome between 4 and 12 months and active CMV proliferation in previously asymptomatic infants appears to increase the likelihood of CMV as the remediable cause of the nephrotic syndrome. Neither the severity of proteinuria or hypoalbuminemia at presentation, nor (estimated) GFR or hematological parameters (anemia or thrombocytopenia)–where reported–appear to have discriminatory power in this small cohort.

Table 3 juxtaposes the histological findings of CMV-associated congenital and infantile NS. All but one patient demonstrating sustained remission of proteinuria following

TABLE 3 | Renal biopsy findings (Literature review and proband).

#	Onset/Bx (age in mo)	Glomerular compartment/BFM	IF/IH	EM	Tubulo-interstitial compartment	CMV-related findings	Genetic findings[a]	References
1	5/5.5	Enlarged glomeruli Mild mesangial cell proliferation	minimal mesangial IgM & C1q	Podocyte vacuolization w/o enlargement Foot process effacement Normal GBM Minor mesangial deposits Enlarged endothelial cells/single platelet thrombus/ scattered fibrin strands	NR	Endothelial tubulo-reticular arrays	ND	(27)
2	5/5	Mod mes cell & matrix increase in 25% of glomeruli w/o sclerosis	Diffuse mes IgM 2+	GBM normal	Normal w/o infiltrates or atrophy	No CMV inclusions Tissue PCR CMV+	ND	(28)
3a	1.5/<2	Normal-appearing glomeruli	NR/normal	NR	Normal (and normal vasculature)	Single inclusion body in distal tubule	*NPHS2*	(14)
3b	1.5/16	3/13 glomeruli globally sclerosed Remainder w/ mild mesangial proliferation & expansion	NR/normal	Foot process effacement	Normal (and normal vasculature)	Inclusion bodies absent Tissue CMV PCR & culture neg		
4	2/∼2.3	Increased mes matrix & cell proliferation Some glomeruli w/ global, the remainder w/ seg mes sclerosis	Absent staining for IGs & C	NR	Focal tubular atrophy and fibrosis w/ tubular dilatation. Some tubules w/ Inflammatory infiltrates	Cytomegalic inclusion bodies in tubules and some glomeruli	ND	(29)
5	6/6	Mild endocapillary proliferation[b]	NR	NR	Atypical heavy tubular lesions, interstitial edema	NR	ND	(32)
6	5/5	Mild mes cell hypertrophy No mes sclerosis	NR	ND	Non-specific tubular lesions Interstitial edema	Absent viral cytopathic inclusions	Negative	(31)
7	0.5/2	Mild mesangial proliferation and Matrix increase. Multiple immature glomeruli with cellular crescents	Mesangial deposits of IgM (++) and C3 (+)	Mild mesangial cell/ matrix hyperplasia Podocyte foot process fusion Normal GBM No electron dense deposits	Vacuolar & granular degeneration tub epithelial cells, small focal atrophy Focal lymphoid interstitial mononuclear cells Eosinophil infiltrates w/ fibrosis Thickened small arterial walls	No CMV inclusions, CMV DNA neg	*NPHS1* *COL4A5*	(12)
8	1.7/2.6	Global or segmental sclerosis in 10% Focal mesangial proliferation Few cellular & fibrocellular crescents Mild cortical fibrosis (<5%)	Negative (non-specific trapping of IgM and C3)	Diffuse podocyte foot process effacement w/ microvillous transformation Intact endothelial fenestration	IF/TA Patchy, moderate tubule-interstitial infiltrates (CD3+, CD20+)	No endothelial tubulo-reticular arrays Staining for CMV negative	*NPHS1*	This report

[a] *Homozygous or compound heterozygous (except for COL4A5). For genetic details, see* ***Table 2****.*

[b] *Very brief pathology description.*

BFM, brightfield microscopy; Bx, kidney biopsy; C, complement; CKD, chronic kidney disease; d, day(s); EM, electron microscopy; ESRD, end stage renal disease; GCV, ganciclovir (IV); IF/IH, immunofluorescence/immuno histological stain; IF/TA, interstitial fibrosis/tubular atrophy; IG(s), immunoglobulin(s); mes, mesangial; mo, month(s); ND, not done; neg, negative; NR, not reported; w, week(s).

antiviral therapy, presented after 3 months of age. One of the common features of all patients with CMV-associated congenital or infantile nephrotic syndrome appears to be (usually mild) mesangial proliferation with or without matrix accumulation. Segmental and global glomerulosclerosis (40) and crescent formation is only noted in the two *NPHS1* patients. Given the paucity of completely defined cases and the heterogeneity of renal pathology among patients with autosomal recessive forms of CNS, the histological differentiation between CMV-induced congenital or infantile NS and patients with an underlying genetic variant remains challenging.

The *NPHS1* mutation identified in our patient is apparently homozygous, although a large deletion of the second allele could not be ruled out as parental testing was not possible. The recurrence risk would remain the same [25%] if the variant was homozygous, or compound heterozygous with a pathogenic deletion. Identifying the exact variant(s) allows for targeted variant analysis either prenatally or via *in vitro* fertilization and pre-implantation genetic diagnosis) for any future pregnancies.

The strength of this report is the complete characterization of the case and others identified in a comprehensive literature survey. Its limitation—and reason for publication—is the small number of fully described cases.

CONCLUSIONS

Genetic testing is warranted in all patients presenting with isolated or syndromic nephrotic syndrome immediately after birth or within the first 2–3 months of life, whether CMV is present or not. To further clarify the specific contributions of CMV and CMV disease to the histological changes in the kidney and severe, persistent nephrotic syndrome, a correlation (tetrad) of clinical and laboratory findings, kidney biopsy with all staining modalities and/or molecular tools, and genetic work-up are needed.

ETHICS STATEMENT

Written informed consent was obtained from the minors' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

AJ collected the data, reviewed the literature, and wrote the first draft of the manuscript. SH, ES, JFS, and AJ participated in the care of the patient. LH read and interpreted the kidney biopsy and provided the photographs. AT and AA were responsible for genetic counseling, performed whole exome sequencing, and analyzed and interpreted the molecular data. WA provided expert infectious disease consultation. MB supervised the care of the patient, reviewed all clinical and laboratory data, performed an independent comprehensive literature review, and wrote the final version of the manuscript. All authors critically reviewed and edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2020.580178/full#supplementary-material

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BRIEF RESEARCH REPORT
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Home Albumin Infusion Therapy, Another Alternative Treatment in Patients With Congenital Nephrotic Syndrome of the Finnish Type

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Background: Congenital nephrotic syndrome of the Finnish type (CNF) is a rare, severe glomerular disease caused by mutations in the NPHS1 gene, which codes for nephrin. It is characterised by massive proteinuria and severe edoema. Progression to end-stage kidney failure occurs during early childhood and the only curative treatment is kidney transplantation. Nowadays, patients need aggressive medical treatment, which includes daily albumin infusions (for months) until they get clinical stability to receive transplant.

Objective: In our paediatric hospital, we implemented a multidisciplinary program for the home infusion of albumin with outpatient follow-up. The aim of the study was to assess the safety and efficacy of this program for the first four years of its implementation.

Material and Methods: Retrospective observational study of CNF paediatric patients treated with home albumin infusion therapy from March 2014 to July 2018 at a tertiary care paediatric hospital. Information on albumin administration was obtained from the electronic prescription assistance program and details on clinical and care-related variables from the hospital's electronic information systems.

Results: Four patients with CNF received albumin infusions for 18, 21, 22 months, and 3 years. The treatment was safe, and the complication rates were to be expected considering the severity of disease. Patients required a median of two hospital admissions a year (19 in total); 47% due to catheter-related complications, but there were just three catheter infections.

Conclusions: In our experience, home albumin infusion therapy is safe and effective and helps to improve children health and quality of life.

Keywords: congenital nephrotic syndrome, finnish type, CNF, NPHS1, albumin, home albumin infusion therapy

INTRODUCTION

Nephrotic syndrome is the most common primary glomerular disease in the paediatric population. It is characterised by a glomerular lesion that leads to massive proteinuria (urine protein/creatinine ratio >2 mg/mg), resulting in hypoalbuminemia and generalised edoema. Nephrotic syndrome can be idiopathic or hereditary. Younger patients are more likely to have the hereditary form, and genetic diagnosis is recommended in all young patients with nephrotic syndrome who fail to respond to conventional treatment (1, 2). The most severe subgroup of nephrotic syndrome is congenital nephrotic syndrome (CNS), which is characterised by disease onset within the first 3 months of life, resistance to corticosteroids and immunosuppressive agents, and a very poor prognosis (3–5).

The prototype of severe hereditary nephrotic syndrome is CNS of the Finnish type (CNF) (OMIM: #256300), which, as its name suggests, is particularly prevalent in Finland, where it has an incidence of 1 case per 8,200 live births (4). However, CNF has been described in populations of different ethnic backgrounds around the world. It is an autosomal recessive disorder caused by loss-of-function mutations in the NPHS1 gene, leading up to alterations to the glomerular filtration barrier, responsible for the massive proteinuria typically seen in CNS (6, 7). Albumin is the main protein lost, but larger plasma proteins, such as immunoglobulins, may also be lost. Protein loss is correlated with hypoalbuminemia, severe malnutrition, hyperlipidemia, and an increased risk of thrombosis and infection (7).

Unlike idiopathic nephrotic syndrome, CNF is caused by a structural alteration. As such, it is resistant to corticosteroids and immunosupressants, and these treatments can even be harmful considering that patients are highly susceptible to infection (8).

As CNF is rare and challenging to manage, most patients are treated in specialised hospitals. Although prognosis has improved thanks to the introduction of early kidney transplantation and advances in dialysis techniques (9), patients still require an aggressive treatment approach to ensure that they survive until they attain an adequate weight and height for kidney transplantation (8).

In a study of 41 children diagnosed with CNF between 1953 and 1982, a team from the University of Minnesota led by Mahan et al. (10) found that all the children diagnosed before 1971 (before the introduction of early kidney transplantation) died. Those diagnosed later, however, showed 2-year survival rates of 82%.

As kidney transplantation is the only curative treatment for CNF, medical treatment is necessary to sustain good health and appropriate growth and development until the child is ready to undergo kidney transplantation (11). The goal of treatment thus is to control clinical manifestations to maintain clinical stability and prevent complications until the child attains a sufficient weight and height for transplantation.

The specific treatment aims are to control proteinuria and massive edoema through a high-calorie and high-protein diet and to prevent intercurrent infections and thromboembolic complications (3–12).

Various palliative treatments exist to control massive proteinuria and sustain clinical stability until the child is ready to undergo kidney transplantation.

The classic treatment for CNF, established by a group of Finnish experts two decades ago, is elective bilateral nephrectomy followed by dialysis until the moment of transplantation. This aggressive approach is justified by the presence of severe *NPHS1* mutations in the Finnish population (4, 5).

A more conservative approach, used at our hospital and other centres, consists of repeated intravenous albumin infusions associated with unilateral nephrectomy and/or treatment with antiproteinuric drugs to ensure clinical stability and normal glomerular filtration, thereby postponing the need for dialysis and transplantation and increasing the chances of success (11, 13–15).

This approach compensates for the massive loss of proteins and maintains intravascular oncotic pressure through daily intravenous infusions of albumin 20% (1 g/kg/day) in combination with furosemide (0.5 mg/kd/day) through a central venous catheter (CVC). In addition to a high-calorie and high-protein diet, patients are concomitantly prescribed drugs such as indomethacin (1–3 mg/kg/day) and angiotensin-converting enzyme inhibitors, to reduce glomerular filtration rate, and consequently proteinuria, and control severe edoema (15, 16).

Patients who remain clinically stable usually need to follow the above treatment for several months, i.e., until they are ready to undergo kidney transplantation. During this time, they typically need to be hospitalised or to make daily visits to the hospital for albumin infusions. This can have a major impact on the child's development and on family quality of life (5).

There have been growing reports of home infusion therapy programs being used in different fields that have proven to be both safe and effective. Examples are parenteral nutrition programs and antibiotic therapy programs for patients with cystic fibrosis (17, 18).

At our hospital we launched a multidisciplinary program for the home administration of intravenous albumin for children with CNF who only needed to be in hospital to receive this treatment. Apart from improving patient and family quality of life and favouring the children's personal and social development, the potential benefits of shortening hospital stays also included cost savings and a reduced risk of hospital-acquired infections.

The aim of this study was to assess the safety and efficacy of a home albumin infusion therapy program for paediatric patients with CNF from a tertiary care hospital during the first 4 years of the program and to evaluate associated morbidity.

METHOD

The program was launched by the paediatric nephrology, pharmacy, and nursing departments in March 2014 to educate and train relatives and caregivers of patients with CNF in home albumin infusion therapy. The goal was to promote outpatient care and minimise hospital stays. The program targeted clinically stable patients whose only reason for being in hospital was to

TABLE 1 | Interventions by the nursing in the home albumin infusion therapy program.

Actions	Pre-discharge	Discharge	Weekly follow-up visits for first 6 months	Twice-monthly follow-up visits after first 6 months
Primary caregiver training	Three training sessions on the knowledge, skills, and abilities needed for proper CVC[1] care and albumin infusion	Evaluation of knowledge, skills, and abilities acquired via a checklist	- Consolidation of good home care practises - Clarification of doubts	- Consolidation of good home care practises - Clarification of doubts
Broviac® CVC monitoring	- Supervision of primary caregiver CVC handling - Training in technique for albumin infusion in a sterile environment	Verification of CVC securement and functioning	- CVC dressing - Entry-point dressing - Assessment of permeability reflux	- CVC dressing - Entry-point dressing - Assessment of permeability reflux
Blood tests			Capillary blood sampling	Capillary blood sampling
Anthropometric characteristics			Weight, height, waist circumference, and blood pressure	Weight, height, waist circumference, and blood pressure
Warning signs and actions	- Training in detection of local and systemic warning signs - Actions: differentiation between contact with assigned nurse (Monday to Friday 9:00–17:00) and emergency visits	- Review of knowledge acquired: detection of local and systemic warning signs - Consolidation of knowledge about when and how to act		
Contact person	Ward nurse and assigned nurse	- Assigned nurse from Monday to Friday 9:00–17:00 - Emergency department	- Assigned nurse from Monday to Friday 9:00–17:00 - Emergency department	- Assigned nurse from Monday to Friday 9:00–17:00 - Emergency department

[1]*CVC, Central venous catheter.*

receive intravenous albumin and furosemide infusions. They were admitted to the program once their albumin infusion needs were reduced to a single daily infusion through a Broviac® CVC. This type of catheter was chosen to avoid the need for direct injections at home. Albumin was delivered by small, long-duration infusion pumps (Alaris™ GH Plus syringe pump from BD), provided by the hospital, that can be transported in dedicated backpacks to facilitate administration.

A multidisciplinary team formed by paediatric nephrologists, nursing staff, and pharmacists designed a series of health education and training sessions to teach the children's parents or caregivers to prepare and administer the albumin infusions in a sterile environment using the home infusion pumps.

Prior to discharge, parents also required to learn basic central line care skills to minimise risk of infection. There were also taught about warning signs (local signs in the area of the catheter and general signs such as edoema or fever) that they would need to watch for at home (**Table 1**). The nursing team evaluated the suitability of the home through a visit. In cases where the patient lived far from the hospital, the nursing team worked with the social worker of the area to assess the home.

Once the home program was launched, the multidisciplinary team worked together to ensure that the follow-up visits with the different teams coincided to minimise the number of scheduled visits to the hospital.

The nursing staff also set up communication channels with the patients' primary care teams to optimise management and follow-up. Weekly visits with nursing staff and members of the paediatric nephrology team were also arranged to coincide. The aim of these visits was to cheque weight, height, waist circumference, blood pressure, CVC securement and dressings, and to adjust overall treatment as necessary. The protocol also envisaged a switch to twice-monthly visits after an initial period of 6 months (**Table 1**).

Medical supplies, ordered through an online system, were sent to the patient's home on a monthly basis, ensuring thus that the caregivers only needed to pick up the medication from the hospital pharmacy twice a month. These trips were designed to coincide with the follow-up visits.

This was a retrospective observational study of paediatric patients with genetically confirmed CNF treated with home albumin infusion therapy from March 2014 (launch of program) to July 2019 at a tertiary care paediatric university hospital.

Information was collected on biodemographic characteristics (sex, age, and weight); clinical data (date of diagnosis, infusion-related complications, and date of kidney transplantation); and care-related variables (number of CVC dressing changes, hospital admissions, and successive visits to the outpatient clinic to cheque the catheter).

Information on albumin administration and dosage was obtained from the Electronic Prescription Assistance Program used in our centre. Details on clinical and care-related variables were obtained from the hospital's electronic information systems.

RESULTS

Four patients, all boys, were included in the home albumin infusion therapy program. They were from four unrelated

TABLE 2 | Clinical and demographic characteristics of patients.

	Patient			
	1	**2**	**3**	**4**
Age at diagnosis, days	2	71	23	7
Genetic diagnosis	c.1379G>A (p.Arg460Gln)	c.[1379G>A] + [2883G>A] (p.[Arg460Gln]+[Trp961*])	c.1135C>T(p.Arg379Trp)	c.3250_3251insG (p.Val1084Glyfs*12)
	Homozygosity	Compound heterozygosity	Homozygosity	Homozygosity
Post-diagnosis hospital stay, days	192 (6.5 months)	43 (1.5 months)	71 (2 months and 1 week)	83 (2.5 months)
Age at start of home albumin infusion therapy	6.5 months	4 months	2 months and 24 days	2 months and 23 days
Serum albumin at start of home albumin infusion therapy (g/dl)	2.7	2	2.18	2.1
Proteinuria prior to home albumin infusion therapy (mg/dl)*	3607.8	618	2,381	3,859
Time receiving home albumin infusion therapy	1 year and 6 months	3 years and 6 months	1 year and 10 months	1 year and 9 months
Age at time of kidney transplantation	2 years and 2 months	4 years and 10 months	2 years and 1 month	2 years
Dialysis before transplantation	No	No	No	No
Estimated glomerular filtration rate at time of kidney transplantation, mL/min (Schwartz equation)	15.55	19.5	6.82	14.57
Weight at the day of kidney transplantation, kg	10.4	18.9	11.1	11.5
Height at the day of kidney transplantation, cm	82.5	110.5	85	84
Weight percentile at the day of kidney transplantation	6.7	40	18.4	30.8
Height percentile at the day of kidney transplantation	2.3	44	18.4	11.5
First placement of *Broviac* CVC[1], days	33	18	18	13
Number of CVC[1]	5	3	2	3
Current glomerular filtration rate, mL/min (Schwartz equation)	106.42	36.87	121.31	103.77
Current creatinine level, mg/dL	0.39	1.12	0.32	0.39
Mechanical complications of CVC[1]s	2	3	3	1
Catheter-associated infections	1	1	0	2
Other complications: pyelonephritis	0	1	0	1
Number of hospital admissions from diagnosis to transplantation	7	5	4	5
Number of admissions due to CVC complications	3	4	1	3

[1]*CVC, central venous catheter; NA, not applicable.*

**The value corresponds to the last determination prior to hospital discharge (1–2 months before discharge).*

families, although three of them had a history of consanguinity. Two patients had been diagnosed with CNF during the first week of life and the other two were diagnosed between the ages of 1 and 2 months. The clinical diagnosis of CNF was confirmed genetically in all cases. Home albumin infusion therapy was started before the age of 4 months in all patients except the first boy recruited, who started when he was 6.5 months old (patient #1 in **Table 2**). The children were discharged from hospital when they required just a single daily infusion of albumin for <6 h. Median hospital stay during the children's first admission was 83 (range 43–192) days (**Table 2**).

All the patients had a suitable family and home environment. Two of the families were Moroccan and two were Spanish. They were from very different sociocultural backgrounds, but this had no influence on their ability to acquire the necessary knowledge and skills covered in the training sessions. We also observed no

association between lower sociocultural level and an increased rate of complications.

Albumin 20% was daily administered intravenously at a dose of 1 g/kg over 4 h (0, 1–0.5 ml/min). Furosemide (0.5 mg/kg/day) was administered as two doses: 50% halfway through the albumin infusion and 50% at the end. In all cases the drugs were delivered by a continuous-flow syringe pump through a *Broviac*® CVC (**Table 2**).

All the patients were also treated with high doses of indomethacin, and the antiproteinuric drug captopril.

The home infusion treatment goal (serum albumin levels of around 2 g/dL) was achieved in all cases and edoema was well-controlled. The median number of outpatient follow-up visits with the nursing staff and the paediatric nephrology team was 56 (range 48–96). Nursing advice was given by telephone on a median of 8 (range 2–14) occasions. Overall, 176 visits were made to the hospital pharmacy [median 44 (range 34–62) per patient] and 68 home medical supply deliveries were made [median 17 (range 6–36)].

A median of 3 (range 2–5) CVC changes per patient was required; these were due to partial dislodgement, breakage, or blockage (**Table 2**). Twenty one hospital admissions were required over the study period. This corresponds to a median of 2.5 admissions per patient per year; 52.4% of the admissions were due to CVC complications.

One of the admissions was due to infection of the CVC exit site by methicillin-resistant *Staphylococcus aureus* and required treatment with topical mupirocin and oral co-trimoxazole for 14 days. The same patient was admitted on two other occasions for catheter-related sepsis, caused by *Enterococcus cloacae* in one case (treated with meropenem) and methicillin-sensitive *S. aureus* in the other (treated with cloxacillin). One additional patient admitted due a respiratory infection episode and catheter exit site infection, was treated with oral cefadroxil as no germen was isolated. Of the remaining admissions, two were due to influenza B virus (in two vaccinated patients), two were due to acute pyelonephritis, and one was due to kidney failure resulting from acute gastroenteritis. One patient with hyporexia also required admission for percutaneous endoscopic gastrostomy (**Table 2**).

All patients, aged 24, 25, 26 months, and 4 years and 10 months, respectively, were discharged from the program when they underwent kidney transplantation and currently, they remain with functional grafts. In every case they received preemptive kidney transplantation without prior dialysis. The patient who was managed with medical treatment up to almost 5 years of age, had a less severe *NHPS1* mutation (**Table 2**).

DISCUSSION

Based on our experience, the home albumin infusion therapy program implemented at our hospital is safe and effective. The complication rates observed were no higher than expected considering the patients' condition, and none of the complications were related to the administration of albumin. None of the patients experienced life-threatening events and they progressed favourably up to the moment of kidney transplantation. The program resulted in shorter hospital stays. The patients were discharged home after the age of 4–6 months and remained in the program until they underwent transplantation (at ∼2 years of age). Management by a multidisciplinary team formed by nursing staff, paediatric nephrologists, nutritionists, and pharmacists ensured the efficacy of treatment. One patient started home therapy later than the others (at 6.5 months of age) even though he had been diagnosed with CNF at just 2 days of age. This was because he was the first patient included and his parents needed to provide care to another child of theirs who had recently undergone kidney transplantation because of same disease.

Home infusion therapy programs for paediatric patients are increasingly being used in different fields, and based on the reports in the literature, they do not result in a significant increase in CVC-related complications. In addition, they have a favourable impact on patient and family quality of life as they reduce hospital admission times and enable a faster return to normal life (19).

Our results are consistent with those reported by a team at the North Children's Hospital in the UK, which has been running a paediatric home albumin infusion program for 25 years. Based on their experience with seven children with CNF, their program is safe and has not resulted in an increased rate of catheter-related complications (20).

We have already included four children since the launch of our program just 4 years ago. Like the UK program, our home albumin infusion therapy program is safe and has not resulted in any serious complications. Considering their risk profile (young age, chronic hypoalbuminemia, hypogammaglobulinemia, daily catheter use, etc.), patients with CNF can be expected to experience a high number of complications, as described by many specialist hospitals (8). In our series, there were very few infection-related complications (one entry-point infection and two cases of sepsis), attesting to the quality of nursing care provided and the success of the training sessions in which caregivers were taught how to administer the drug in a sterile environment. There were no thrombotic complications, although it should be noted that home infusion of alteplase has been found to significantly reduce the risk of intraluminal thrombosis (21). We, however, do not have any experience in this area.

Although we observed a high number of hospital admissions ($N = 21$) over the study period, again, this is to be expected given the severity of the patients' condition. Moreover, over half of the admissions were due to events unrelated to the albumin infusion program, such as viral infections and/or morbidity associated with the primary kidney disease. No life-threatening events occurred.

Further, with our conservative therapeutic approach, we avoided potential complications associated with early dialysis initiation children with CNF (5, 9), mainly in those patients who maintained native kidney renal function for extended period of time, as described (8).

It should be noted that implementation of the program increased the outpatient care burden for both the paediatric nephrologist and nursing teams, who had to closely monitor the patients, and the hospital pharmacists, who had to dispense the

medications required and arrange for other supplies to be sent to the patients' homes.

This type of home programs is, especially, important in the current state of the SARS- CoV-2 pandemic since they allow reducing patient's or relative's exits from the home and hospital visits, thus reducing the possibility of contagion. In this scenario, recently another infant boy with CNF has successfully entered at the home albumin infusion therapy program in our centre.

Limitations

The main limitation of this study is the small number of patients admitted to the program, but again, this is to be expected given the rarity of the disease. Nonetheless, the results obtained suggest that the program results in substantially shorter hospital stays and improved quality of life without compromising treatment efficacy and patient safety. Although we did not undertake a formal study of the impact of the program on quality of life, both the patients and their parents reacted very positively to being able to continue treatment at home.

A second limitation of our study is that we were unable to compare the economic impact of the home program with in-hospital care and treatment, as we did not have a control group.

Conclusions

Based on our experience, a paediatric home albumin infusion therapy program run under the supervision of an expert team is both safe and effective. In our opinion, this treatment modality should be offered to all paediatric patients with CNF who have reached the requirement of a single daily dose of albumin within the framework of a structured caregiver training program. We observed no significant increase in catheter-related complications, adverse drug effects, or albumin infusion reactions.

Our home albumin infusion therapy program allows patients and their families to continue treatment in the comfort of their home. It favours growth and development (and may even improve general health), reduces the need for long hospital stays, and offers an opportunity to optimise healthcare resource utilisation. Finally, it can reduce the psychological and social sequelae associated with this disease in early childhood.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

ES, AF-P, MMo, LG-G, and CC-R: data collection. ES, MMu, AF-P, LG-G, and CC-R: data analysis. ES, MMu, and AF-P: manuscript writing. AF-P and GA: review. All authors contributed to the article and approved the submitted version.

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Persistent Abnormalities of Fatty Acids Profile in Children With Idiopathic Nephrotic Syndrome in Stable Remission

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Steroid-sensitive nephrotic syndrome is an immunological disorder mediated by still poorly defined circulating factor(s) that target the podocyte and damage the filtration barrier. Fatty acids (FA) have several biological roles and, in particular, are strictly involved in cell to cell communication, inflammatory processes and regulation of lymphocyte pools. Studies of FAs during INS have been mainly focused on biochemical changes during the phase of proteinuria; while no information is available about FA profile in patients with idiopathic nephrotic syndrome (INS) on stable remission. Aim of this study is to assess differences in blood FA profile between pediatric patients with INS during the phase of stable remission. Blood fatty acid profile of 47 pediatric patients on stable remission and 47 matched healthy controls were evaluated with gas chromatography. Patients with INS on stable remission had significantly higher levels of PUFA and omega-6 than controls (40.17 vs. 37.91% and 36.95 vs. 34.79%), lower levels of SFA and MUFA. Considering the single fatty acids, levels of omega-6 18:2n6 linoleic acid and omega-6 20:4n6 arachidonic acid were significantly higher in patients with INS than in controls (23.01 vs. 21.55%, *p*-value 0.003 and 10.37 vs. 9.65%, *p*-value 0.01). Moreover, patients with INS showed lower levels of SFA 14:0 (0.74 vs. 0.92%) and 18:0 (10.74 vs. 11.74%) and MUFA 18:1n9 oleic acid (18.50 vs. 19.83%). To the best of our knowledge this is the first study assessing FAs profile in children with INS in stable remission. In a population of 47 patients, we were able to demonstrate a higher blood level of linoleic and arachidonic acid, and consequently of omega-6 and PUFA, compared to controls. Persistently higher than normal levels of either linoleic or arachidonic acid, could be viewed as candidate biomarker for a state of risk of relapse in children with idiopathic nephrotic syndrome.

Keywords: idiopathic nephrotic syndrome (INS), fatty acids (FA), arachidonic acids, stable remission, omega-6 (ω-6) PUFA

INTRODUCTION

Childhood idiopathic nephrotic syndrome (INS) is characterized by the presence of heavy proteinuria (≥50 mg/kg/day), serum albumin <25 g/L, and edema (1–5). Children who respond to corticosteroids are defined as steroid-sensitive and have a favorable long-term prognosis; however, among them, those with relapsing episodes of proteinuria may require additional immunosuppressant therapy, which includes calcineurin inhibitors (6), mycophenolate mofetil, and more recently rituximab (7).

Steroid-sensitive nephrotic syndrome is an immunological disorder (1), mediated by still poorly defined circulating factor(s) that target the podocyte and damage the filtration barrier, causing proteinuria. There are some hypotheses on the genesis of the various proposed serum circulating factor(s) (8): T cells, B cells, and podocytes have been indicated as putative secretory cells (9), regulating B-lymphocytes homeostasis (10). A role of inflammation in childhood INS, driven by the activity of IL-17A (11) is supported by the fact that renal epithelial cells synthesize C-Reactive Protein (CRP) in a proteinuria independent manner (12); moreover, an increased expression of Tumor Necrosis Factor-α (TNF-α) in CD4-lymphocytes, with a concomitant decrease of expression of Interferon gamma (IFN-γ) in CD8-lymphocytes, regardless the proteinuria level (13), and an expression dysfunction of Toll Like Receptor-3 (TLR-3) (14) were observed in patients with INS.

Fatty acid metabolism is an emerging field of research in healthcare, not only under the nutritional aspect, but also within a more comprehensive and holistic approach, aimed at finding possible biomarkers for the cardiovascular risk (15, 16). Fatty acids have several biological roles and, in particular, are strictly involved in cell to cell communication, inflammatory processes and regulation of lymphocyte pools (17–21).

Fatty acids are compounds that can be classified either by chain length or saturation, resulting in the saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), and polyunsaturated fatty acid (PUFA) categories.

PUFA include the essential fatty acids (EFAs) linoleic acid (18:2n6) and α-linolenic acid (18:3n3), which can be only taken with a diet (22). Among PUFA, the most relevant biologic role is played by the omega-3 and the omega-6 series, as they may regulate multiple gene expression and play a fundamental role in cell signaling and inflammation pathways (23, 24). In particular, the pivotal omega-6 arachidonic acid is released from membrane phospholipids when cells are under stress, and becomes the precursor of several pro-inflammatory bioactive compounds, like prostaglandins, thromboxanes, leukotrienes, lipoxines, epoxyeicosatrienoic acids, and hydroxyeicosatetranoic acids (21).

Studies of fatty acids during INS have been mainly focused on biochemical changes during the phase of proteinuria (25). While a decrease in palmitic and arachidonic acids and an increase of oleic acid have been described by Aldamiz Echevarria (26), Das found an increase in palmitic acid and a decrease of 18:0, alpha linolenic acid and EPA (27). This discrepancy may be explained by the fact that Das studied the effect of PUFA n3 supplementation on fatty acid levels, while Aldamiz Echevarria merely described the changes in fatty acid profile. Interestingly, for both authors the pivotal point was the change in the ratio between pro- and anti-oxidant PUFA metabolites, driving to a pro-inflammatory state. However, a pathogenetic meaning cannot be reasonably attributable to these observational findings.

If the changes in fatty acids levels during proteinuria are well-established, to the best of our knowledge no information is available about fatty acids profile in patients with INS on stable remission, although it is well-known that INS is characterized by periods of remission alternating with relapses of proteinuria, mainly in the course of infections, and that fatty acids are strictly involved in the inflammatory processes and in lymphocyte regulation.

The aim of this study is therefore to measure fatty acids levels in patients with INS on stable remission, comparing them to those of a group of matched healthy controls.

PATIENTS AND METHODS

The subjects participating in the study were enrolled according to the following criteria:

Patient group: (1) Pediatric patients with a previous diagnosis of idiopathic nephrotic syndrome, based on the following criteria: ratio of proteinuria to creatininuria UPr/UCr >2 mg/mg, and serum albumin <2.5 g/dl. (2) Steroid dependent or frequently relapsing course, according to the definition of the ISKDC: steroid dependent nephrotic syndrome is defined as steroid-sensitive NS with 2 or more consecutive relapses during tapering or within 14 days of stopping steroids; frequently relapsing NS is defined with 2 or more relapses within 6 months, or 4 or more relapses within a 12-months period. (3) Stable remission if UPr/UCr was <0.2 mg/mg, total serum protein >6.4 gr/dl, serum albumin >4 gr/dl (28), and duration of remission more than 30 days from the end of the nearest proteinuric event.

Control group: healthy controls matched for age and gender with neither concomitant inflammatory disease, nor a metabolic or genetic disease, enrolled from a group of children who performed a blood test in preparation to minor urologic corrective surgery.

Body Mass Index of both patients' and controls' groups was calculated, to exclude overweight or obese subjects from the study.

Patients' blood samples were collected for fatty acid profile, serum total protein, albumin, total cholesterol, and triglycerides during scheduled routine visits and obtained after parental consensus and following Helsinki declaration. Blood samples from controls were obtained after parental consensus. The related study protocol was approved by our local Ethical Committee.

Urine was collected from the first morning sample for urine protein and creatinine measurement and urine protein to creatinine ratio (mg/mg) was calculated.

Abbreviations: PUFA, polyunsaturated fatty acids; MUFA, monounsaturated fatty acids; SFA, saturated fatty acids; EFA, essential fatty acids; DHA, docoexaenoic acid; COX, cyclooxygenase; LOX, lipid oxygenase.

A 3-days dietary diary was collected in a random sample of 10 patients and 10 controls to assess differences in dietary fatty acid intake.

Blood samples were collected on Whatman 903 collection cards BHT (Sigma-Aldrich) pre-treated and stored at temperature of −20°C. Cards were cut and transferred into vials (one vial for each sample) for methylation as described by Marangoni et al. (29). Afterwards, 2 ml of KCl solution (Sigma Aldrich) and 330 μl hexane (Sigma-Aldrich) were added. Samples were first vortexed and then centrifuged 3,000 rpm for 10 min. Finally, hexane layer (the upper layer) was collected from each vial and transferred into gas chromatography vial for fatty acids profile evaluation with fast gas-chromatographer Master GC fast (Dani), equipped with a 15 m fused silica capillary column Omegawax™ 100 (Supelco). The gas chromatography results were analyzed using Clarity software (Data Apex).

Fatty acids were evaluated as percent on the total of fatty acids; the obtained values were used for statistical analysis. Data are provided by clustering fatty acids on the basis of their saturation state (PUFA, MUFA, SFA) and considering among PUFA the omega-3 and omega-6 series.

The activity of the enzymes involved in FA synthesis pathway was evaluated by the product/precursor ratio (30).

Statistical analysis: *t*-test (SPSS 21.0 IBM) was conducted to assess differences in fatty acid profile between patients and controls after a normality test; a p-value ≤ 0.05 was considered significant.

In the absence of published data, the power of the test to calculate cohort sample size was not performed.

RESULTS

Forty seven pediatric patients, aged 8 ± 4 years, 51% males, with steroid dependent or frequently relapsing nephrotic syndrome were enrolled into the study in the period 01/01/2016–31/12/2019 (**Figure 1**) and had a blood sampling for fatty acid profile during stable remission (median time elapsed from the nearest occurrence of proteinuria of 60 days, with a range of 32–150 days). Their serum total protein in remission was 7 ± 0.34 g/dl, serum albumin 4.7 ± 0.31 g/dl, and UPr/Cr 0.14 ± 0.04 mg/mg. Thirty-two out of 47 patients were in immunosuppressive therapy, 11 with cyclosporine A, 3 with tacrolimus, and 18 with mycofenolate mofetil.

Sixty healthy children matched for age and gender (age 8 ± 5 years, 51% males) were enrolled as a control group.

None of the patients and controls was overweight or obese. The two groups did not show any statistically significant difference in fatty acid intake at the analysis of the 3-days dietary diaries, as shown in **Table 1**, and their cholesterol and triglycerides levels were in the normal range.

Considering the main groups of fatty acids (**Figure 2**), patients with INS on stable remission had significantly higher levels of PUFA and omega-6 than controls (40.17 vs. 37.91% p-value 0.0004 and 36.95 vs. 34.79%, p-value 0.0005), with consequent lower levels of SFA (37.22 vs. 38.15%, p-value 0.02) and MUFA (22.57 vs. 23.94%, p-value 0.01). Omega-3 levels were not significantly different in the two groups (3.12 vs. 3.01%, p-value 0.53).

Considering the single fatty acids (**Figure 3** and **Table 2**), levels of omega-6 18:2n6 linoleic acid and omega-6 20:4n6 arachidonic acid were significantly higher in patients with INS during the phase of stable remission than in controls (23.01 vs. 21.55%, p-value 0.003 and 10.37 vs. 9.65%, p-value 0.01). Moreover, patients with INS, compared to healthy controls, showed significantly lower levels of SFA 14:0 (0.74 vs. 0.92%, p-value 0.008) and 18:0 (10.74 vs. 11.74%, p-value 0.00002) and MUFA 18:1n9 oleic acid (18.50 vs. 19.83%, p-value 0.011).

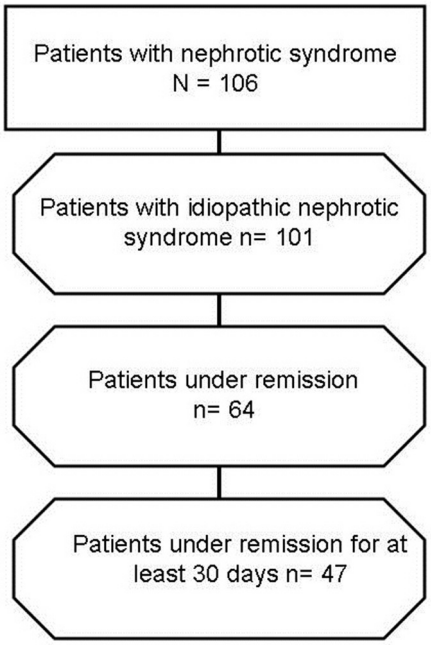

FIGURE 1 | Patients selection flow.

TABLE 1 | Fatty acids dietary intake (g/day) in a random sample of patients and healthy controls.

Fatty acids dietary intake		
	Patients (n = 10)	**Controls (n = 10)**
SFA	5.851	7.23
18:1n9	5.896	4.74
16:0	2.366	3.12
18:2 n-6	1.478	1.10
18:3 n-3	0.198	0.23
20:4 n-6	0.068	0.063
20:5 n-3	0.040	0.043
22:6 n-3	0.058	0.079

Values are expressed in g/day; dietary intake was calculated on the basis of a 3-days diary record.
n.s., not significant p-value at T-test.

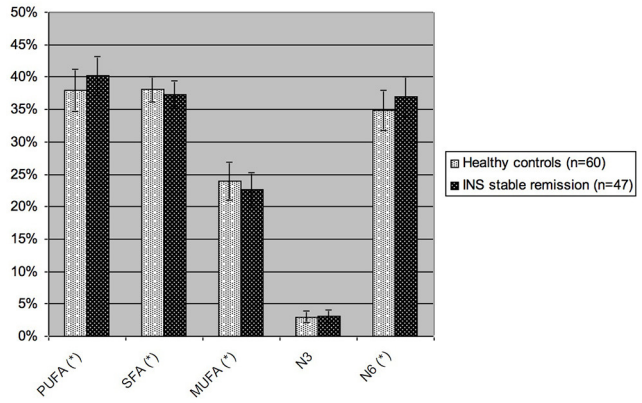

FIGURE 2 | Blood levels of the different groups of fatty acids in children with INS on stable remission and in healthy controls. PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; N3, omega-3 fatty acids; N6, omega-6 fatty acids. Values are expressed as percent of total blood fatty acids. *p-value <0.05, statistically significant at T-test.

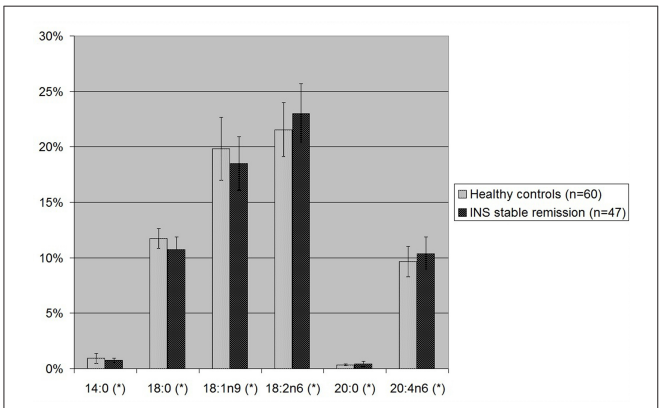

FIGURE 3 | Statistically significant single fatty acids blood levels in children with INS on stable remission and in healthy controls. 18:1n9, oleic acid; 18:2n6, linoleic acid; 20:4n6, arachidonic acid. *p-value <0.05 is statistically significant at T-test.

Finally, a significant increase in estimated enzymatic activity in the arachidonic acid synthesis pathway was found in the last step of the omega 6 pathway (**Figure 4**).

TABLE 2 | Blood levels of single fatty acids and different groups of fatty acids.

	Healthy controls		INS stable remission	
Fatty acid	**Average**	**s.d.**	**Average**	**s.d.**
14:0 (%)	0.92	0.43	0.74	0.23
16:0 (%)	22.61	1.36	22.94	1.37
16:1n7 (%)	0.92	0.29	1.01	0.31
18:0 (%)	11.74	0.92	10.74	1.12
18:1n9 (%)	19.83	2.85	18.50	2.40
18:1n7 (%)	1.26	0.16	1.25	0.21
18:2n6 (%)	21.55	2.43	23.01	2.69
18:3n6 (%)	0.27	0.13	0.30	0.12
18:3n3 (%)	0.22	0.09	0.22	0.06
20:0 (%)	0.34	0.07	0.42	0.23
20:3n9 (%)	0.12	0.04	0.11	0.05
20:3n6 (%)	1.59	0.28	1.58	0.39
20:4n6 (%)	9.65	1.37	10.37	1.52
20:5n3 (%)	0.28	0.16	0.32	0.21
22:0 (%)	1.03	0.20	1.05	0.24
22:4n6 (%)	1.22	0.31	1.14	0.41
22:5n6 (%)	0.50	0.25	0.62	0.38
22:5n3 (%)	0.60	0.14	0.62	0.15
24:0 (%)	1.52	0.29	1.47	0.43
22:6n3 (%)	1.91	0.63	1.90	0.70
24:1n9 (%)	1.68	0.33	1.55	0.44
PUFA	37.91	3.30	40.17	2.97
SFA	38.15	1.93	37.22	2.12
MUFA	23.94	2.88	22.57	2.63
Omega-3	3.01	0.84	3.12	1.03
Omega-6	34.79	3.11	36.95	3.08

Fatty acid levels are expressed as percent of total fatty acids. P-value calculated at T-test.

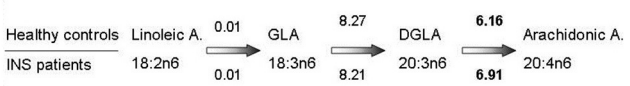

FIGURE 4 | Estimated enzymatic activity through omega-6 pathway. Estimated enzymatic activity from 18:2n6 linoleic acid (precursor) to 20:4n6 arachidonic acid (product). Numbers on the upper side of the figure indicate the estimated activity, calculated as the ratio of product to precursor, in healthy controls, while numbers on the lower side indicate the estimated activity in patients with INS under stable remission. Statistically significant data (p-value 0.03) are in bold. GLA, gamma linoleic acid; DGLA, dihomo gamma linoleic acid.

DISCUSSION

While previous reports have only been focused on the level of fatty acids in patients with proteinuria (26, 27), to the best of our knowledge this is the first study assessing FA profile in children with INS in stable remission. In contrast to the contradictory data on the fatty acids profile available in the literature (26, 27), we were able to demonstrate, in a population of 47 patients, a higher blood level of linoleic and arachidonic acid, and consequently of omega-6 and PUFA, compared to controls.

Immune system derangements resulting in an inflammatory state have been described during proteinuria in nephrotic syndrome and include a few mediators of inflammation, like IL17 A, TNF-α, IFN-γ, TLR-3, and CRP (11–14). Notably, for some of them, the expression was independent from the degree of proteinuria.

Our findings of high omega-6 levels, in particular arachidonic and linoleic acid, are on the same line and can be considered the expression of an inflammatory state, as it involves

arachidonic acid (21). Indeed, omega-6 arachidonic acid is the precursor of several pro-inflammatory bioactive compounds, like eicosanoids (21).

However, such findings refer to patients in stable remission, not to patients with proteinuria, and this is the novelty of our study. A high blood level of arachidonic acid, and more generally omega-6 compounds, may be considered a biomarker of a persistent pro-inflammatory state in children with INS even during remission, possibly acting as co-factor involved in the relapse of proteinuria. Availability of larger pool of arachidonic acid, upon a trigger (for instance, an infection) could further contribute to activation of the omega 6 turnover, leading to increased synthesis of pro-inflammatory eicosanoids (the major end-products of arachidonic acid), possibly associated with relapsing proteinuria.

In our patients in stable remission, the high levels of arachidonic acid may be explained by an increase of the estimated enzymatic activity possibly supported by the described higher availability of linoleic acid, observed in the patients, but not in controls, in the last step of the omega 6 pathway, from DGLA to arachidonic acid.

The increased levels of arachidonic acid were associated with increased levels of linoleic acid, the arachidonic acid precursor. The increase in arachidonic and linoleic acid levels cannot be simply explained by a higher dietary intake of omega-6 in children with INS than in controls, as our 3-days dietary diary did not show any statistical difference between the two groups. On the contrary, the origin of the delta in linoleic acid levels between patients and controls could be explained in the first case by a different release from endogenous pools of fatty acids, as part of the general picture of non-communicable chronic diseases with inflammatory expression, in the second case by a reduced activity of metabolizing enzymes of linoleic acid LOX and COX in the patient's group (21). Furthermore, the fatty acids changes that we described did not depend on the use of immunosuppressive agents, as fatty acids levels are not affected by this group of medications (26).

Finally, the existing inverse correlation between blood levels of oleic acid and arachidonic acid (31) may account for the lower levels of MUFA oleic acid in patients than in controls. This association may be mechanistically explained by the biologic balance within families of unsaturated fatty acids (32, 33).

Considering the fatty acid profile described in the literature during the proteinuric phase (26, 27), and the data of this study relating to the remission phase, we can hypothesize that the imbalance of the PUFA n-3/PUFA n-6 ratio reported in the course of proteinuria is a phenomenon that persists even in the course of remission.

Strong points of this work are the homogenous and well-characterized cohort of patients and controls and the novelty of information about a so far scarcely investigated aspect of INS, with possible implications for the management of the disease.

A few limitations should also be recognized. First, the lack of direct measurement either of fatty acid metabolites or of the real enzymatic activity, possibly confirming our explanation of the mechanisms underlying fatty acids profile in patients with INS, otherwise based on the estimated product to precursor ratio. Second, the fact that the dietary record, as regards fatty acids intake, was performed only in 10 INS patients and 10 healthy controls, but the risk of potential inequality in fatty acids dietary intake in the two groups is low, considering that patients with INS on stable remission had no diet limitation and they came from the same geographical area as that of healthy, matched controls. The third limit is the absence of patients' fatty acids profile during relapse, that could help in better evaluating the fatty acids changes during remission, but this would imply longitudinal data, which were not available in all our patients. Also the absence in our series of infrequent relapser patients on full remission without immunosuppressive agents can be seen as a limit of our study, since their fatty acid profile might be similar to controls and serve as a proof of concept of the role of fatty acids as candidate biomarkers for a risk of relapse in children with INS.

In conclusion, our results suggest that patients with INS in stable remission could not be considered "healed," if they still show an abnormal fatty acid profile compared to that of healthy subjects. Persistently higher than normal levels of either linoleic or arachidonic acid, could be viewed as candidate biomarker for a state of risk of relapse in children with idiopathic nephrotic syndrome.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

Material preparation, data collection, and analysis were performed by ST and M-LS. CT, ED, and AB selected and screened the subjects cohort. VD analyzed the dietary data. The first draft of the manuscript was written by ST. Manuscript was reviewed and edited by AE, LG, and WM. CA, GMa, and GMo critically revised the article and supervision. All authors contributed to the study conception and design, commented on previous versions of the manuscript, read, and approved the final manuscript.

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CASE REPORT
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Case Report: Complete Remission of C1q Nephropathy Treated With a Single Low-Dose Rituximab, a Reality or Coincidence?

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C1q nephropathy is a glomerulopathy that is characterized by large amount of C1q deposits in the glomerular mesangium. It is a diagnosis of exclusion after ruling out systemic lupus erythematosus and membranoproliferative glomerulonephritis by systemic and serological examination. The pathogenesis of C1q nephropathy is unclear. In addition, there is very little generalizability in the treatment and prognosis for pediatric C1q nephropathy due to diversities in clinical manifestations and pathological types. Rituximab is a human/mouse chimeric monoclonal antibody against CD20, which is primarily used for treating lymphomas and, most recently, has been used to treat certain kidney diseases including C1q nephropathy. In this report, we used one quarter of the typical dose of rituximab for lymphoma treatment to achieve complete remission in a C1q nephropathy patient, significantly reducing deposition of immune complexes and glomerular damage. This case indicates that dosage reconsiderations may be necessary for rituximab in treatment of pediatric C1q nephropathy.

Keywords: C1q nephropathy, nephrotic syndrome, rituximab, complete remission, B lymphocyte depletion

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INTRODUCTION

C1q nephropathy is characterized by large amounts of mesangial immunoglobulin and complement deposition with predominant appearance of C1q, after exclusion of systemic lupus erythematosus and membranoproliferative disease (1). The incidence of C1q nephropathy varies in different reports ranging from 0.2 to 16% with no gender differences (1–4). It is speculated that complement activation and glomerular antigen–antibody complex formation underlie pathogenesis of C1q nephropathy, with additional involvement of alternative complement pathway and lectin pathway (5).

The clinical manifestations of C1q nephropathy are diverse. Most of the patients present with nephrotic syndrome, some with acute or chronic glomerulonephritis, and, occasionally, the only presentation may be hematuria. The disease also presents with various nephropathologies, the most common being minimal change nephropathy, focal segmental glomerulosclerosis nephropathy, and immune complex-mediated mesangial proliferative glomerulonephritis (5). These distinctions are especially important, as prognosis of C1q nephropathy in patients depends on their pathological type and clinical manifestations. Notably, children with C1q nephropathy have higher rate of recurrence and shorter recurrence interval compared to nephrotic syndrome without C1q

deposition (6). There are no disease-specific treatment guidelines for C1q nephropathy. Currently, treatment guideline for C1q nephropathy follows the general guidelines for treatment of primary nephrotic syndrome, with corticosteroid as first-line treatment. If a patient is corticosteroid-dependent or corticosteroid-resistant, a second-line medication, e.g., cyclosporine, is selected with criteria based on patient condition. Of interest to the case presented here, a small number of cases have reported the efficacy of rituximab for treating C1q nephropathy, with significant improvement of renal function and clinical manifestations (7–10). Rituximab is a human/mouse chimeric monoclonal antibody targeting CD20 (11). It was originally used to treat B-cell non-Hodgkin's lymphoma but has since been widely used in autoimmune anemia, rheumatic diseases, and more recently used for the treatment of autoantibody-related kidney diseases, including antineutrophil cytoplasmic antibodies (ANCA)-associated nephritis and membranous nephropathy (12–14). The target of rituximab is CD20, a tetra-transmembrane protein expressed in pre-B cells. Rituximab depletes B cells via direct signal-induced apoptosis, complement-dependent cytotoxicity, antibody-dependent cell-mediated cytotoxicity, and antibody-dependent phagocytosis (11, 12, 14). Depletion of B cells results in the reduction in antibody and immune complex formation and, ultimately, reduces C1q deposition.

The standard dosage of rituximab for treating nephrotic syndrome is adapted from existing guidelines for lymphoma treatment (10, 12, 15, 16). No disease-specific guidelines has been made available for treatment of C1q nephropathy using rituximab. In this report, we show complete remission achieved via a significantly reduced dose of rituximab in a pediatric patient with C1q nephropathy. Our results suggest the need for further investigation into disease-specific dosage for C1q nephropathy, especially in the pediatric setting, with benefits of lower risks of adverse events as well as cost considerations in uninsured and underinsured patients.

CASE PRESENTATION

The patient is a 7-year-old Asian girl who was diagnosed with nephrotic syndrome in June 2017 in a local primary care clinic before transferring care to our department 9 months later. Prior to diagnosis, patient was healthy, with no significant birth history and no history of surgery, trauma, or blood transfusion. The patient visited the local clinic due to cold/flu-like symptoms, swelling eyelids and lower limbs, and abdominal pain that was eventually diagnosed as nephrotic syndrome. No family history was reported. Based on the records provided by the patient's parents, the patient underwent a course of oral corticosteroid, and subsequently, urine protein was negative, indicating corticosteroid-sensitive response. However, patient's urine protein increased once again after tapering corticosteroid, and her steroid dosage was increased. Subsequently, her urinalysis was once again negative for protein, but each time steroid taper was attempted, patient relapsed with significant proteinuria. Although her disease was progressively steroid dependent, corticosteroid was discontinued per the request of the patient's parents after several courses due to concern for adverse effects of long-term therapy. Instead, the patient was switched to traditional Chinese medicine (ingredients unknown) for nearly 4 months (**Figure 1A**). During the treatment with traditional Chinese medicine alone, the patient visited her local primary care clinic for several urinalyses that consistently demonstrated 3+ proteinuria and occult blood. Meanwhile, the patient showed progressive clinical decline with severe complications including urinary tract infections, systemic edema, shortness of breath,

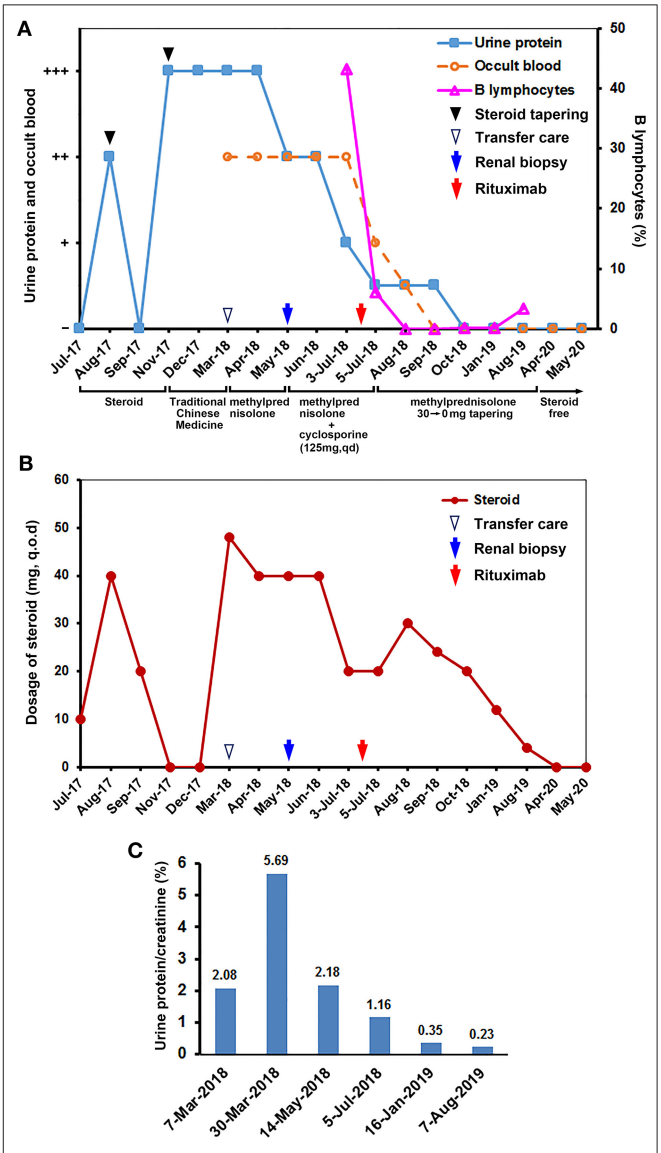

FIGURE 1 | The clinical course for the C1q nephropathy patient. **(A)** Selected urinalysis results and clinical course for the patient. Proteinuria and urinary occult blood have been negative for >18 months since administration of rituximab, indicating long-term remission. No relapse occurs after withdrawing maintenance dose of corticosteroid. **(B)** The course of steroid treatment is shown for corresponding time points in **(A)**. **(C)** Urinary protein/creatinine ratios during the care provided by us.

frequent urination, and dysuria. Due to significant disease progression, patient was referred to our hospital in March 2018.

On presentation to our hospital, the patient was admitted to the Pediatric Intensive Care Unit (PICU) and found to have a fungal infection, heart failure, hypertension, ascites, and persistent oliguria. Routine examination and laboratory tests showed body weight of 33 kg, body surface area of 1.11 m^2, serum albumin of 14.3 g/L, total cholesterol of 20.78 mol/L, and triglyceride of 11.13 mmol/L, indicating hypoproteinemia and hyperlipidemia. Renal function tests showed serum uric acid of 545 μmol/L, creatinine of 121.0 μmol/L, blood urea nitrogen (BUN) of 22.4 mmol/L, and glomerular filtration rate (GFR) of 37.86 ml/min, indicating azotemia and renal dysfunction. Initial treatment included correcting electrolyte disorder, diuresis, anticoaglutant therapy, and fluconazole (3.6 mg/kg) for fungal treatment. During this time, she was also treated with methylprednisolone (2 mg/kg daily) and aldehyde oxystarch, without significant efficacy (**Figures 1A,B**). Therefore, the patient underwent continuous renal replacement therapy (CRRT) with significant improvement of disease. Laboratory tests showed blood creatinine of 46 μmol/L, BUN of 10 mmol/L, and GFR of 99.59 ml/min. Patient was then transferred from PICU to our department for subsequent therapy. Autoimmune antibody panel was ordered, and all resulted negative. These included antinuclear antibody (ANA), antidouble-stranded DNA (anti-dsDNA), anti-Smith, antihistone antibody (AHA), anti-Sjögren's-syndrome-related antigen A (anti-SSA), anti-SSB, rheumatoid factor, ANCA, cardiolipin autoantibody, antimitochondrial antibody (AMA), smooth muscle antibody (SMA), anticentromere antibody, and antibasement membrane antibody. With negative immunological workup, we recommended a renal biopsy, but this was declined by her parents at that time with concerns regarding invasiveness of biopsy. During that admission, patient experienced repeated hypertension that was difficult to control on nifedipine and, subsequently, developed convulsions accompanied by right hemiplegia concerning for hypertensive encephalopathy. She subsequently underwent aggressive sodium nitroprusside (1–5 μg/kg/min), diuretic (spironolactone 20 mg and hydrochlorothiazide 10 mg daily), and steroid therapy (methylprednisolone 40 mg, q.o.d). Following these, her blood pressure returned to normal, neurological symptoms resolved, and systemic edema also improved. Laboratory tests showed that urine protein and occult blood decreased to 2+, blood creatinine was 42.0 μmol/L, and hypoproteinemia was corrected. Patient was discharged with continuation on methylprednisolone (40 mg daily in divided doses), losartan potassium (20 mg daily), and spironolactone (20 mg daily).

During her outpatient follow-up, patient's urine protein consistently returned 2–3+, suggesting steroid resistance. The need for tissue diagnosis via biopsy was reiterated, but patient's family continued to refuse at that time. Therefore, cyclosporine 5 mg/kg PO was added to her regimen. Subsequent labs showed improved proteinuria without significant decrease in serum creatinine despite the addition of cyclosporine. Due to frequency of relapses, renal biopsy was discussed again with the patient's parents for which consent was given in May 2018,

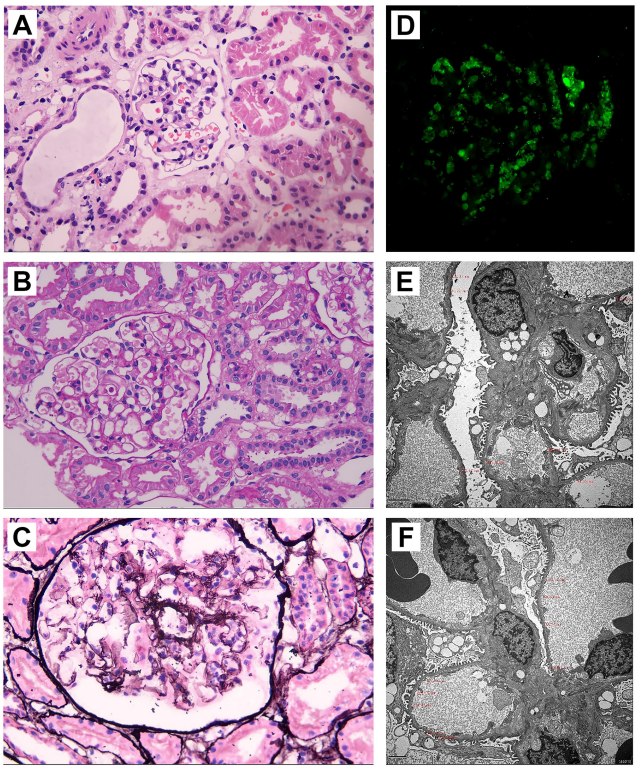

FIGURE 2 | Pathology evaluation for the C1q nephropathy patient. **(A)** Hematoxylin–eosin staining shows increased number of cells in glomerulus. No glomerular crescent, focal segmental glomerular sclerosis, and fibrosis are noted. However, renal tubule dilation and vacuolar degeneration of epithelial cells are present, and renal interstitium is slightly infiltrated with inflammatory cells, indicating a minimal change disease (MCD). **(B)** Periodic acid–Schiff (PAS) staining shows no remarkable cellular proliferation of mesangium and stroma. **(C)** Periodic Schiff–methenamine (PASM) staining shows normal capillary loops and no significantly thickened basement membrane. **(D)** Immunofluorescent staining confirms large amount of C1q deposition. **(E,F)** Transmission electron microscopy shows vacuolar degeneration of epithelial cells, podocyte foot processes effacement, proliferation of mesangium and stroma, and thin basement membrane.

and microscopy results were consistent with C1q nephropathy (**Figures 2A–F**).

Based on the pathological findings, a trial of rituximab was initiated after lymphocyte subset analysis showed B lymphocyte ratio of 43.2%. The patient received a single intravenous dose of 100 mg rituximab (93 mg/m^2). During infusion, patient experienced facial flushing but no other significant discomfort. B lymphocyte subset fell to 6.1% after 1 day. After 2 weeks of rituximab treatment, B cells were completely depleted, and renal function was significantly improved with a GFR of 93 ml/min (**Figure 1A**). Patient was then given prednisone starting at 30 mg every other day and gradually tapered at a rate of 5 mg/month. At 1-year follow-up in August 2019, patient presented with symptoms of urinary tract infection. Urinalysis at that time showed 4+ white blood cells (WBCs) but was negative for protein and blood. Serum tests showed normal kidney function and 3.30% of total B lymphocyte subset. At present, patient has

maintained normal kidney function off steroid therapy and has had no relapses since rituximab therapy. The treatment courses and selected laboratory results are shown in **Figure 1**.

DISCUSSION

Although C1q nephropathy currently has no discrete clinical considerations from nephrotic syndrome, its pathogenesis and disease course do indicate disparate treatment guidelines. Patients with C1q nephropathy are frequently steroid dependent, more likely to relapse, and have a shorter recurrence period than nephrotic syndrome patients with no C1q deposition (4).

Typically, pathological findings in children with nephrotic syndrome are mostly small lesions, and in these cases, steroid therapy is preferred. If treatment is inefficacious, an immunosuppressor is added, with risk of detriment to renal function. As aforementioned, rituximab has been used to treat C1q nephropathy and shown to partially restore kidney function (9). At present, the dose of rituximab for treating C1q nephropathy is based on guidelines for lymphoma treatment, i.e., four doses of 375 mg/m^2 at weekly intervals (7). Recent studies reported complete remission for patients with C1q nephropathy by using a single dose or two doses of rituximab at 375 mg/m^2 (8, 10). In malignant disease such as lymphoma, abnormal lymphocytes are generally significantly elevated, while the number of lymphocytes in C1q nephropathy does not have similar magnitude of elevation. Therefore, we posited that the dosage of rituximab for treating C1q nephropathy may not need to be as high as that for lymphoma. Several studies have used low-dose rituximab (a fixed dose of 100 mg) to treat adult patients with idiopathic autoimmune hemolytic anemia and primary immune thrombocytopenia and showed comparable efficacy of standard dose (17, 18).

In this case, we reported the first successful use of a single low dose of rituximab (100 mg or 93 mg/m^2) for C1q nephropathy treatment, a quarter that of the dosage indicated for lymphoma. After treatment, our patient has shown normal renal function, negative urine protein, and occult blood for >18 months and is currently steroid-free, suggesting that successful remission was achieved at this significantly reduced dosage. The side effects of rituximab have not been clarified, with some occurring early in administration, including nausea, rash, and bronchospasm, although adverse effects can be minimized by controlling infusion rate (15). Viral infections and pyelonephritis accompanied by neutropenia can also occur during the rituximab treatment (15). As shown in the literature, low-dose rituximab may reduce risks of severe side effects such as neutropenia and pulmonary toxicity (19). A lower dose can also be cost saving and shortens infusion time, which are important considerations in making biologics accessible to indigent populations. In the setting of good treatment efficacy with better controlled adverse effect profile, reduced dosage treatment with rituximab in C1q nephropathy warrants additional examination.

Our report features only one case, which is a limitation presented by the rarity of C1q nephropathy in our practice. Although the response of this patient to such a low dose of rituximab may occur on occasion and could be race dependent, the overall outcomes for this patient is remarkable. These results suggest that the dosage of rituximab for treating pediatric nephrotic syndrome, particularly C1q nephropathy, may be significantly reduced. To further investigate, a prospective case-controlled clinical trial recruiting larger, more diverse populations will be needed.

Although the pathogenesis of C1q nephropathy still remains unclear, it is speculated that complement activation and glomerular antigen–antibody complex formation play a central role. Studies show that C1q molecules have affinity to various substances including DNA, RNA, viral proteins, Gram-negative bacteria, and various immune cells (5). C1q has been demonstrated to enhance B-cell response to antigens, which underlies the reason for use of rituximab to deplete B cells and reduce deposition of immune complexes. Previous studies have suggested that dosage of rituximab should be determined according to serum CD19 level, which is expressed in pre-B cells prior to differentiation (10). This method of quantitatively metered administration may be a focus of clinical trials in the future. Some Literatures also report that rituximab is less effective in patients with hormones and cyclosporine-dependent nephrotic syndrome (12). In consideration of increased complications with multidrug treatment, administration of rituximab in the early stages for steroid-dependent patients can effectively prevent progression of disease while also mitigating the myriad of harmful side effects of steroid treatment. There are also some reports suggesting that subcutaneous injection of rituximab is more time saving and less labor intensive compared to intravenous administration (11, 13). In addition, rituximab can be subcutaneously injected with recombinant human hyaluronidase that increases the dispersion and absorption of rituximab (11). Given the multitude of areas for development, we expect to continue investigating the mode of administration and dosage of rituximab to achieve maximum efficacy and minimize adverse outcomes.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethical Committee of Lanzhou University Second Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

RM collected the data and prepared the manuscript. DW participated in the patient's care. ZH collected and analyzed the data. QC participated in the patient's clinical care. YY

participated in the patient's care, supervised the study, analyzed the data, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Pediatric Immunization Practices in Nephrotic Syndrome: An Assessment of Provider and Parental Knowledge

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Background: Children with nephrotic syndrome (NS) are at high risk for vaccine-preventable infections due to the immunological effects from the disease and concurrent treatment with immunosuppressive medications. Immunizations in these patients may be deferred due to their immunosuppressive treatment which may increase the risk for vaccine-preventable infections. Immunization practices in children with NS continue to vary among pediatric nephrologists. This raises the question of whether children with NS are receiving the recommended vaccinations at appropriate times. Therefore, it is critical to understand the practices and patient education provided by physicians to patients on the topic of vaccinations.

Methods: After informed consent, parents/guardians of 153 pediatric patients (<18 years old) diagnosed with NS from 2005 to 2018 and 50 pediatric nephrologists from 11 participating centers completed anonymous surveys to evaluate immunization practices among pediatric nephrologists, assess the vaccine education provided to families of children with NS, assess the parental knowledge of immunization recommendations, and assess predictors of polysaccharide pneumococcal vaccine adherence. The Advisory Committee on Immunization Practices (ACIP) Immunization 2019 Guideline for those with altered immunocompetence was used to determine accuracy of vaccine knowledge and practices.

Results: Forty-four percent of providers self-reported adherence to the ACIP guidelines for inactive vaccines and 22% to the guidelines for live vaccines. Thirty-two percent of parents/guardians reported knowledge that aligned with the ACIP guidelines for inactive vaccines and 1% for live vaccines. Subjects residing in the Midwest and provider recommendations for vaccines were positive predictors of vaccine adherence ($p < 0.001$ and p 0.02, respectively).

Conclusions: Vaccine recommendation by medical providers is paramount in vaccine adherence among pediatric patients with NS. This study identifies potential educational opportunities for medical subspecialty providers and family caregivers about immunization recommendations for immunosuppressed patients.

Keywords: nephrotic syndrome, children, immunization, immunosuppression, education

INTRODUCTION

Nephrotic syndrome (NS) is caused by renal diseases that affect the permeability of the glomerular filtration barrier resulting in massive proteinuria, including immunoglobulins and complement proteins (1–4). Given the immunological effects from the disease and concurrent treatment with immunosuppressive medications, children with NS are at high risk for severe bacterial infections, especially with encapsulated bacteria (5). *Streptococcus pneumoniae* is a common encapsulated bacterial pathogen that is known to cause serious infections in children with NS (6, 7). Given the risk for this infectious pathogen, the pneumococcal polysaccharide vaccine (PPSV 23) has been specifically recommended for pediatric patients with certain medical conditions/diseases (i.e., NS, chronic renal failure, and immunosuppression medications). This susceptibility to infection extends beyond bacterial infections and includes serious viral infections such as Varicella, which can result in severe disseminated infections in immunocompromised hosts.

Children with NS have a high burden of healthcare utilization with a mean charge per hospitalization of $26,500 that exceeds many other chronic illnesses (8). Gipson et al. evaluated hospitalization costs in a cohort of children with NS and clearly showed that serious complications of NS, including infection, occur commonly and increase healthcare costs. In this study, 16% of 9,934 discharges in 2006 and 2009 had at least one severe complication (pneumonia, sepsis, peritonitis, thromboembolism, or diabetes) attributable to NS or its treatment. Infection-related complications were the most common including pneumonia, sepsis, or peritonitis. In 2019, Carpenter et al. investigated the prevalence of infection and venous thromboembolism in hospitalized pediatric patients with NS (9). This group demonstrated high rates of infection in hospitalized pediatric NS patients, with *Streptococcus pneumoniae* being the most common pathogen. Appreciating the infectious susceptibility associated with nephrotic syndrome is critical since improved vaccination practices would potentially aid in the prevention of many of these infections (10, 11).

This raises the question of whether children with NS are receiving the recommended vaccinations at appropriate times. A critical first step in this process is to understand the practices and patient education provided by physicians to patients on the topic of vaccinations. The aims of this are: (1) evaluate the immunization practices among pediatric nephrologists, (2) assess the education provided to families of children with NS by the pediatric nephrology providers, (3) assess the parental knowledge and understanding of these immunization recommendations, and (4) assess predictors of PPSV 23 adherence.

Abbreviations: ACIP, Advisory Committee on Immunization Practices; AAP, American Academy of Pediatrics; CDC, Center for Disease Control; NS, nephrotic syndrome; PPSV 23, pneumococcal polysaccharide vaccine; PCP, primary care provider.

METHODS

Parents/guardians of pediatric patients (<18 years old) diagnosed with NS from 2005 to 2018 from 11 institutions in the Pediatric Nephrology Research Consortium and Kidney Research Network participated in the study. Patient inclusion criteria were children <18 years old with primary NS diagnosed between 2005 and 2018 who were seen in the pediatric nephrology practice at the participating center on at least one occasion. All pediatric nephrologists at the participating study centers were also included in the study. A parent/guardian survey was distributed, and a separate pediatric nephrology provider survey was distributed to pediatric nephrologists at each center. This study was performed in line with the principles of the Declaration of Helsinki. Institutional Review Boards of each participating center approved the study. Consent and assent were obtained per institutional guidelines.

Survey and Measures

The parent/guardian survey contained 24 questions which were created by the authors and included demographic data, immunosuppression history, parental knowledge of immunization recommendations and their intent to follow the recommendations, and potentially vaccine-preventable hospitalizations for infections. The provider survey contained 21 questions which were created by the authors and included demographic data, provider immunization recommendation practices, knowledge of the current immunization guidelines, and hospitalizations for infections at any time during their practice that may have been vaccine-preventable. Family caregivers/study coordinators provided redacted copies of patient immunization records. Validation studies of self-administered surveys have shown this type of survey methodology to be a promising tool across medical disciplines (12–14). Surveys were self-administered by the families and providers in the clinic office. The surveys are provided in the **Supplementary Material 1, 2**.

The Advisory Committee on Immunization Practices (ACIP) Recommended Child and Adolescent Immunization Schedule was used to determine accuracy of parent and provider knowledge of current immunization guidelines (**Figure 1**) (15). In brief summary, the ACIP recommends inactive vaccines for any pediatric patient who is not receiving immunosuppression

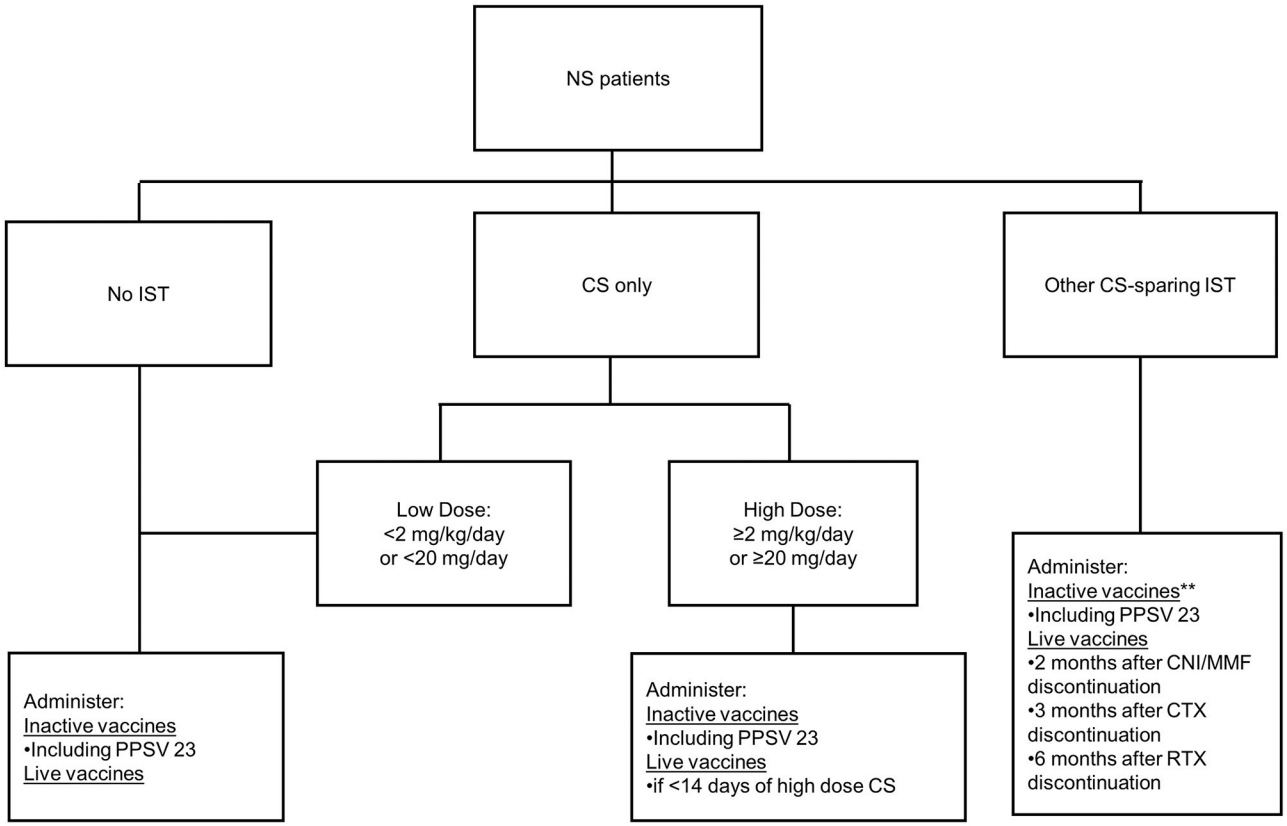

FIGURE 1 | Flow diagram of abbreviated vaccine guidelines. Information for this was adapted from the Advisory Committee on Immunization Practices 2019 vaccine guidelines. Abbreviations: NS, nephrotic syndrome; IST, immunosuppression; CS, corticosteroids; PPSV 23, polysaccharide pneumococcal vaccine; CNI, calcineurin inhibitor; MMF, mycophenolate mofetil; CTX, cyclophosphamide; RTX, rituximab. **Defer 6 months after RTX discontinuation, or administer if no finite endpoint for RTX and assess titers after discontinuation of RTX.

medications as well as those on most immunosuppression therapies. There is an exception to consider deferring inactive vaccines for 6 months after receiving B-cell depleting therapy, or may consider administration of inactive vaccines while receiving B-cell depleting therapy with assessment of titers after discontinuation of the B-cell depleting agent. With regards to live vaccines, the ACIP recommends administration of live vaccines in patients who are not on immunosuppression therapy; patients who have received <14 days of high dose corticosteroids; and patients who have been off of Calcineurin inhibitors (CNI)/Mycophenolate Mofetil (MMF) for 2 months, off of cytotoxic agents (i.e., Cyclophosphamide) for 3 months, and off of B-cell depleting agents (i.e., Rituximab) for 6 months. A more detailed summary of this guideline is provided in **Supplementary Material 3**.

Patient demographic variables included state of residence, patient age, race, parent/guardian level of education, and household income. Provider demographic variables included institution, type of practice (academic vs. private), type of subspecialty training (Pediatric Nephrology, Internal Medicine-Pediatric Nephrology, or Internal Medicine Nephrology), and percent effort allocated to clinical care.

Statistical Analysis

Descriptive statistics included frequencies for categorical variables and mean ± standard deviation (SD) or median (IQR) for continuous variables, as appropriate. Fischer's exact tests were used to compare knowledge and guidelines across regions, and evaluate predictors of PPSV 23 vaccination. Multivariable logistic regression was used to test independent predictors of PPSV 23 vaccination. Variables with unadjusted $p < 0.2$ entered a multivariable model and were removed in descending order until all remaining terms were significant. For all analyses, $p \leq 0.05$ were considered statistically significant.

RESULTS

Study Population Demographics

Between November 2015 and December 2018, 175 pediatric patients diagnosed with NS between January 2005 and December 2018, from 11 North American centers, were eligible for the study, of which 153 parents/guardians of pediatric subjects consented to the study and completed the anonymous survey (parent/guardian response rate 87.4%). Fifty-eight pediatric nephrologists at participating centers were provided an

TABLE 1a | Demographic description of pediatric nephrotic syndrome subject population.

Variable	NS subject study participants *n* = 153
Current Age (y), n (%)	
0–5	46 (30)
6–11	70 (46)
12–22	37 (24)
Age at Diagnosis (y), n (%)	
0–5	117 (76)
6–11	30 (20)
12–18	6 (4)
Race, n (%)	
Asian/Pacific Islander	21 (14)
Black/African American	27 (18)
Hispanic/Latino	25 (16)
Native American	4 (3)
White/Caucasian	62 (40)
Other	14 (9)
Region of care, n (%)	
Coastal	28 (18)
Midwest	64 (42)
South	61 (40)
Highest level of parent education, n (%)	
No schooling to 8th grade	3 (2)
Some high school to high school graduate	30 (20)
Some college, college graduate, or vocational training	87 (57)
Some postgraduate work to postgraduate degree	30 (20)
Decline to answer	3 (2)
Household annual income, n (%)	
<$25,000	32 (21)
$25,000–$39,999	22 (14)
$40,000–$49,999	7 (5)
$50,000–$74,999	15 (10)
$75,000–$99,999	20 (13)
>$100,000	44 (29)
Decline to answer	13 (8)
Immunosuppression exposure, n (%)	
Calcineurin inhibitor	94 (61)
Cyclophosphamide	19 (12)
Mycophenolate mofetil	55 (36)
Prednisone	143 (93)
Rituximab	23 (15)
Other	3 (2)
None	2 (1)
Number of times seen by pediatric nephrology provider in the past year	
Once	9 (6)
2–3 times	63 (41)
≥4 times	81 (53)

TABLE 1b | Demographic description of pediatric nephrology provider population.

Variable	Pediatric nephrology provider participants *n* = 50
Region, n (%)	
Coastal	12 (24)
Midwest	21 (42)
South	17 (34)
Type of practice, n (%)	
Academic	43 (86)
Private	4 (8)
Both academic & private	3 (6)
Type of subspecialty training, n (%)	
Pediatric nephrology	47 (94)
Internal medicine-pediatric nephrology	3 (6)
Provider percent effort, median (IQR)	
Clinical care to pediatric patients	70 (55–86)
Clinical care to adult patients	0 (0)
Administration	4 (0–20)
Research	5 (0–16)
Education	5 (0–10)

anonymous survey, of which 50 (86.2%) completed the survey. All subjects were stratified by region [Coastal (3 centers), Midwest (4 centers), and South (4 centers)]. Due to the small number of study centers on either coast, the current study grouped these centers into one "Coastal" region to avoid identifying a single site and its study participants.

At study enrollment (**Table 1a**), the median age at NS diagnosis was 3 years of age (IQR 2-5); nearly half of the pediatric patients (46%) were 6–11 years of age at study enrollment; 40% were White; majority of parents/guardians had some post high school education (77%); and nearly all pediatric patients had exposure to immunosuppression (99%). In the pediatric nephrology provider population (**Table 1b**), the majority practiced in an academic setting (86%); majority trained specifically in Pediatric Nephrology (94%); and all provided care to pediatric patients with the median percent effort allocated to clinical care of 70% (IQR 55–86%).

Pediatric Nephrology Provider Vaccine Recommendations

Table 2 displays the pediatric nephrology provider recommendations for inactive vaccines. All providers indicated that they provide recommendations for inactive vaccines to patients. Eighty-two percent recommended inactive vaccines while off of steroids and in remission, and 78% recommended inactive vaccines on low dose steroids. However, only 44% recommended inactive vaccines while on high dose steroids with 58% recommending inactive vaccines when off of other immunosuppressive medications. Only 44% recommended inactive vaccines at any time regardless of immunosuppression status. Overall, 56% of providers had at least one response inconsistent with guidelines.

Table 3 displays the pediatric nephrology provider recommendations for live vaccines. Of the 50 providers surveyed, only 1 (2%) indicated they did not provide live vaccine recommendations to patients. Seventy-eight percent recommended live vaccines while off steroids and in remission; however, only 30% recommended live vaccines while on low dose steroids. All providers recommended avoiding live vaccines while on daily high dose steroids, and 76% recommended

TABLE 2 | Provider inactive vaccine recommendation.

Inactive immunizations	Total $N = 50$ (%)	Coastal $N = 12$ (%)	Midwest $N = 21$ (%)	South $N = 17$ (%)	Fischer's exact p
I do not provide recommendations regarding INACTIVE immunizations	0 (0)	0 (0)	0 (0)	0 (0)	—
I provide/recommend when the patient is…					
a. Off steroids and in remission	41 (82)	10 (83)	16 (76)	15 (88)	0.73
b. On low dose (every other day) steroids	39 (78)	10 (83)	17 (81)	12 (71)	0.75
c. On daily high dose steroids	22 (44)	5 (42)	7 (33)	10 (59)	0.28
d. Off other immunosuppressive medications (i.e., cyclosporine, tacrolimus, mycophenolate mofetil, cyclophosphamide, rituximab)	29 (58)	9 (75)	9 (43)	11 (65)	0.18
I would not alter the immunization schedule for INACTIVE immunizations in patients with NS. They can receive them at any time	22 (44)	5 (42)	7 (33)	10 (59)	0.28
I would never provide an INACTIVE immunization to a NS patient regardless of therapy or remission status	0 (0)	0 (0)	0 (0)	0 (0)	—
All responses consistent with guidelines	22 (44)	5 (42)	7 (33)	10 (59)	0.28

Data are shown as number (percent).
NS, nephrotic syndrome.

receiving live vaccines while off other immunosuppressive medication. Overall, only 22% of provider's responses were in complete agreement with the ACIP guidelines for live vaccines. The main deviance from the ACIP guidelines was with respect to low dose steroids. Of the 39 providers that did not follow guidelines, 35 (90%) indicated they would not recommend live vaccines while on low dose steroids.

Pediatric NS Parent/Guardian Vaccine Knowledge

Table 4 shows the parent/guardian knowledge of recommended inactive vaccines. Out of 153 respondents, 13% indicated that no one told them when it was acceptable to receive inactive immunizations. Of the remaining, only 48% indicated that inactive vaccines could be administered while off steroids and in remission with 38% indicating that inactive vaccines could be given on low dose steroids. Only 33% indicated that inactive vaccines could be administered while on high dose steroids with 33% indicating that inactive vaccines could be given when off other immunosuppressive medications. Overall, 32% of parents/guardian responses suggested understanding of immunization practices that aligned with ACIP guidelines for inactive vaccines.

Table 5 shows the parent/guardian knowledge of recommended live vaccines. Twenty percent of parents/guardians indicated that no one told them when it was acceptable to receive live immunizations. Of the remaining, 27% indicated that live vaccines could be administered while off steroids and in remission; 8% indicated that live vaccines could be given while on low dose steroids; and 4% of parents/guardians indicated that live vaccines could be administered on high dose steroids. Twenty-one percent indicated that live vaccines could be administered when off other immunosuppression medications. Overall, 1% of parent/guardian responses suggested an understanding of current live vaccination best practices.

Pediatric NS Patient Immunization Status Validation and Predictors

One-hundred and 52 parents/guardians of pediatric NS subjects (99%) indicated that their child had been vaccinated and one pediatric NS subject (1%) had never been vaccinated. Records of vaccinations were available from 141 (93%) participants for validation of immunization status. Immunization status was considered up to date if the subject received all ACIP recommended vaccines based on immunosuppression status, age at time of survey, and vaccine release date. One-hundred and twenty-two subjects (87%) were up to date on the recommended vaccines. Fifty-three percent received the 7-valent pneumococcal vaccine, 71% received the 13-valent pneumococcal vaccine, and 48% received the PPSV 23.

Subjects residing in the Midwest was a positive predictor of subjects receiving the PPSV 23 ($p < 0.001$). Additionally, receiving immunization recommendations from the pediatric nephrology provider was a positive predictor of subjects receiving the PPSV 23 as well (p 0.02) (**Table 6**). Multivariable logistic regression of the differences in **Table 6** revealed that region and immunization recommendations were independent predictors of vaccinations. Those in the Coastal region were less likely to be vaccinated than the Midwest (OR = 0.30 95% CI = 0.13–0.66) and those with recommendations from the pediatric nephrologist were more likely to be vaccinated than those without recommendations (OR = 1.95 95% CI = 1.05–3.63) (**Table 7**). When provider responses were reviewed, providers in the Coastal region were less likely to review immunization records on a periodic basis ($p < 0.01$) and were more likely to state that they were unsure if they would recommend the

TABLE 3 | Provider live vaccine recommendation.

Live immunizations	Total *N* = 50 (%)	Coastal *N* = 12 (%)	Midwest *N* = 21 (%)	South *N* = 17 (%)	Fischer's exact *p*
I do not provide recommendations regarding LIVE immunizations	1 (2)	0 (0)	1 (5)	0 (0)	0.99
I provide/recommend when the patient is…					
a. Off steroids and in remission	39 (78)	9 (75)	17 (81)	13 (76)	0.91
b. On low dose (every other day) steroids	15 (30)	3 (25)	7 (33)	5 (29)	0.93
c. On daily high dose steroids	0 (0)	0 (0)	0 (0)	0 (0)	—
d. Off other immunosuppressive medications (i.e., cyclosporine, tacrolimus, mycophenolate mofetil, cyclophosphamide, rituximab)	38 (76)	10 (83)	15 (71)	13 (76)	0.91
I would not alter the immunization schedule for LIVE immunizations in patients with NS. They can receive them at any time	0 (0)	0 (0)	0 (0)	0 (0)	—
I would never provide a LIVE immunization to a NS patient regardless of therapy or remission status	0 (0)	0 (0)	0 (0)	0 (0)	—
All responses consistent with guidelines	11 (22)	3 (25)	5 (24)	3 (18)	0.83

Data are shown as number (percent).
NS, nephrotic syndrome.

TABLE 4 | Parent inactive vaccine recommendation.

Inactive immunizations	Total *N* = 153 N (%)	Coastal *N* = 28 N (%)	Midwest *N* = 64 N (%)	South *N* = 61 N (%)	Fischer's exact *p*
No one ever told me when it was ok to receive INACTIVE immunizations.	20 (13)	6 (21)	4 (6)	10 (16)	0.07
I was told it was OK to receive INACTIVE immunizations when my child is…					
a. Off steroids and in remission	74 (48)	12 (43)	39 (61)	23 (38)	0.03
b. On Low dose (every other day) steroids	58 (38)	8 (29)	30 (47)	20 (33)	0.15
c. On daily high dose steroids	50 (33)	8 (29)	24 (38)	18 (30)	0.57
d. Off other immunosuppressive medications (i.e., cyclosporine, tacrolimus, mycophenolate mofetil, cyclophosphamide, rituximab)	51 (33)	7 (25)	24 (38)	20 (33)	0.56
I was told that my child could receive INACTIVE immunizations at any time regardless of type of medication or remission status.	49 (32)	7 (25)	24 (38)	18 (30)	0.47
I was told that my child could never receive INACTIVE immunizations.	0 (0)	0 (0)	0 (0)	0 (0)	—
Parent had correct knowledge of guidelines	49 (32)	7 (25)	24 (38)	18 (30)	0.47

Data are shown as number (percent).

PPSV23 if the patient had previously received the 7-valent pneumococcal conjugate vaccine (PCV) or PCV-13 (p 0.03) (**Supplementary Material 4**).

DISCUSSION

While there have been published studies investigating vaccine practices and vaccine adherence patterns, these studies have evaluated the medical provider population alone or the parent population alone. Our study investigates both the subspecialty medical provider and parent population from the same institution and region simultaneously. This study demonstrates gaps in vaccine knowledge of both pediatric nephrology providers and parents of children with NS who are immunosuppressed.

The decision to vaccinate a child can be influenced by parental knowledge, parental understanding of the benefits of vaccination, provider knowledge, and other complex factors related to their child's underlying disease and treatment. Deficiencies in vaccinations directly impact the health of the individual child and of the larger pediatric community if herd protection is not achieved due to poor vaccine adherence. Children most at risk for severe vaccine-preventable infections are those who are immunocompromised, which includes children with NS.

TABLE 5 | Parent live vaccine recommendation.

Live immunizations	Total $N = 153$ N (%)	Coastal $N = 28$ N (%)	Midwest $N = 64$ N (%)	South $N = 61$ N (%)	Fischer's exact p
No one ever told me when it was ok to receive LIVE immunizations.	30 (20)	10 (36)	9 (14)	11 (18)	0.05
I was told it was OK to receive LIVE immunizations when my child is…					
a. Off steroids and in remission	41 (27)	6 (21)	21 (33)	14 (23)	0.37
b. On low dose (every other day) steroids	12 (8)	0 (0)	9 (14)	3 (5)	0.05
c. On daily high dose steroids	6 (4)	0 (0)	4 (6)	2 (3)	0.56
d. Off other immunosuppressive medications (i.e., cyclosporine, tacrolimus, mycophenolate mofetil, cyclophosphamide, rituximab)	32 (21)	2 (7)	21 (33)	9 (15)	0.007
I was told that my child could receive LIVE immunizations at any time regardless of type of medication or remission status.	5 (3)	0 (0)	4 (6)	1 (2)	0.27
I was told that my child could never receive LIVE immunizations.	18 (12)	5 (18)	7 (11)	6 (10)	0.57
Parent had correct knowledge of guidelines	1 (1)	0 (0)	0 (0)	1 (2)	0.58

Data are shown as number (percent).

The Center for Disease Control (CDC), ACIP, and the Committee on Infectious Diseases of the American Academy of Pediatrics (AAP) release an updated recommended immunization schedule for healthy children annually (16). This immunization schedule is available to medical providers online (http://www.cdc.gov/vaccines/recs/schedules/child-schedule.htm). In addition to the yearly updated recommendations, health care providers should be aware of guidelines for vaccinating immunocompromised patients, which are readily available online (https://www.cdc.gov/vaccines/hcp/acip-recs/general-recs/immunocompetence.html and https://www.cdc.gov/vaccines/schedules/downloads/child/0-18yrs-child-combined-schedule.pdf). Despite these guidelines and published comprehensive reviews outlining vaccine guidelines in various immunosuppressed states, there remains a discrepancy in vaccine practices among pediatric nephrologists in the management of children with NS on immunosuppressive therapy (17–19).

The discrepancy in vaccine practice amongst providers and low compliance in following ACIP guidelines is likely multifactorial. It could be in part due to the provider's misinterpretation of vaccine guidelines due to the complex immunosuppression regimens that these NS patients may require, or the provider's disagreement of vaccination guidelines based on anecdotal experience with NS relapses and/or belief that immunosuppressed patients may not mount a robust vaccine response. In this study, only 44% of provider responses were in complete alignment with the ACIP guidelines for inactive vaccines and 22% for live vaccines. The CDC general principles for those with altered immunocompetence, state that inactivated vaccines could be deferred during a time of immunosuppression since vaccines may be less effective during this period. However, if an inactivated vaccine is given during the time that a patient is immunosuppressed, then the inactive vaccine may need to be repeated when immune function has improved. Yet, the CDC guidelines emphasize the need for immunocompromised patients to receive certain inactive conjugated and polysaccharide-based vaccines (i.e., pneumococcal vaccines, *Haemophilus influenzae* type b, and meningococcal vaccines) due to the increased risk for disease if the vaccine is withheld. Live vaccines are typically deferred until immune function has improved due to the risk of uninhibited growth of the attenuated live virus or bacteria in those with altered immunocompetence (20–23). It is clear that the vaccine guidelines for those who are immunosuppressed can be nuanced.

Since 80% of pediatric patients with new onset NS are steroid-sensitive and eventually taper off of steroid therapy, some pediatric nephrologists may opt to withhold vaccines until these patients have return of immune function to help mount a more effective response to the vaccine (24). In our study, 44% of the pediatric nephrologists surveyed reported withholding inactive vaccines when a NS patient is on any dose of steroids due to concern that the patient may not mount an immune response. Ten percent of the pediatric nephrologists surveyed observed a NS relapse after immunization administration. However, published studies have demonstrated vaccine efficacy in NS patients who are immunosuppressed. Aoun and Ulinski exhibited good serologic response to the PPSV 23 in children with NS on high dose prednisone in the short term and in the long term (25). They also demonstrated that the long term pneumococcal antibody response was not impacted by other immunosuppressive agents (26). Hsu et al. demonstrated good vaccine efficacy of the pneumococcal vaccine in children with NS on high dose steroids and no differences in rate of NS relapse (27). In addition, other inactive polysaccharide-based vaccines, such as meningococcal C conjugate vaccine has not been shown to be associated with increased NS relapses (28). Other studies have demonstrated that the Varicella vaccine has been well-tolerated in children with NS and highly immunogenic in those on low-dose, every other day prednisone (29, 30). Despite these groups demonstrating vaccine efficacy without differences in

TABLE 6 | Pediatric NS patient PPSV 23 vaccine adherence and predictors*.

Variable	N (% NS children vaccinated to PPSV 23)	N (% NS children unvaccinated to PPSV 23)	Fischer's exact *p*
Parent income (*N* = 125)			0.30
<$25,000 (*n* = 29)	17 (58.6)	12 (41.4)	
$25,000–39,999 (*n* = 19)	8 (42.1)	11 (57.9)	
$40,000–49,999 (*n* = 7)	14 (57.1)	3 (42.9)	
$50,000–74,999 (*n* = 15)	11 (73.3)	4 (26.7)	
$75,000–99,999 (*n* = 18)	10 (55.6)	8 (44.4)	
≥$100,000 (*n* = 37)	15 (40.5)	22 (59.4)	
Parent education (*N* = 134)			0.07
No schooling to 8th grade (*n* = 2)	2 (100)	0 (0)	
Some high school to high school graduate (*n* = 25)	10 (40)	15 (60)	
Some college, college graduate, or vocational training (*n* = 82)	47 (57.3)	35 (42.7)	
Some postgraduate work to postgraduate degree (*n* = 25)	9 (36)	16 (64)	
Race (*N* = 135)			0.83
White (*n* = 53)	28 (52.8)	25 (47.1)	
Black (*n* = 25)	12 (48)	13 (52)	
Hispanic (*n* = 24)	14 (58.3)	10 (41.7)	
Asian (*n* = 17)	8 (47.1)	9 (52.9)	
Native American (*n* = 4)	1 (25)	3 (75)	
Other (*n* = 12)	5 (41.7)	7 (58.3)	
Region (*N* = 135)			<0.001
Coastal (*n* = 17)	1 (5.9)	16 (94.1)	
Midwest (*n* = 58)	38 (65.5)	20 (34.4)	
South (*n* = 60)	29 (48.3)	31 (51.7)	
Number of times seen by pediatric nephrology provider in the past year (*N* = 135)			0.99
Once	4 (44.4)	5 (55.6)	
2–3 times	28 (50.9)	27 (49.1)	
≥4 times	36 (50.7)	35 (49.3)	
Immunization recommendations provided by Pediatric nephrologist (*N* = 118)			0.02
Yes (*n* = 100)	55 (55)	45 (45)	
No (*n* = 18)	4 (22.2)	14 (77.8)	

NS, nephrotic syndrome; PPSV 23, polysaccharide pneumococcal vaccine 23-valent.

**Excluded the following from analysis: (1) Centers in Coastal region without vaccine records to validate PPSV 23 status, (2) Pediatric NS subjects who were unable to receive the PPSV 23 due to age eligibility, (3) Parents/guardians who declined to answer in the parent income and parent education variables, and (4) Parents/guardians who answered "I am not sure" in Immunization recommendations provided by pediatric nephrologist variable.*

NS relapse rates, there has been a wide variation of vaccine practices as evidenced by two studies conducted in 1993 and 2001 (17, 18). Our study similarly observed varied vaccine practices by providers, which translates to the gap in parental knowledge and understanding of the timing of vaccine administration in their children with NS as seen in the parental/guardian survey responses.

NS can be a difficult disease to treat especially in patients who have a steroid-dependent, frequently-relapsing, or steroid-resistant clinical course. These patients may have no or very brief periods of time off of immunosuppression medications to keep the disease in control. As a result, opportunities to vaccinate with inactive and/or live vaccines become limited if providers choose to withhold immunizations until full recovery of immune function. Withholding vaccines in this high-risk population can lead to an increased risk of vaccine-preventable disease (9). It is known that infection is a common trigger for NS relapses which can result in the need for hospitalization to help manage the complications of the relapse, thus leading to significantly higher hospitalization charges (8).

Factors associated with immunization adherence have been studied by various groups. Our group specifically evaluated the adherence to PPSV23 administration since it is recommended by the ACIP for pediatric patients with NS and immunosuppressed states. Cost of vaccines, lack of parental knowledge of the benefits of vaccines, and lack of vaccine recommendation by medical providers has been reported to adversely impact immunization adherence (31, 32). Our study similarly showed that provider vaccine recommendations were associated with vaccine adherence to PPSV 23. However, we did not investigate

TABLE 7 | Adjusted logistic regression model of PPSV 23 vaccine (Complete case analysis $n = 110$).

Variable	Odds ratio [95% confidence interval]	*p*-value
Region		0.01
Coastal	0.30 [0.13–0.66]	0.003
Midwest	Reference	Reference
South	0.56 [0.24–1.30]	0.18
Immunization recommendations provided by pediatric nephrologist		0.04
Yes	1.95 [1.05–3.63]	0.04
No	Reference	Reference

PPSV 23, polysaccharide pneumococcal vaccine 23-valent.

the cost of vaccines as a potential factor of immunization adherence in this survey study. Schuller et al. demonstrated an increased likelihood of vaccination if the child's caregiver received higher education, lived in the Northeast, had private insurance, and was of Hispanic race among US children (33). We found that subjects residing in the Midwest were more likely to receive the PPSV 23, which may be due to a higher number of participating study sites in the Midwest region compared to other regions surveyed in North America for our study, and therefore the Midwest region may be more representative than a region with fewer sites. Additionally, parent income, level of parent education, and race were not positive predictors of subjects receiving the PPSV 23 in the current study. The differences in the factors associated with vaccines adherence in our study compared to other published studies may also be explained by our small sample size. Our study also evaluated other possible contributing factors to vaccine adherence including the ability of the subspecialty clinic to provide vaccines (i.e., pneumococcal vaccine) and the communication of vaccine recommendations from the pediatric nephrology provider to the patient's primary care provider (PCP). Seventy-six percent of providers indicated that their subspecialty clinic can administer pneumococcal vaccines. The remaining providers who do not have this capability may defer the responsibility of vaccinations to the PCP. Ninety-two percent of providers indicated that they communicate their vaccine recommendations to their patient's PCP with a letter as the most common modality of communication.

The current study demonstrates the need for a more uniform vaccine practice in the pediatric NS population among pediatric nephrologists, which will in turn better educate and help parents/guardians understand the importance of vaccination and when it is appropriate for their child to receive certain vaccines.

Limitations and Strengths

The limitations of this study include the heterogeneity of survey responses among the parent/guardian cohort and pediatric nephrology providers with regards to knowledge of the ACIP guidelines. The discrepant responses amongst both groups could be explained by comprehension and communication between parent and provider or alternatively by survey interpretation. Since the surveys were anonymous for both cohorts, we were unable to associate the parent/guardian and provider response resulting in the inability to directly correlate responses. While this study evaluated the communication of vaccine recommendations from pediatric nephrologist to PCP, an assessment of the knowledge and practice of the patient's PCP was not evaluated which could have identified another area for improvement of vaccine adherence in this cohort. Lastly, the small sample size of this study may have played a role in the differences in factors associated with vaccine adherence compared to other published studies.

The strengths of this study include the high response rate. This study also investigated the immunization knowledge from both pediatric nephrology providers and parent/guardians of children with NS simultaneously from their shared institutions, which helped to evaluate for any institution/region-specific immunization practices.

CONCLUSION

Our findings support the growing evidence that vaccine recommendation by medical providers is paramount in vaccine adherence among pediatric patients with NS. The disparate survey responses among the pediatric nephrology providers and parents/guardians likely reflect the individual interpretations of the ACIP guidelines by the medical providers and possibly their anecdotal experiences with treatment of NS. Additionally, some pediatric nephrology practices do not have the capability to administer certain vaccines (i.e., PPSV 23) and therefore rely on vaccinations through the patient's PCP. Ensuring primary care establishment with a PCP as well as being a champion to clarify and communicate vaccination provisioning guidance with the patient's PCP may help to improve vaccine adherence. Future studies assessing the knowledge and practice of vaccine recommendations by PCPs for pediatric patients with NS may also highlight other areas for improvement of vaccine adherence such as communication between healthcare providers to assure vaccinations occur in a timely and complete fashion in the child's primary care office. Lastly, it is important that the ACIP immunization guidelines are reviewed by the pediatric nephrologist and primary care physicians yearly to be informed of any change in the immunization practice recommendations for those with altered immune competence.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by each participating center. Written informed consent

to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

Data analysis was performed by JT and CT. All authors read and approved the final manuscript, contributed to the study conception, design, and data collection.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2020.619548/full#supplementary-material

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ORIGINAL RESEARCH
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Function of miR-24 and miR-27 in Pediatric Patients With Idiopathic Nephrotic Syndrome

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Purpose: We investigated the pathogenesis of idiopathic nephrotic syndrome (INS) by measuring the effects two specific miRNAs on Th2 cells in children with this disease.

Methods: After informed consent, we enrolled 20 children with active INS before steroid initiation, 20 children with INS in remission after steroid therapy, and 20 age-matched healthy controls. Flow cytometry was used to measure the levels of Th2 cells and a cytometric bead array was used to measure the levels of IgE, interleukin (IL)−4, and IL-13. RT-PCR was used to measure the levels of miR-24 and miR-27 in $CD4^{+}TCD25^{-}$ cells. PBMCs were isolated using Ficoll density gradient centrifugation, and transfected with different mimic or inhibitor miRNAs. RT-PCR was used to measure the expression of different RNAs, and flow cytometry was used to determine the percentage of Th2 cells.

Results: Relative to healthy controls, children with active INS had higher percentages of Th2 cells ($P < 0.05$), but there was no significant difference in controls and children in remission. The plasma levels of IgE, IL-4, and IL-13 were significantly increased in children with active INS ($P < 0.05$). There were lower levels of miR-24 and miR-27 in children with active non-atopic INS ($P < 0.05$). Transfection experiments indicated that upregulation of each miRNA decreased the percentage of Th2 cells and the level of IL-4 ($P < 0.05$), and down-regulation of each miRNA had the opposite effects ($P < 0.05$).

Conclusion: Children with active INS, with or without atopy, had higher levels of IgE, possibly related to their higher levels of IL-13 and IL-4 due to a drift toward Th2 cells. miR-24 and miR-27 suppressed the expression of Th2 cells and have a critical function regulating Th2 cell expression in INS.

Keywords: idiopathic nephrotic syndrome, miR-24, Th2 cells, IL-4, IL-13, miR-27

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INTRODUCTION

Idiopathic nephrotic syndrome (INS) is the most common renal disease in children, and a main cause of chronic renal failure in children from China (1). The clinical manifestations of INS are proteinuria, low level of plasma albumin, hyperlipidemia, and edema. The prevalence is greatest in preschool children who are 3–5 years-old. About 80–90% of patients with kidney disease who are under 10 years-old have minimal change disease (MCD), a common cause of INS (2). However, the specific etiology and mechanism of INS in children remain unclear. Previous studies showed that

INS is associated with immune system dysfunctions, including humoral immune disorders, T cell subset dysfunction, and abnormal secretion of cytokines, especially due to T cell dysfunction (3–8).

T helper type 2 (Th2) cells, which mainly produce IL-4, IL-5, and IL-13, play a major role in responses to parasitic infections and allergic inflammatory diseases (9–11). A seminal 1959 study reported an association of pollen sensitivity with seasonal proteinuria, and that about 30% of children with INS had atopic manifestations, such as allergic rhinitis and idiopathic dermatitis (12). Subsequent studies confirmed elevated levels of immunoglobulin E (IgE) during the active phase of INS (13–15), but it is unknown whether the increased levels of IgE in these children are pathogenic or coincidental.

Previous researchers proposed a "two hit" hypothesis for the pathogenesis of INS. The "first hit" occurs when microbial products, allergens, or T-cell cytokines (such as IL-13) damage the glomeruli, resulting in the overexpression of CD80 by podocytes and temporary proteinuria (16–18). In normal settings, regulatory cytokines produced by T regulatory cells (Tregs) terminate the CD80 overexpression, so that the proteinuria is transient and mild (18–20). However, a "second hit" occurs when MCD is present, and this leads to a failure to block CD80 expression by podocytes due to a disruption of autoregulatory responses in Tregs or even in the podocytes themselves. After this second hit, CD80 expression remains continuously elevated, leading to nephrotic syndrome (20). Th2 related cytokines such as IL-13, which stimulates IgE-mediated responses, can promote proteinuria in patients with MCD because it can directly induce CD80 expression in podocytes (13–15, 18). Recent studies found increased levels of IL-13 and IL-4 during the active phase of INS, and that IL-13 functions in the pathogenesis of kidney disease (13–15, 18), but the mechanisms responsible for the increased level of IL-13 are still unclear. Several studies also reported that Th2 cells were over-active in INS (8, 18, 21, 22), but the mechanisms leading to over-activation of these cells are also unknown. Studies of these topics may help to elucidate the role of altered immune responses in the pathogenesis of INS.

MicroRNAs (miRNAs) are short (20–23 nucleotides) non-coding RNAs that can alter the expression of targeted genes (23, 24). In particular, a specific miRNA binds to the 3′-untranslated region (3′-UTR) of its target mRNA, and this is followed by inhibition of translation or increased mRNA degradation (23, 24). Initially, most studies of miRNAs were in the field of oncology, but recent studies have also examined their role in kidney disease (25, 26). For example, several studies showed that multiple miRNAs can inhibit the differentiation and function of Th2 cells (27–29). In 2016, researchers studying allergies found that miR-24 and miR-27 also inhibited the function of Th2 by inhibiting the production of cytokines such as IL-4 (28, 29). However, the effects of these two miRNAs on Th2 expression in patients with active INS, and their specific mechanisms need further study.

Atopic patients have abnormally increased proportions Th2 cells and related factors (9–11). However, it is not clear whether these alterations in children with INS are related to atopy and the pathogenesis of nephropathy. Thus, we systematically measured the dynamics of multiple cytokines, miR-24, and miR-27 in the regulation of Th2 cell differentiation during the active and remission phases of INS, and measured changes in the number and function of Th2 cells isolated from children with non-atopic INS under conditions of altered miRNA expression. Our general purpose was to clarify the role of immune system alterations in the pathogenesis of INS, and to identify potential therapeutic targets and new ideas for the treatment of kidney disease.

PATIENTS AND METHODS

Subjects

Forty children with INS (23 males and 17 females; median age: 38 months; age range: 22–92 months) were enrolled as patients and were divided into two groups: before steroid initiation (11 males and nine females; median age: 35 months; age range: 22–84 months) and after steroid therapy (12 males and eight females; median age: 41 months; age range: 26–92 months). There were also 20 healthy volunteers of similar age (11 males and nine females; median age: 31.3 months; age range: 25–105 months) enrolled as controls (Ctrl) who visited the hospital for physical exams.

All patients were examined at Shenzhen Children's Hospital from September 2015 to October 2016. The inclusion criteria were diagnosis of INS based on the 2010 criteria determined using evidence-based diagnosis and treatment guidelines for common kidney diseases in children from China (30). The 40 children were divided into four groups: an active phase (first-onset) group with atopic constitution (AA, $n = 6$), an active phase group with non-atopic constitution (ANA, $n = 14$), a remission group with atopic constitution (ReA, $n = 6$), and a remission group with non-atopic constitution (ReNA, $n = 14$). Atopy was diagnosed based on family history, the presence of relevant symptoms (asthma, recurrent urticaria, eczema, and allergic rhinitis), and elevated serum IgE concentration. The results of the skin-prick tests were positive for all atopic children. Prednisone therapy was initiated at a dose of 2 mg/kg/day. All patients were steroid-sensitive, had negative test results for urinary protein within 4 weeks of treatment, completed the treatment protocol, and received no other immunosuppressants. In addition, none of the patients had a secondary kidney disease (secondary nephrotic syndrome, nephrotic syndrome, congenital kidney disease, etc.) and none had other systemic visceral syndromes. Blood samples were collected for analysis before steroid initiation in the active NS group and 4 weeks after steroid discontinuation in the remission group.

All parents or legal guardians provided informed consent prior to study enrollment, and the study was performed after approval by the local Medical Ethics Committee.

Blood Samples

Venous blood was collected from patients and healthy controls in EDTA tubes. Then, Ficoll density gradient centrifugation was used to isolate peripheral blood mononuclear cells (PBMCs) for analysis by flow cytometry. Plasma samples were collected after centrifugation and were frozen (at −80°C) prior to using the

cytometric bead array (CBA; kit no. 11363D, Dynal, Invitrogen, USA) for isolation of $CD4^+CD25^-$ T cells). Cell purity was based on a threshold of 97% from flow cytometry. Cell activity (determined using the trypan blue exclusion assay) was based on a threshold of 95%.

Extraction of Total RNA Extraction and Synthesis of cDNA

Total RNA (including miRNAs) samples were isolated from $CD4^+CD25^-$ T cells using the miRNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Following confirmation of purity (average $OD_{260nm}/OD_{280nm} = 1.98$), cDNA was synthesized using oligo-dT primers and RevertAid™ H Minus reverse transcriptase (Fermentas, Lithuania). The miScript II RT kit (Qiagen) was used for synthesis of miRNA cDNAs. Negative controls (no first-strand synthesis) were synthesized using reverse transcription without reverse transcriptase.

LightCycler Real-Time PCR

The level of IL-4 was determined using real-time PCR with the Quantitect™ SYBR green PCR Kit (Takara, Japan) and a LightCycler® 2.0 (Roche Molecular Biochemicals, Switzerland). The primers were: 5′-TCATTTTCCCTCGGTTTCAG-3′ (forward) and 5′- ATAGGTGTCGATTTGCAGTG-3′ (reverse). Real-time PCR with the miScript SYBR Green PCR Kit (Qiagen) and the LightCycler® 2.0 were used to quantitate the levels of miR-24 and miR-27. The primers were synthesized from recommendations in the miRNA miScript Primer Assay (Qiagen). The second derivative maximum method was used to identify the crossing point (Cp) with LightCycler software version 3.5.30 (Roche Molecular Biochemicals). After normalization using Relative Quantification Software version 1.0 (Roche Molecular Biochemicals), the levels of the PCR products were presented relative to those of *GAPDH* (target genes) or *U6* (miRNAs).

Flow Cytometry Analysis of Th2 Cells

To measure expression of cytoplasmic markers of Th2 cells, cells were cultured in a 37°C/5% CO_2 incubator, with ionomycin (250 ng/mL; SigmaAldrich), PMA (25 ng/mL; Sigma-Aldrich), and monensin (20 ng/mL; eBio-science, San Diego, CA, USA) added as stimulation for 24 h. Then, cells were stained for different markers, and were fixed and permeabilized using the Cytofix/Cytoperm kit (eBioscience). The cells were stained with specific antibodies (or corresponding isotype-matched controls) and then analyzed using a FACS Canto II flow cytometer (BD Biosciences, Mississauga, ON, Canada). Staining with the fixable viability stain 450 (FVS450, BD Biosciences) was used to determine viability. Validated antibodies (CD3-eFour450A, CD8-FITC, and IL-4-PE) were purchased from eBioscience.

CBA Detection of Plasma IgE, IL-4, and IL-13

A CBA kit (e Bioscience) was used to measure the plasma levels of IgE, IL-4, and IL-13. Each sample was measured twice.

Cell Transfection and Culture

The roles of miR-24 and miR-27 in Th2 cells were examined using transfection experiments in PBMCs. First, PBMCs were isolated from healthy controls or patients using Ficoll density gradient centrifugation. Then mimics (Ctrl-m, miR-24-m, or miR-27-m) or inhibitors (Ctrl-i, miR-24-i, or miR-27-i) from RiboBio (Guangzhou, China) were transfected into the PBMCs using the riboFECT CP Transfection Kit (Guangzhou, China). Cells were cultured in RPMI-1640 medium (Gibco, CA, USA) that was supplemented with 15% fetal calf serum (Gibco, CA, USA) and maintained at 37°C/5% CO_2 in 24-well plates (3×10^6 cells per mL). Cells were then harvested for flow cytometry and RT-PCR analyses.

Statistical Analysis

Statistical analyses were performed using SPSS software for Windows version 13.0 (SPSS Inc., USA). Data are expressed as means ± standard deviations. For comparisons of multiple groups, a one-way analysis of variance was used. For comparison of two groups, Student's *t*-test was used. *P*-values below 0.05 were considered significant.

RESULTS

Patients With Atopic and Non-atopic INS Have Increased Serum IgE Levels

We first measured the plasma levels of IgE in the five different groups using a CBA (**Figure 1**). Analysis of IgE concentration indicated significantly greater levels in the AA group (1350.67 ± 837.39 IU/mL, $P < 0.05$) and the ANA group (441.01 ± 357.45 IU/mL, $P < 0.05$) than in the Ctrl group (57.76 ± 48.25 IU/mL). In addition, the AA group had higher levels of IgE than the other groups (all $P < 0.05$). Compared with the ANA group, the Ctrl, ReNA (62.97 ± 27.31 IU/mL), and ReA (237.75 ± 95.15 IU/mL) groups had significantly reduced levels of IgE (all $P < 0.05$). However, the ReA and ReNA groups had no significant difference ($P > 0.05$).

Patients With Active Phase INS Have Over-Expression of Th2 Cells and Associated Cytokines

We quantified the Tregs from whole blood samples in the five groups using flow cytometry (**Figures 2A,B**). The percentage of peripheral Th2 cells in the AA group (5.60% ± 1.21) and the ANA group (4.23% ± 0.92) were significantly greater than in the Ctrl group (3.29% ± 1.02, both $P < 0.05$). As with IgE, there was no significant difference between the ReA (3.60% ± 0.78) and ReNA (3.40% ± 0.64) groups ($P > 0.05$). Also as with IgE, the AA group had a higher percentage of Th2 cells than all other groups (all $P < 0.05$).

We then measured the plasma levels of IL-4 and IL-13 in all five groups using CBA (**Figures 2C,D**). Relative to the control group, the AA group had higher levels of IL-4 (66.66 pg/mL ± 43.85 vs. 23.29 pg/mL ± 13.91, $P < 0.05$) and IL-13 (65.15 pg/mL ± 16.50 vs. 15.66 pg/mL ± 8.16, $P < 0.05$). Also relative to the control group, the ANA group had higher levels of IL-4 (37.04 pg/mL ± 17.39 vs. 23.29 pg/mL ± 13.91, $P < 0.05$)

and IL-13 (33.15 pg/mL ± 14.09 vs. 15.66 pg/mL ± 8.16, $P < 0.05$). However, there was no significant difference between the ReA and ReNA groups in these cytokines (IL-4: 35.91 pg/mL ± 15.75 vs. 23.73 pg/mL ± 11.71, $P > 0.05$; IL-13: 22.33 pg/mL ± 8.87 vs. 16.53 pg/mL ± 7.76, $P > 0.05$). Compared with the AA group, all other groups had significantly decreased levels of both cytokines (all $P < 0.05$).

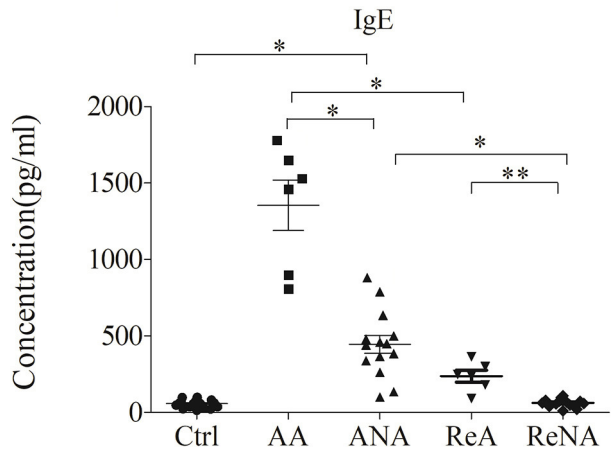

FIGURE 1 | Children with atopic and non-atopic INS have increased serum levels of IgE. Here and below: all data are shown as means ± SDs; *$P < 0.05$ and **$P > 0.05$; and abbreviations are healthy control (Ctrl, $n = 20$), active phase atopic (AA, $n = 6$), active phase non-atopic (ANA, $n = 14$), remission phase atopic (ReA, $n = 6$), and remission phase non-atopic (ReNA, $n = 14$).

Patients With Non-atopic INS Have Altered Expression of miR-24 and miR-27

Previous research showed that miR-24 and miR-27 affected Th2 cell expression. Because we identified increased expression of Th2 cells in the ANA group, we also determined whether these miRNAs were also decreased (**Figure 3**). The results showed that the levels of miR-24 and miR-27 were significantly lower in the ANA group than in the Ctrl group (both $P < 0.05$; **Table 1**). The levels of these two miRNAs were greater in the ReNA group than in the ANA group, but not up to the levels in the Ctrl group ($P < 0.05$).

miR-24 and miR-27 Suppress the Expression of Th2 Cells and IL-4

We further examined the relationship between miR-24, miR-27, and Th2 cells by transfection of PBMCs isolated from healthy volunteers and INS patients with ANA using six different miRNAs: negative control (Ctrl-m); miR-24 mimic (miR-24-m); miR-27 mimic (miR-27-m); miRNA inhibitor negative control (Ctrl-i); miR-24 inhibitor (miR-24-i); or miR-27 inhibitor

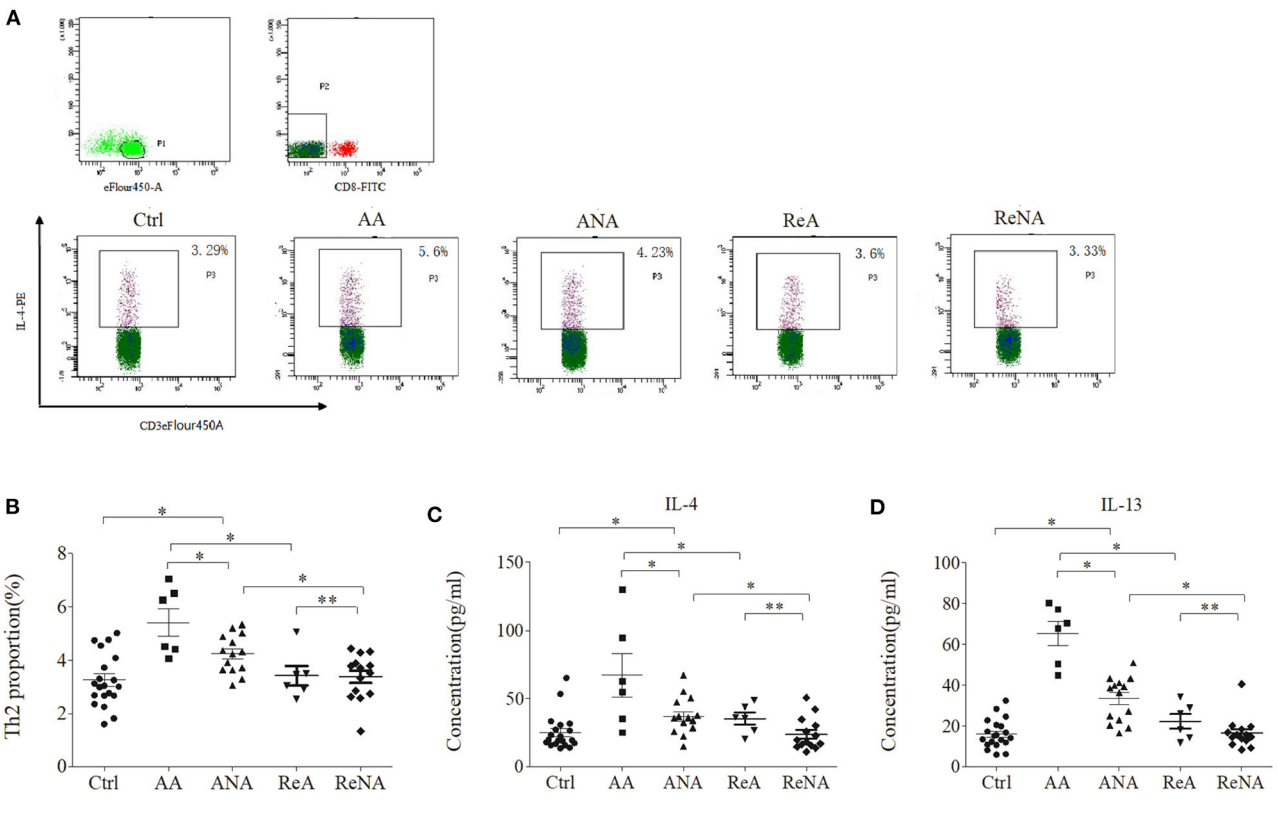

FIGURE 2 | Children with INS have increased levels of Th2 cells and Th2-related cytokines. **(A)** Flow cytometric analysis of Th2 cells. **(B)** Percentages of Th2 cells. **(C)** Plasma concentrations of IL-4. **(D)** Plasma concentrations of IL-13. *$P < 0.05$ and **$P > 0.05$.

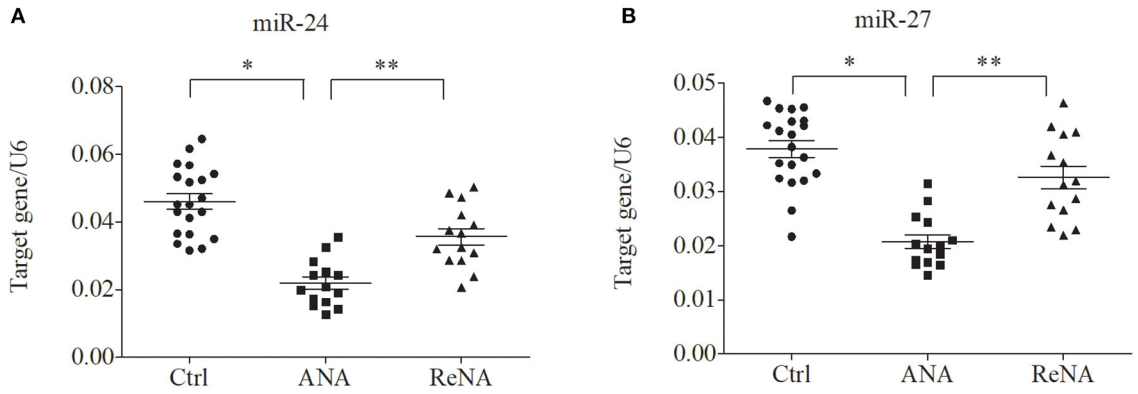

FIGURE 3 | Children with INS have reduced expression of miR-24 and miR-27 in $CD4^+CD25^-$ T cells. Expression of miRNAs (relative to U6) were determined by real-time PCR. **(A)** Expression levels of miR-24. **(B)** Expression levels of miR-27. $^*P < 0.05$ and $^{**}P > 0.05$.

TABLE 1 | Expression of miR-24 and miR-27 in patients with non-atopic INS and healthy controls.

	Ctrl ($n = 20$)	ANA ($n = 14$)	ReNA ($n = 14$)
miR-24	$(46.03 \pm 10.08)\times10^{-3}$	$(21.84 \pm 6.86)\times10^{-3a}$	$(35.59 \pm 9.10)\times10^{-3b}$
miR-27	$(37.83 \pm 6.83)\times10^{-3}$	$(20.72 \pm 4.93)\times10^{-3a}$	$(32.55\pm7.86)\times10^{-3b}$

Values are expressed as mean ± SD.
[a]P < 0.05 for one-way ANOVA vs. Ctrl group.
[b]P < 0.05 for one-way ANOVA vs. ANA group.
Ctrl, healthy control; ANA, active phase non-atopic; ReNA, remission phase non-atopic.

(miR-27-i). The results showed that up-regulation of miR-24 (**Figures 4A,B**) and miR-27 (**Figures 5A,B**) reduced the percentage of Th2 cells (**Figures 4C,D**, **5C,D**) and the expression of IL-4 mRNA (**Figures 4E,F**, **5E,F**). In agreement, down regulation of miR-24 (**Figures 6A,B**) and miR-27 (**Figures 7A,B**), increased the percentage of Th2 cells (**Figures 6C,D**, **7C,D**) and the expression of IL-4 mRNA (**Figures 6E,F**, **7E,F**).

DISCUSSION

A large body of evidence has demonstrated that the immune system may play a crucial role in INS (3), although the pathogenic details of this disease remain mostly unknown. Previous research suggested that INS may be due to an abnormal T cell response or the dysregulation of T lymphocytes (3–8). MCD is the most common cause of INS (1, 2, 18, 19), and Th2 cells play an important role in many allergic and inflammatory diseases (9–11, 27, 31). More than 60 years ago, Hardwicke et al. reported an association of seasonal proteinuria with pollen sensitivity (12). Patients with MCD often have allergy-like symptoms, such as bronchial asthma, allergic rhinitis, atopic dermatitis, and urticaria. Some studies also confirmed that patients with INS may have increased levels of serum IgE, especially children with INS recurrence (13–15, 18). However, the causal relationship between the increased serum level of IgE or atopy and the pathogenesis of INS remains uncertain. The present study confirms that children in the active phase of INS with or without atopy had increased plasma levels of IgE compared with healthy controls, and they also had higher levels than children in the remission phase of INS. Among children in remission, the IgE levels of non-atopic children were nearly normal, but the IgE levels of atopic children were high. Thus, children with atopic and non-atopic INS have increased serum levels of IgE. However, further investigations of the underlying cytokine regulatory network are needed to more completely understand the relationship between INS and IgE production.

Th2 cells secrete IL-13 and IL-4, and this can promote the production of IgE in B cells (9–11). In particular, IL-13 induces a change from expression of IgM to IgE in B cells, and also induces podocytes to increase their synthesis of CD80. Increased CD80 expression by podocytes is associated with proteinuria (13–15, 18). Elevated levels of IL-13 are present in the urine and serum of patients who have kidney disease, and are also associated with a proteinuria (13–15, 18). These previous studies thus suggest that IL-13 functions in the pathogenesis of kidney disease, but the mechanism responsible for the increased IL-13 level is still unclear. Recent studies reported that Th2-related factors, IL-13 and IL-4, have increased levels in patients with active INS (13–15, 18). Other studies noted that patients with INS have increased proportions of Th2 cells (8, 18, 21, 22), but it was uncertain whether this alteration contributed to the pathogenesis of INS. This led us to examine the expression of Th2 cells in peripheral blood and the plasma concentrations of IL-13 and IL-4 in patients with different INS disease states. Our results demonstrated significantly increased levels of Th2 cells, IL-13, and IL-4 in patients with active phase INS with or without atopy. These results thus suggest that the proteinuria and increased IgE levels that occur during the active phase of INS, regardless of the presence of atopy, might be due to the increased levels of IL-13 and IL-4, which were caused by a drift toward Th2 cells. However, the mechanism responsible for this drift toward Th2 cells needs further study to elucidate the role of the immune system in the pathogenesis of INS.

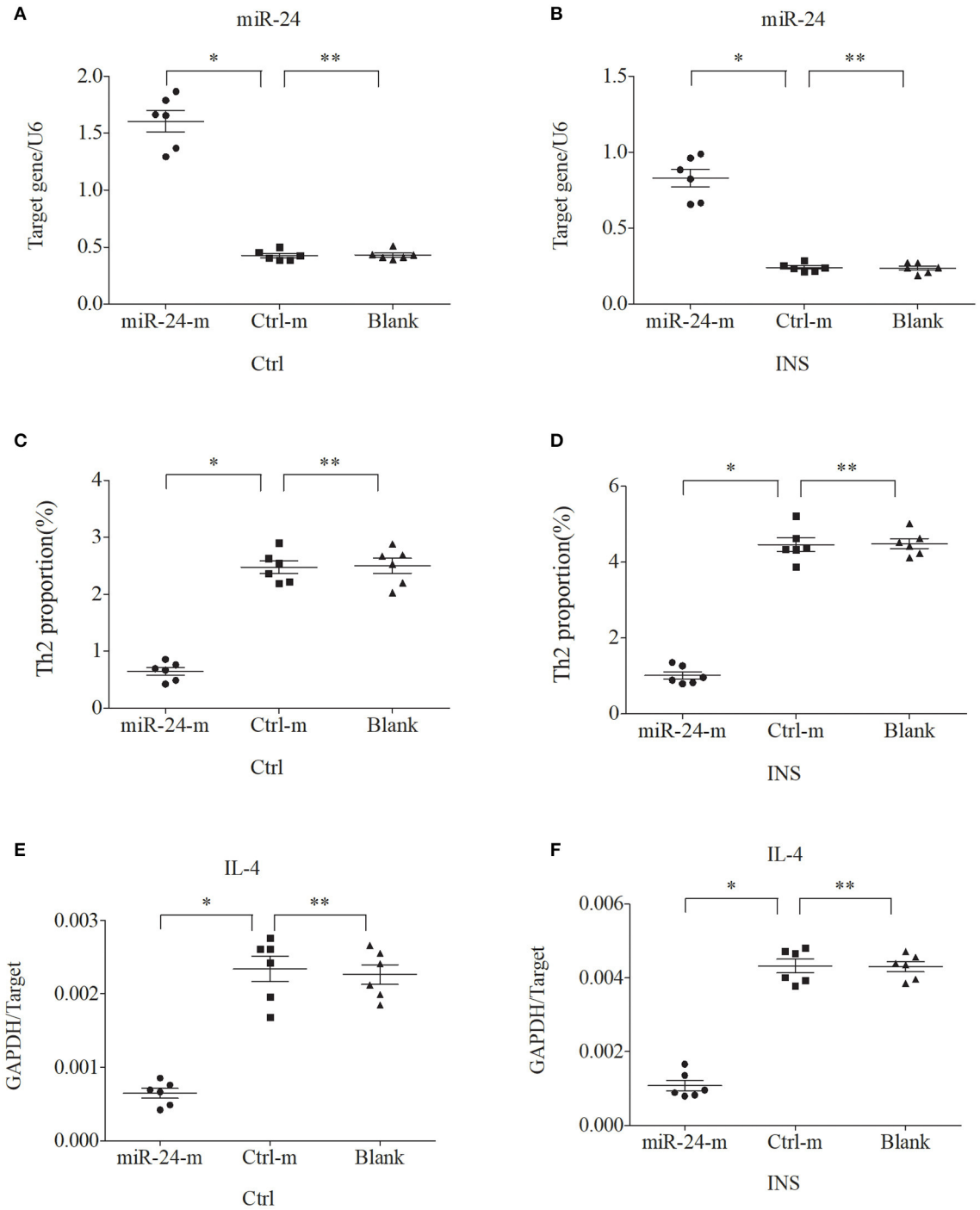

FIGURE 4 | Up-regulation of miR-24 in PBMCs from controls and INS patients **(A,B)** reduces the percentage of Th2 cells **(C,D)** and the expression of IL-4 mRNA **(E,F)**. Here and in **Figures 5–7**: expression of miRNAs (relative to U6) and IL-4 (relative to GAPDH) were determined by real-time PCR; ANA ($n = 6$), Crtl ($n = 6$); and abbreviations are Ctrl-m (miRNA negative control), miR-24-m/miR-27-m (miR-24/miR-27 mimic), Ctrl-i (miRNA inhibitor [negative control]), and miR-24-i/miR-27-i (miR-24/miR-27 inhibitor). $^*P < 0.05$ and $^{**}P > 0.05$.

miRNAs are subtle "master controllers" of gene expression, and function in the pathogenesis of many human diseases, especially chronic and multifactorial diseases (23, 24). Previous studies reported that some specific miRNAs were abnormally expressed in patients with various allergic and autoimmune diseases, such as asthma, systemic lupus erythematosus (SLE),

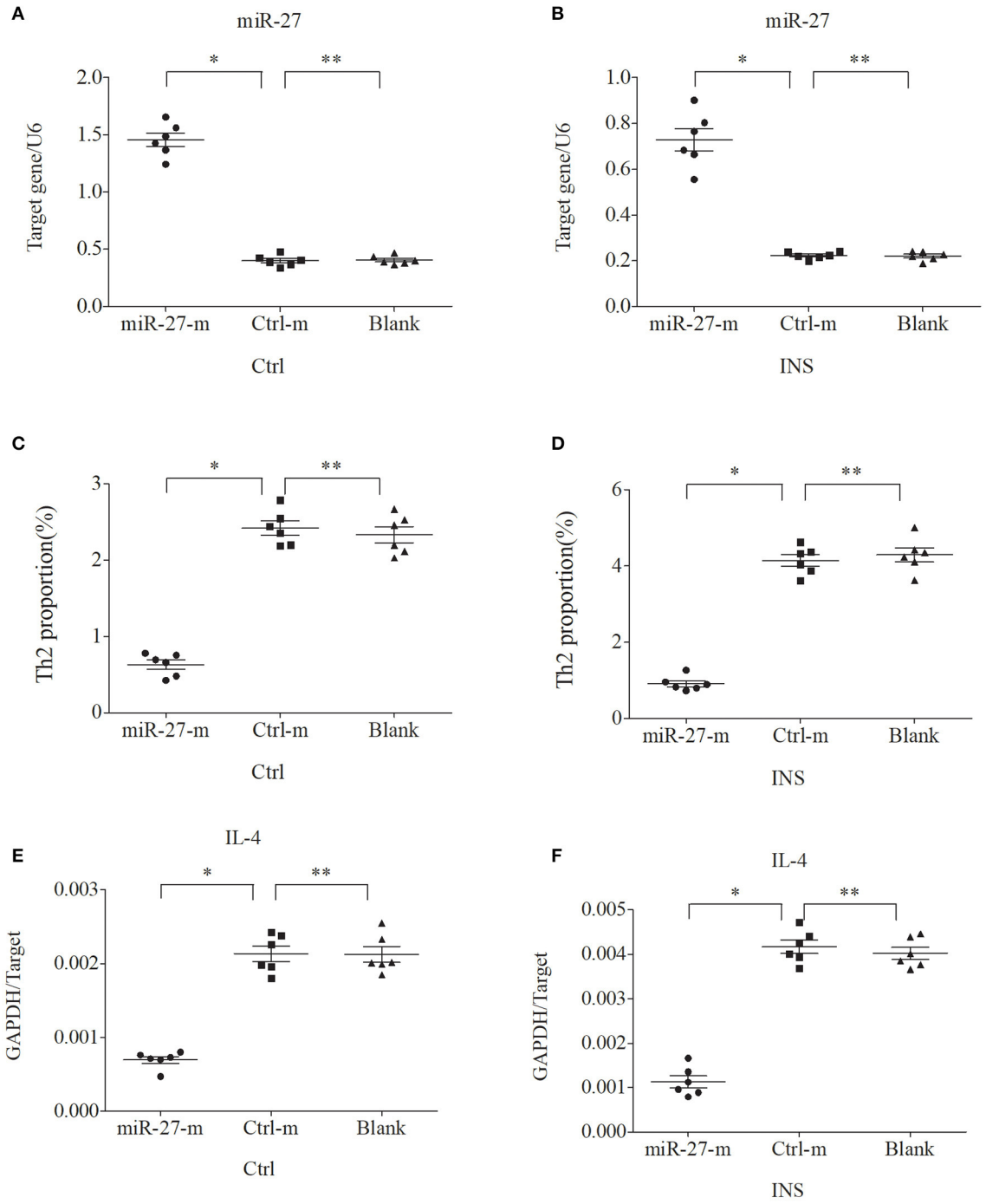

FIGURE 5 | Up-regulation of miR-27 in PBMCs from controls and INS patients **(A,B)** reduces the percentage of Th2 cells **(C,D)** and the expression of IL-4 mRNA **(E,F)**. *$P < 0.05$ and **$P > 0.05$.

and lupus nephritis, and that their expression also increased with disease activity (27, 32, 33). Other specific miRNAs are important for Th2 cell proliferation, differentiation, and immune function (27–29). In particular, miR-24 and miR-27 suppress allergic inflammation and target a network of regulators of Th2 cell-associated cytokine production (28, 29). Thus, the abnormal increase of Th2 cells and its related factor IL-13 in children with INS is not related to atopy, but is related to the pathogenesis of nephropathy. However, it is unclear whether alterations in the levels of different miRNAs lead to alterations in Th2 expression in patients with active INS. Therefore, we determined the expression of miR-24 and miR-27 and examined their possible

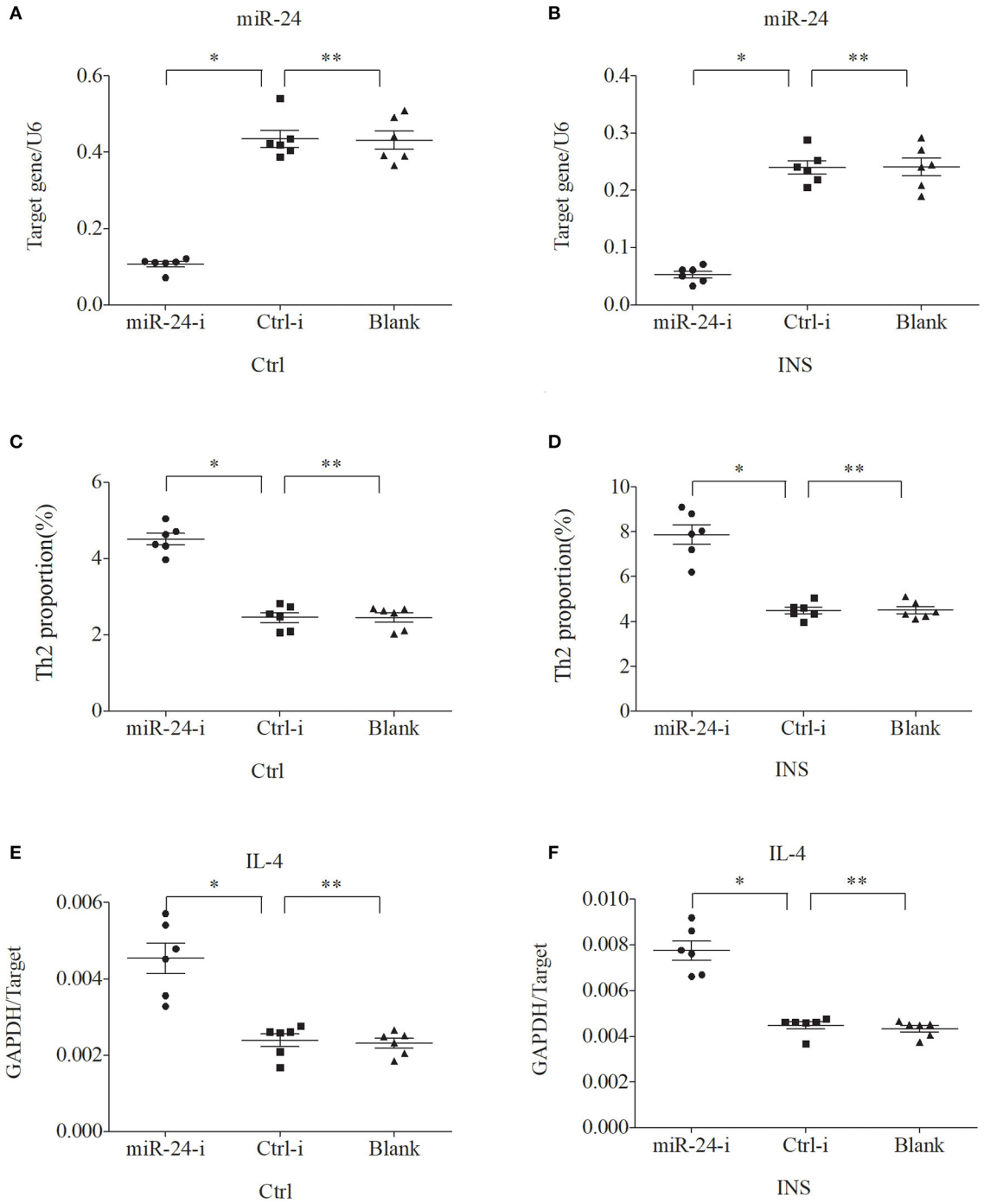

FIGURE 6 | Down regulation of miR-24 in PBMCs from controls and INS patients **(A,B)** increases the percentage of Th2 cells **(C,D)** and the expression of IL-4 mRNA **(E,F)**. *$P < 0.05$ and **$P > 0.05$.

role in regulating Th2 cell differentiation. Our results clearly showed that these two miRNAs had low expression in pediatric patients with active non-atopic INS. This suggests that the drift toward Th2 cells might be related to the low expression of these miRNAs in these children.

To verify this hypothesis, we first isolated PBMCs from healthy controls and children with non-atopic INS. Then, we transfected these cells with miR-24 or miR-27 mimics to increase expression, or with miR-24 or miR-27 inhibitors to reduce expression. The results showed that the percentage

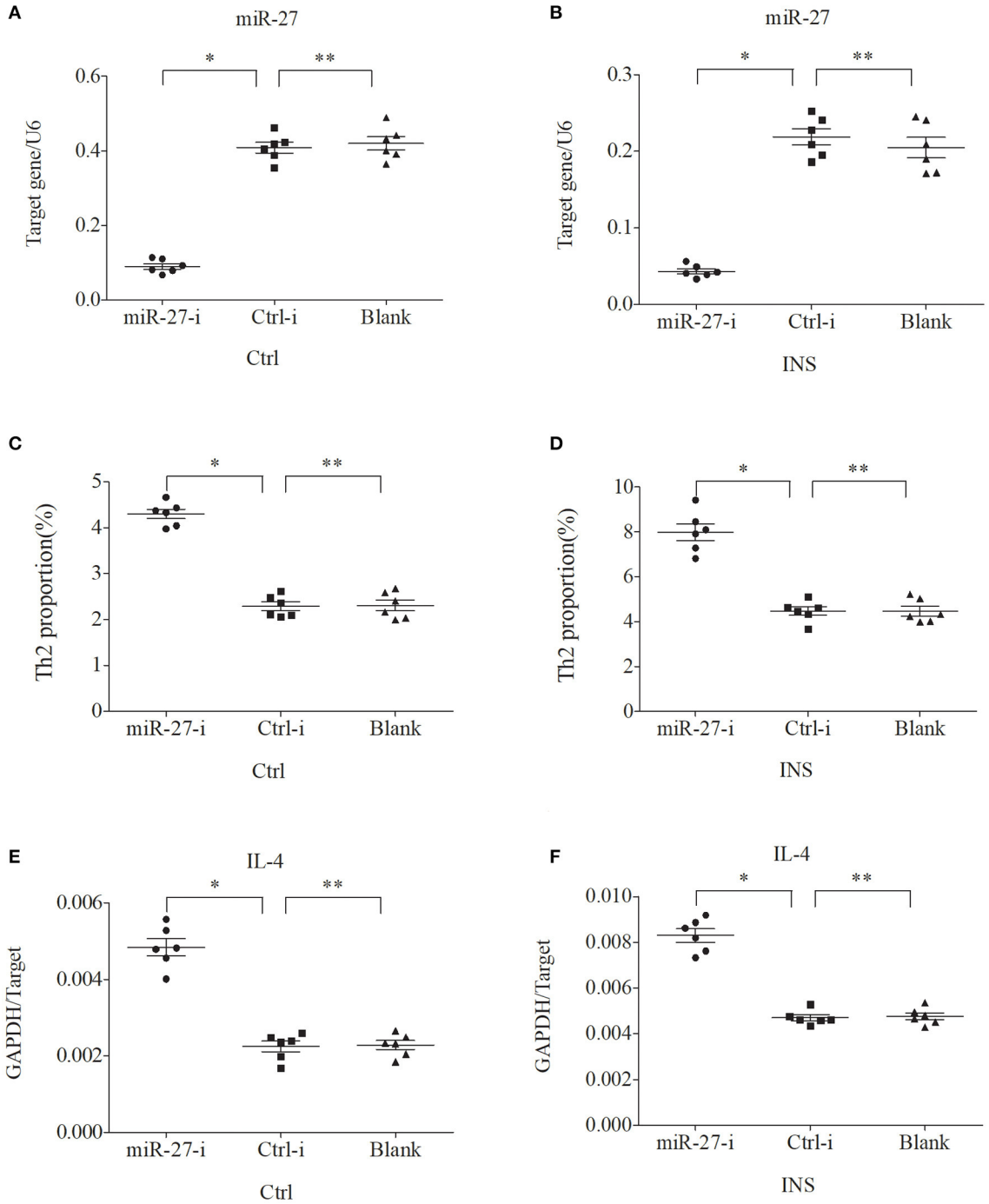

FIGURE 7 | Down regulation of miR-27 in PBMCs from controls and INS patients **(A,B)** increases the percentage of Th2 cells **(C,D)** and the expression of IL-4 mRNA **(E,F)**. *$P < 0.05$ and **$P > 0.05$.

of Th2 cells and the level of IL-4 decreased when the levels of these miRNAs was increased. In agreement, the percentage of Th2 cells and the expression of IL-4 increased when the levels of these miRNAs was decreased. These results support our initial hypothesis that miR-24 and miR-27 reduce the percentage of Th2 cells, and suggest they might play an important role in Th2 expression during active INS.

In conclusion, our results indicate that the Th2 related-cytokine IL-13 is related to the pathogenesis of kidney disease but is not related to atopy, although the mechanisms responsible for the increased level of this cytokine remain unclear. Our results

also indicate that pediatric patients with active phase INS that is either atopic or non-atopic have higher levels of IgE, and this might be related to their higher levels of IL-13 and IL-4 due to a drift toward Th2 cells. Children with active phase non-atopic INS had reduced levels of miR-24 and miR-27, an increased percentage of Th2 cells, and an increased level of IL-4, and this led to IL-13 over-expression and increased IgE levels. In other words, these two miRNAs suppress the expression of Th2 cells and play an important role in the drift toward Th2 cells in children with active INS. Therefore, we speculate that these miRNAs should be considered as potential targets for treatment of kidney disease. Our research thus provides a new research direction and suggests a possible novel treatment for INS.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Shenzhen Children's Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

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

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CASE REPORT
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Case Report: A Rare Presentation of NSAID-Induced Secondary Membranous Nephropathy in a Pediatric Patient

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Background: Membranous nephropathy (MN) is a common cause of nephrotic syndrome in adults, but it is responsible for <5% of nephrotic syndrome cases in children. MN has primary and secondary forms. Secondary MN is caused by viral infections, autoimmune diseases like lupus, or drugs. Non-steroid anti-inflammatory drug (NSAID)-induced secondary MN is rarely described in the pediatric population. Thus, the clinical presentation and time to recovery are vastly unknown in the pediatric subgroup.

Clinical Presentation: We report a case of a 15-year-old female who presented with acute onset of nephrotic range proteinuria, significant hypoalbuminemia, hyperlipidemia, and lower extremity edema related to the presence of nephrotic syndrome. She had a history of ibuprofen use periodically for 6 months before presentation because of menstrual cramps and intermittent lower abdominal pain. After the presentation, we performed a renal biopsy that reported stage 1–2 MN, likely secondary. The phospholipase A2 receptor (PLA2R) antibody on the blood test and PLA2R immune stain on the renal biopsy sample were negative. We performed a comprehensive evaluation of the viral and immune causes of secondary MN, which was non-revealing. She had stopped ibuprofen use subsequent to the initial presentation. She was prescribed ACE inhibitor therapy. After 6 months of ACE inhibitor treatment, the proteinuria had resolved.

Conclusion: Proteinuria can last for several weeks when NSAID induces secondary MN and nephrotic syndrome. With the widespread use of NSAIDs prevalent in the pediatric community, further studies are needed to evaluate and study the role of NSAIDs in this condition.

Keywords: NSAID, membranous nephropathy, proteinuria, nephrotic syndrome, ACE inhibitor

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INTRODUCTION

Membranous nephropathy (MN) is a rare cause of nephrotic syndrome in the pediatric population and contributes to <5% of childhood nephrotic syndrome cases (1). Primary MN is a renal-specific autoimmune glomerular disease characterized by proteinuria, primarily mediated by antibodies against phospholipase A2 receptor (PLA2R). It is characterized by specific histological findings on renal biopsy, such as a pathognomic "spike pattern" of the glomerular basement membrane on light microscopy (LM); positive PLA2R immunostaining, IgG and complement C3

distributed in a delicate granular pattern distributed across a subepithelial portion of glomeruli on immunofluorescence microscopy (IFM); and electron-dense deposits confined to the subepithelial space on electron microscopy (EM), with the absence of mesangial deposits (2, 3). Secondary MN has previously been shown to be caused by viral infections (e.g., Hepatitis B, Hepatitis C, or HIV), autoimmune conditions (e.g., lupus), drugs (e.g., penicillamine, gold, or NSAIDs), hematological malignancies, and solid organ tumors (1, 2). The histological findings of secondary MN can vary depending on the cause, and PLA2R immunostaining is negative (2). Primary MN is a more common form of MN in adults in the literature review, comprising ∼80% of total MN cases, but secondary MN may be more common in children (1). Due to the rarity of secondary MN in the pediatric population and the lack of controlled studies, there are no reliable treatment guidelines or predictions of outcomes for this condition.

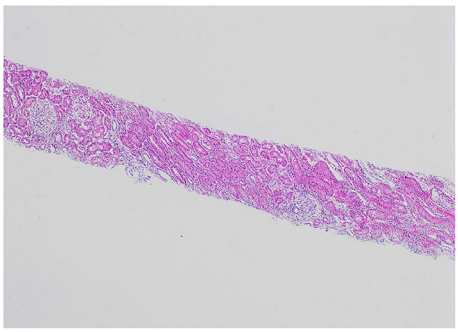

FIGURE 1 | H&E, 4X: The renal biopsy shows glomeruli with normal cellularity and no significant tubular atrophy or interstitial fibrosis.

PATIENT INFORMATION

We report the case of a 15-year-old female (weight 68.3 kg, BMI of 22.56) who presented with acute onset bilateral lower extremity edema and fatigue. She had gained nine pounds of weight at presentation compared to her previous weight 1 month earlier. She denied using any medications, except she said she had been taking ibuprofen at high doses (1,600–2,400 mg/day) intermittently for the past 6 months because of menstrual cramps and intermittent lower abdominal pain. She rejected any illicit substance use and was living at home with her parents and sibling.

CLINICAL FINDINGS

The comprehensive metabolic panel suggested the presence of hyponatremia, with a serum sodium level of 132 meq/L (reference range: 135–145 meq/L), and significant hypoalbuminemia, with a serum albumin level of 2.4 g/dL (reference range: 3.6–5.1 g/dL). Renal function was normal, based on blood urea nitrogen (BUN) of 11 mg/dL (reference range: 4–20 mg/dL) and serum creatinine of 0.5 mg/dL (reference range: 0.5–1 mg/dL). The complete blood count was acceptable, with a WBC count of 13.62 $\times$ 10^3/uL, hemoglobin of 12.4 g/dL, and a platelet count of 407 $\times$ 10^3/uL. The serum lipid profile was abnormal, with a total cholesterol level of 366 mg/dL (reference range: <170 mg/dL) and elevated serum LDL of 249 mg/dL (reference range: <110 mg/dL). The urine dipstick showed +3 protein and was negative for blood. The random urine albumin-to-creatinine ratio was high: 5,229 mcg/mg of creatinine (reference normal <30 mcg/mg of creatinine). The renal ultrasound was normal.

DIAGNOSTIC ASSESSMENT

A clinical diagnosis of nephrotic syndrome was made based on hypoalbuminemia, hyperlipidemia, and heavy proteinuria. Although minimal change disease (MCD) is a common cause of nephrotic syndrome in pediatric patients, the risk of focal segmental glomerulosclerosis (FSGS) increases when the onset of nephrotic syndrome occurs in late childhood or adolescence (4).

Our patient was 15 years of age at presentation; hence, an early renal biopsy was performed to determine the cause. The glomeruli appeared normal overall on light microscopy (LM), and there were no "spikes" or "holes" in the glomerular basement membrane (**Figures 1**, **2**). There was no significant tubular atrophy, interstitial inflammation or interstitial fibrosis. Even though MN is rare among pediatric patients, primary MN was in the differential given the patient's age. We performed PLA2R immunostain on renal biopsy specimens to differentiate primary and secondary MN as there are differences in treatment in these two conditions. On immunofluorescence microscopy (IFM), the PLA2R immunostain was negative, but there was positive mesangial and glomerular diffuse capillary loop staining for IgG, kappa, and lambda (**Figure 3**). There was also positive staining for segmental granular mesangial C1q. Further, we saw negative staining for IgA and C3, and there was no full-house pattern on IFM, which made lupus less likely as a plausible diagnosis. Electron microscopy (EM), revealed the presence of mesangial and diffuse subepithelial electron-dense deposits (**Figures 4**, **5**). There were no tubule-reticular bodies (as seen with lupus), and podocytes were effaced on EM. Given the presence of subepithelial electron-dense deposits and diffuse capillary loop staining for IgG, a diagnosis of MN was made (1). However, the classical findings of primary MN, such as a "spike pattern" of the glomerular basement membrane, C3 staining on immunofluorescence, and positive PLA2R staining, were absent, leading to an increased possibility of secondary MN. Additionally, there was an additional presence of mesangial electron-dense deposits and positive C1q staining, which is reported with cases of secondary MN (1, 2). Viral and immune studies were performed to evaluate the causes of secondary MN and found to be non-revealing (**Table 1**). Based on the above findings and the patient's history of high- dose ibuprofen use, the diagnosis of NSAID-induced secondary MN was made.

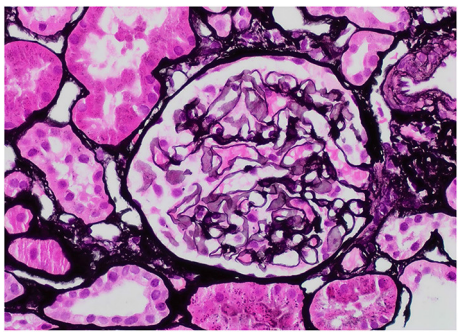

FIGURE 2 | Jones Silver, 40X: The glomerulus lacks well-developed "spikes" or "holes".

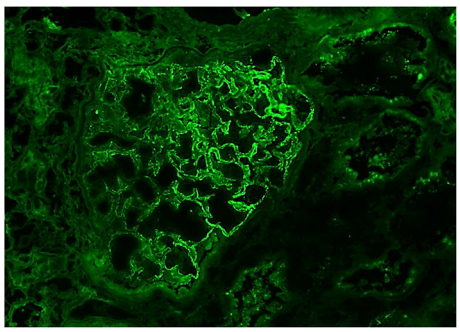

FIGURE 3 | IgG, 40X: The glomerulus demonstrates mesangial and diffuse global, granular capillary loop staining.

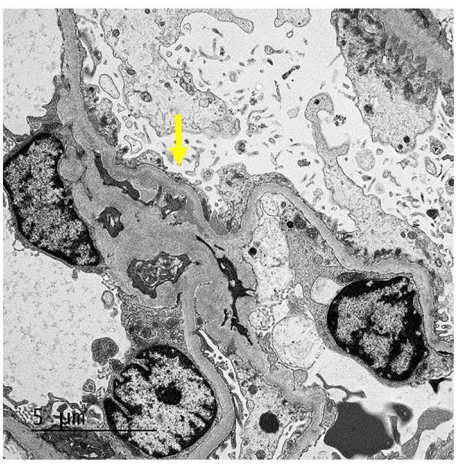

FIGURE 4 | EM: The glomerulus demonstrates mesangial (arrow) and segmental small subepithelial electron-dense deposit.

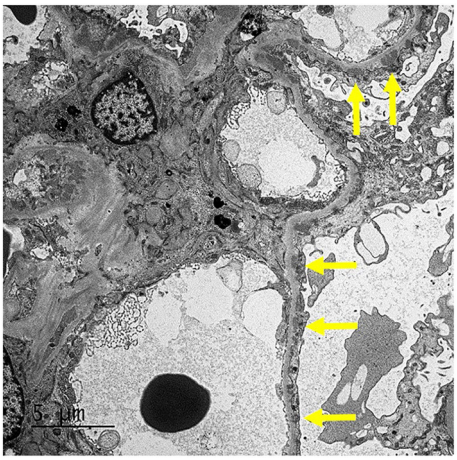

FIGURE 5 | EM: The glomerulus demonstrates mesangial and segmental small subepithelial electron-dense deposit (arrows).

TABLE 1 | Viral and immunological studies.

Test	Result	Reference/ normal
Rheumatoid factor (RF)	<14 IU/ml	< 14 IU/ml
Thyroid stimulating hormone (TSH)	3.78 u(iU)/mL	0.470–4.680 u(iU)/mL
Immunoglobulin G (IgG)	488 mg/dL	500–1,590 mg/dL
Antinuclear antibody (ANA) screen	Negative	Negative
Complement C3	217 mg/dL	83–193 mg/dL
Complement C4	43 mg/dL	15–57 mg/dL
Hepatitis panel (Hepatitis A IgM, Hepatitis B surface Ag, Hepatitis B core Ab, Hepatitis C antibody)	Negative	Negative
HIV screen	Negative	Negative
Cytomegalovirus PCR	Negative	Negative
Epstein-Barr virus PCR	Negative	Negative
Serum protein electrophoresis	Normal	
Serum phospholipase A2 receptor antibody (PLA2R)	Negative	Negative

THERAPEUTIC ASSESSMENT

Our patient stopped using NSAIDs after the initial presentation. She was also initially started on steroids (prednisone) because of her presentation with nephrotic syndrome and based on the available pediatric literature review, but they were weaned over 4 weeks because of a lack of improvement of proteinuria and a strong suspicion of secondary MN (1). Because of the lack of data on treatment options for NSAID-induced secondary MN in pediatric patients, we reviewed the available literature on adult patients, where the treatment varies and includes no treatment, ACE inhibitor use, and steroid use (5–7). Apparently, proteinuria can last for several weeks after stopping NSAIDs in NSAID-induced secondary MN (2, 6). We initiated treatment with an ACE inhibitor and monitored the improvement in proteinuria.

FOLLOW-UP AND OUTCOMES

The swelling on extremities improved in 6–8 weeks, while the patient's serum albumin level took 4 months to return to the

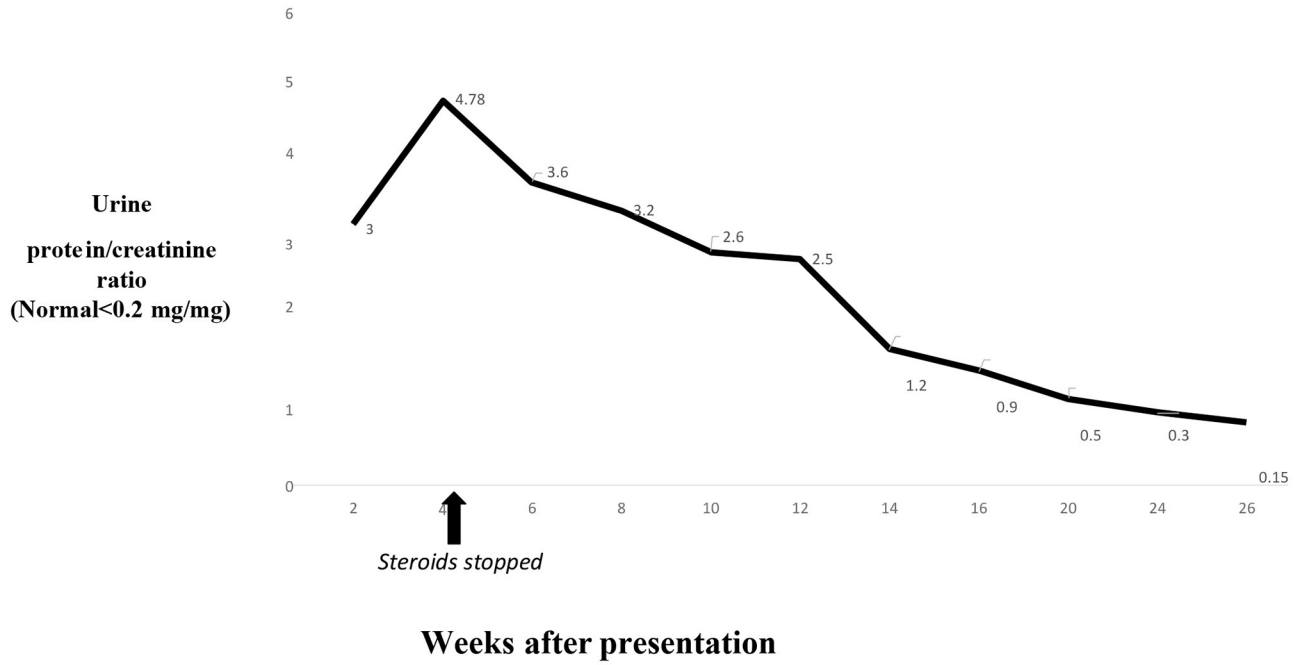

FIGURE 6 | Trends in proteinuria on ACE inhibitor therapy.

normal range after the initial presentation. The proteinuria took over 6 months to resolve completely (**Figure 6**). She had dizziness on 10 mg/day lisinopril, and dose had to be reduced to 7.5 mg/day. Lisinopril was stopped 6 months after presentation. She continues to be in remission and has had no episodes of relapse or significant proteinuria 1 year after presentation.

DISCUSSION

NSAIDs are widely used in the pediatric population to treat pain or fever, and their side effects on kidneys are often not well-recognized. The absence of evident symptoms often leads to a delay in the diagnosis of NSAID-induced kidney disease (8). NSAIDs cause acute kidney injury (AKI) secondary to pre-renal vasoconstriction, acute tubular necrosis (ATN), or acute tubulointerstitial nephritis (ATIN) (8). In hypovolemic states, the production of prostaglandins is upregulated to maintain renal blood flow. NSAIDs inhibit the cyclooxygenase (COX) enzyme, which affects the biosynthesis of prostaglandins, leading to pre-renal vasoconstriction, pre-renal AKI, and ATN in association with hypovolemia (9). It also leads to ATIN and enlargement of kidneys due to an immuno-allergic reaction (8). In terms of nephrotic syndrome, both MCD and MN have been reported with NSAID use (10). MN has been reported with all NSAIDs, including selective COX-2 inhibitors (2, 5). The exact mechanism of NSAID-induced secondary MN is unknown. Different theories have been proposed, including COX inhibition, glomerular deposition of antigens that bind to NSAIDs, or NSAIDs triggering autoimmune reaction against antigens in the glomerular filtration barrier (2, 10).

The duration of NSAID treatment prior to the development of MN is highly variable, from a few weeks to a few months, but there is a rapid development of nephrotic-range proteinuria or presentation with nephrotic syndrome at disease onset (2). Because the symptoms of hypoalbuminemia and edema may be more apparent in NSAID-induced MN, an early renal biopsy is often performed, and this also explains the early stage of MN (stage I or II) often seen on renal biopsies in these patients (2). Our patient also had stage I–II MN on renal biopsy. Since NSAIDs can cause nephrotic syndrome with both MCD and MN presentation, the presence of electron-dense subepithelial deposits and podocyte effacement on EM and the absence of interstitial inflammatory infiltrates on LM are characteristic findings of NSAID-induced MN. In contrast, interstitial infiltrates typically present in NSAID-induced MCD (10). Because of the absence of interstitial infiltrates, renal function can be generally normal in patients with NSAID-induced MN, as also seen in our patient (10). The absence of interstitial infiltrates on renal biopsy could also explain the normal kidney sizes and the lack of enlarged kidneys seen in our patient on renal ultrasound. The lack of a classical "spike" pattern on LM, the absence of PLA2R immune-stain on IFM and negative serum PLA2R antibody screen, and the mesangial presence of deposits along with sub-epithelial deposits on EM led to increased suspicion of secondary MN, as seen in previous reports (1, 2).

A previous retrospective case series published before the availability of PLA2R immune-stain diagnosed the presence of NSAID-induced MN when patients had early-stage I–II MN on renal biopsy; the patients were concurrently taking NSAIDs

at the presentation of nephrotic syndrome, and other causes of MN were not detected (6). This series identified 13 adult patients with NSAID-induced MN, and the duration of NSAID use ranged from 1 to 36 months prior to disease onset, the duration of symptoms, such as edema, ranged from 1 to 24 weeks, and treatment ranged from the withdrawal of NSAIDs to further steroid or ACE inhibitor use (6). Interestingly, the time for improvement of proteinuria (as evaluated by 24-h urine protein <1 g) ranged from 9 to 40 weeks after disease presentation among patients who were regularly followed (6). Our patient was treated with an ACE inhibitor and regularly followed, but proteinuria lasted for 26 weeks after presentation. The improvement criteria for proteinuria in our patient were more stringent, as observed by a random urine protein and creatinine ratio <0.2, and this may have also explained the longer duration of proteinuria. Once the patient was in remission, no relapse was observed, in line with previous reports of NSAID-induced MN (2, 6). A randomized controlled trial comparing the anti-proteinuric effects of lisinopril and losartan treatment for 27 patients with primary MN showed a significant reduction in proteinuria in both groups after 12 months of follow-up (11). However, the role of ACE inhibitors in secondary MN and whether they help to decrease the duration of proteinuria or change the natural course of the disease after stopping NSAIDs needs to be further studied.

There are occasional pediatric case reports of drug-induced secondary MN from pencillamine, but the use of such medicines is now decreasing while NSAID use remains prevalent (12). To the best of our knowledge, this report is one of the initial comprehensive case reports on NSAID- induced MN and its outcome in a pediatric patient, as the literature regarding this topic is limited in this population. Our patient had some unique features on renal biopsy that may provide more insight into this challenging condition.

CONCLUSION

NSAIDs are widely used in children and adolescents and can lead to side effects on kidneys, which often go unrecognized. The diagnosis of NSAID-induced secondary MN is challenging and requires a proper laboratory work-up and histological testing. Proteinuria can last for several weeks, even after the resolution of edema in this condition. Further studies are needed to better understand the natural history and long-term outcomes of NSAID-induced secondary membranous nephropathy. More awareness is needed regarding the effects of NSAIDs on kidneys, and their careful use would help to decrease the incidence of NSAID-induced nephrotic syndrome in children.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The case report was reviewed and approved by the Institutional review Board at Louisville, KY, USA. We obtained written informed consent from the parent to publish any potentially identifiable data included in this report.

PATIENT PERSPECTIVE

Note from Father:

Yes, as a father I can say that for me and her mother it was very hard to come to terms with the fact that something wrong had happened just because of a simple over the counter medication. It took us aback and made us nervous.

AUTHOR CONTRIBUTIONS

SS was the primary pediatric nephrologist involved in diagnosing and treating the patient's presentation with nephrotic syndrome. MA was the pediatric resident involved in the care of the patient. JH was the pathologist involved in examining the renal biopsy sample and diagnosing membranous nephropathy. All authors contributed to the article and approved the submitted version.

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

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SYSTEMATIC REVIEW
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Mycophenolate Mofetil in the Treatment of Steroid-Dependent or Frequently Relapsing Nephrotic Syndrome in Children: A Meta-Analysis

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Objectives: This meta-analysis aims to evaluate the efficacy and safety of the mycophenolate mofetil (MMF) in the treatment of steroid-dependent nephrotic syndrome (SDNS) or frequently relapsing nephrotic syndrome (FRNS) in children.

Methods: We searched for the studies especially the randomized controlled trials in PubMed, Cochrane Library, Embase, China National Knowledge Infrastructure, and Wan Fang database. The data were analyzed by Review Manager 5.3 software. We used the GRADE pro-Guideline Development Tool online software to evaluate the quality of evidence.

Results: Finally, we identified 620 studies, of which we included five randomized controlled trials and one prospective cohort study with 447 children. The results showed the following: (1) the relapse-free survival rate within 1 year—the MMF group was superior to the levamisole group [ratio difference (RD) = 0.13, 95% CI (0.02, 0.24), $P = 0.02$] but not to the calcineurin inhibitors (CNIs) group [RD = −0.27, 95%CI (−0.40, −0.14), $P < 0.0001$]; (2) the number of relapses within 1 year—the MMF group was less than that in the CNIs and levamisole group [mean difference (MD) = −0.26, 95%CI (−0.45, −0.08), $P = 0.005$]; (3) the cumulative prednisone dosage—the MMF group was lower than that in the control group [standardized mean difference (SMD) = −0.32, 95%CI (−0.53, −0.11), $P = 0.003$]; (4) incidence of adverse reactions—there was no significant difference between the MMF group and the control group [RD = 0.02, 95%CI (−0.04, 0.09), $P = 0.46$].

Conclusion: The therapy of mycophenolate mofetil in the treatment of SDNS or FRNS in children has a certain advantage in reducing the number of relapses and cumulative prednisone dosage within 1 year when compared with the CNIs and levamisole. However, due to the limited quantity and quality of the included studies, the conclusions above need to be confirmed by more high-quality randomized controlled trials.

Keywords: mycophenolate mofetil, frequently relapsing nephrotic syndrome, steroid-dependent nephrotic syndrome, children, meta-analysis

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INTRODUCTION

Nephrotic syndrome (NS) is a common glomerular disease in childhood characterized by proteinuria, hypoproteinemia, hyperlipidemia, and edema. The pathogenesis of the disease has not been fully elucidated. At present, it is mainly related to immune imbalance (1), systemic circulatory factors (2), and abnormal podocyte genetic mutations (3). Ninety percent of cases of nephrotic syndrome presenting in childhood are idiopathic (4), and glucocorticoid therapy has been considered a first-line treatment for PNS in children since the 1950s. According to the response to steroid treatment, PNS can be classified as steroid-sensitive nephrotic syndrome (SSNS), steroid-resistant nephrotic syndrome (SRNS), and steroid-dependent nephrotic syndrome (SDNS). SDNS and FRNS are refractory patterns of steroid responsiveness in nephrotic syndrome that account for about 40% of PNS in children whose goal is to choose appropriate second-line immunosuppressants, to induce a response as soon as possible, and to maintain a long-term response. The Kidney Disease: Improving Global Outcomes (KDIGO) guidelines (5) recommend a variety of immunosuppressants for the treatment of SDNS or FRNS in children, including mycophenolate mofetil (MMF), cyclophosphamide, cyclosporine A (CsA), tacrolimus (TAC), rituximab, and the immunomodulator levamisole. However, due to the responsiveness of different patients to different drugs and adverse reactions, making the best plan of pharmacotherapy is still a great challenge for most pediatric nephrologists.

MMF, as a novel immunosuppressant, is a 2-ethyl ester derivative of mycophenolic acid, which is taken off the esterification *in vivo* to form a metabolite with immunosuppressive activity. Mycophenolic acid can selectively act on T and B lymphocytes and the first or second signal in the process of activation to achieve the purpose of immunosuppression. In addition, the inhibitory effect of MMF on other cytokines *in vivo* can also play a role in delaying the progression of the disease. In 1998 (6), American doctors used MMF for the first time in the treatment of adult RNS and achieved good results. Subsequently, the role of MMF in the treatment of kidney disease has received widespread attention. Previous studies have confirmed its positive effect on the treatment of SDNS or FRNS in children. However, most of these studies are based on retrospective analysis, and only a few randomized controlled trials (RCTs) have focused on its efficacy and safety. Therefore, in this study, a meta-analysis was conducted to evaluate the efficacy and safety of MMF in the treatment of SDNS or FRNS in children to provide higher-strength evidence for the usage of MMF.

METHODS

Study Design

In accordance with the principle of "PICOS," it was defined as following: (1) P: the children with SDNS or FRNS; (2) I: treated with MMF or other immunosuppressants (TAC, CsA, and levamisole; (3) C: MMF vs. other immunosuppressants (TAC, CsA, and levamisole); (4) O: relapse-free survival rate within 1 year, the number of relapses within 1 year, cumulative prednisone dosage, and incidence of adverse reactions; (5) S: a meta-analysis of RCTs and a prospective cohort study.

Literature Search

Chinese Literature

We searched the China National Knowledge Infrastructure (CNKI) and Wan Fang database with the keywords including "nephrotic syndrome," "mycophenolate mofetil," and "children."

English Literature

We used the combination of subject words and free words to search PubMed, Embase, and The Cochrane Library. For example, the free words of "nephrotic syndrome" include "nephrotic syndromes," "syndrome, nephrotic," "syndromes, nephrotic," and "nephrotic."

Search Time

Unlimited–December 2020.

Study Selection and Data Extraction

Inclusion criteria

Inclusion criteria were as follows: (1) the prospective studies, especially RCTs, of MMF in the treatment of SDNS or FRNS in children aged from 3 months to 18 years old; (2) the follow-up period of at least 1 year; and (3) the diagnostic criteria refers to the 2012 KDIGO guidelines (7, 8): (a) SDNS, two consecutive relapses during corticosteroid therapy or within 14 days of ceasing therapy; (b) FRNS, >2 relapses within 6 months of initial relapse, or ≥4 relapses in any 12 months. Among them, steroid sensitivity showed that urinary protein turned negative after 4 weeks of treatment with a sufficient amount of prednisone [2 mg/(kg·day) or 60 mg/(m^2·day)].

Exclusion criteria

Exclusion criteria were as follows: (1) all kinds of secondary nephrotic syndrome, such as nephrotic syndrome caused by lupus, hepatitis B virus infection, and antineutrophil-associated glomerulonephritis; (2) retrospective studies, review, meeting, and literature that is not consistent with the purpose of evaluation.

Data Extraction

Based on the inclusion and exclusion criteria, independent duplicate data extraction was performed by two reviewers (Xin Xiang and Shi-Yuan Qiu) using a predesigned data collection form, and the results were reviewed by a third investigator (Mo Wang). In this study, a small number of data in the original literature are expressed by the quartile method, which needs to be converted into mean and standard deviation by using Luo (9) and other methods.

Types of Outcome Measures

Primary Outcomes

Relapse-free survival rate within 1 year, the number of relapses within 1 year, and cumulative prednisone dosage.

Abbreviations: SDNS, steroid-dependent nephrotic syndrome; FRNS, frequently relapsing nephrotic syndrome; SRNS, steroid-resistant nephrotic syndrome; RNS, refractory nephrotic syndrome; SSNS, steroid-sensitive nephrotic syndrome; PNS, primary nephrotic syndrome.

Relapse

Twenty-four-hour urine protein ≥50 mg/kg for 3 consecutive days, urinary protein/creatinine (mg/mg) ≥2.0 in morning urine or morning urine protein changed from negative to positive or (+ + +)–(+ + ++).

Secondary Outcome

Incidence of adverse reactions (mainly considering serious adverse reactions, such as severe infection, agranulocytosis, etc.).

Statistical Methods

Statistical analysis of the data was carried out by RevMan5.3 software provided by The Cochrane collaboration network. Heterogeneity analysis was carried out in selected trials. When $P > 0.05$ and I^2 <50%, the homogeneity of the study was not significant. A fixed-effect model was used. On the contrary, the random effect model was adopted. Ratio difference (RD) and its 95% confidence interval (95%CI) were used for count data. Mean difference (MD) or standardized mean difference (SMD) and its 95% CI were used for measurement data. If there was obvious heterogeneity in the study, its sensitivity was analyzed and postprocessed. Only descriptive analysis was carried out if it cannot be determined.

Evidence Quality Assessment

We used the GRADE pro-Guideline Development Tool online software (GRADEpro GDT, Evidence Prime, Hamilton, ON) to evaluate the quality of evidence.

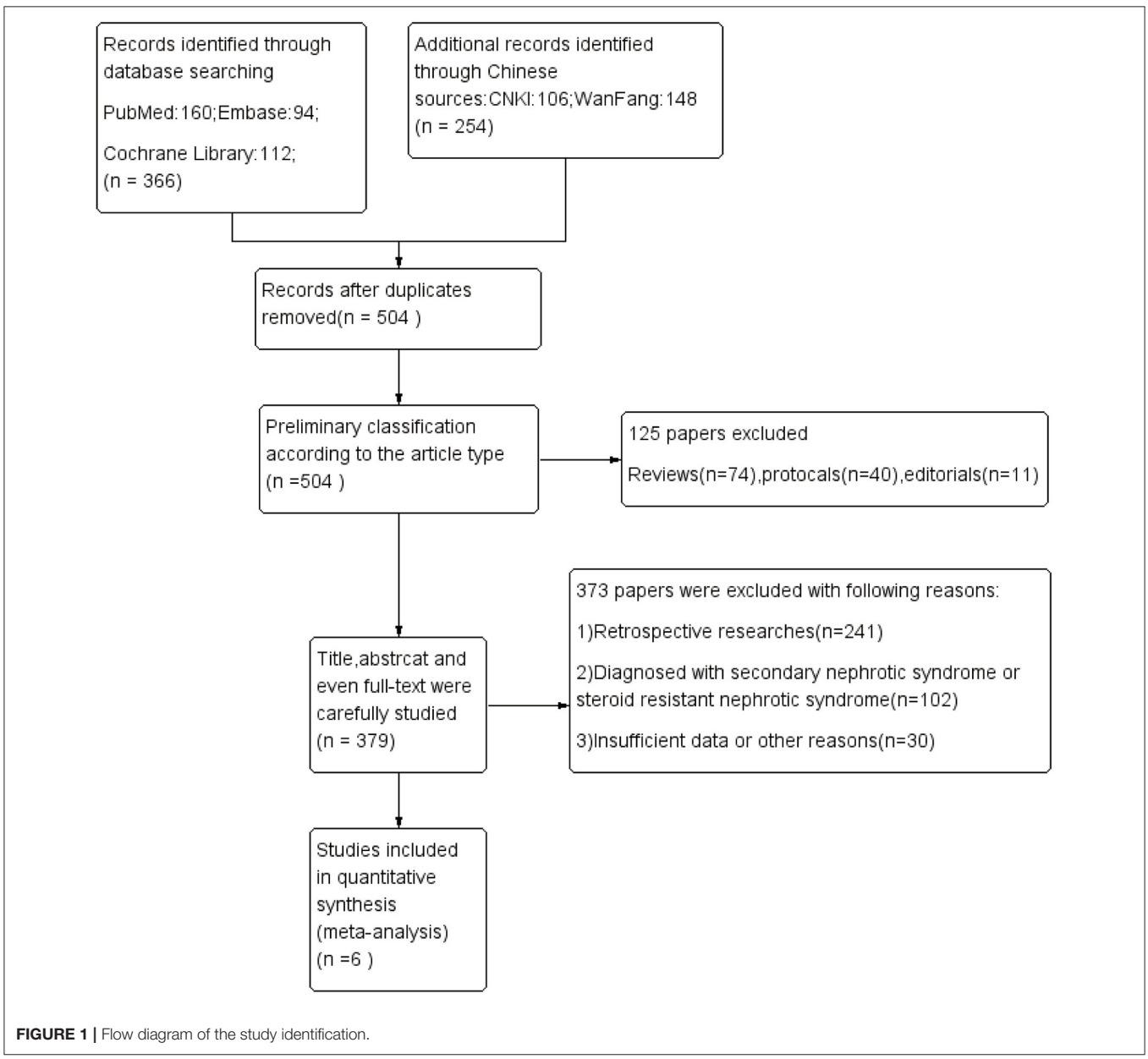

FIGURE 1 | Flow diagram of the study identification.

RESULTS

Literature Retrieval Results

Six hundred twenty articles were retrieved according to the above method. We excluded the repetitions, reviews, retrospective studies, nonclinical studies, and the studies inconsistent with the purpose of evaluation by reading titles, abstracts, and some of the specific contents of the literature. Finally, a total of six articles were included: one Chinese literature (10) and five English articles (11–15). The total number of subjects analyzed was 447 children. **Figure 1** shows the process of literature retrieval. **Table 1** shows the characteristics of the included studies.

Risk of Bias

The Cochrane Collaboration (16) was used for assessing the risk of bias. Of the six articles, one was randomly generated by a computer, one was stratified by randomized numbers in Excel table, one was randomized by central computer minimization, and the other two were randomly grouped, whose random allocation method was not indicated. Two of them were hidden in design allocation but without indicating the specific hiding method. One article adopted the blind method, but others did not mention whether to use blind methods. Follow-up after publication was not mentioned in almost all literature. All the studies clearly explained that there was no significant statistical difference in the baseline data between the experimental group and the control group, which means that they were balanced and comparable. **Figure 2** shows the authors' judgment about each risk bias item presented as percentages across all included studies.

1-Year Relapse-Free Survival Rate

The definition of relapse of SDNS or FRNS in children is as mentioned above. All of the six articles included in this study reported the relapse-free survival rate within 1 year. When analyzing all the data extracted from the literature, we found that there was significant heterogeneity among the studies. Thus, we conducted the subgroups according to the types of immunosuppressants used in the control group. The results

TABLE 1 | Characteristics of the included study.

Trial	Patient number		Intervention		Renal biopsy	Outcome
	E[a]	C[b]	E	C		
Sinha (14)	76	73	MMF 750–1,000 mg/m²/d, 1 year	Levamisole 22.5 mg/kg/qod,1 year	No	1[c] 2[d] 3[e] 4[f]
Basu (15)	56	56	MMF 1,200 mg/m²/d,1 year	Levamisole 2.5 mg/kg/qod,1 year	No	1 2 3 4
Dorresteijn (13)	12	12	MMF 1,200 mg/m²/d,1 year	CsA 4–5 mg/kg/d,1 year	Yes	1 2 3 4
Gellermann (11)	28	30	MMF, target plasma trough level 1.5–2.5 mg/ml	CsA, target plasma trough level 80–100 ng/ml	Yes	1 4
Wang (12)	34	38	MMF 20–30 mg/kg/d, 1 year	TAC 0.05–0.15 mg/kg/d, 1 year	Yes	1 3 4
Geng (10)	14	18	MMF 20–30 mg/kg/d, 1 year	CsA 3–5 mg/kg/d,1 year	Yes	1 2 4

[a] *Experiment group.*
[b] *Control group.*
[c] *1-year relapse-free survival rate.*
[d] *The number of relapses within 1 year.*
[e] *Cumulative glucocorticoid dosage.*
[f] *Incidence of adverse reactions.*

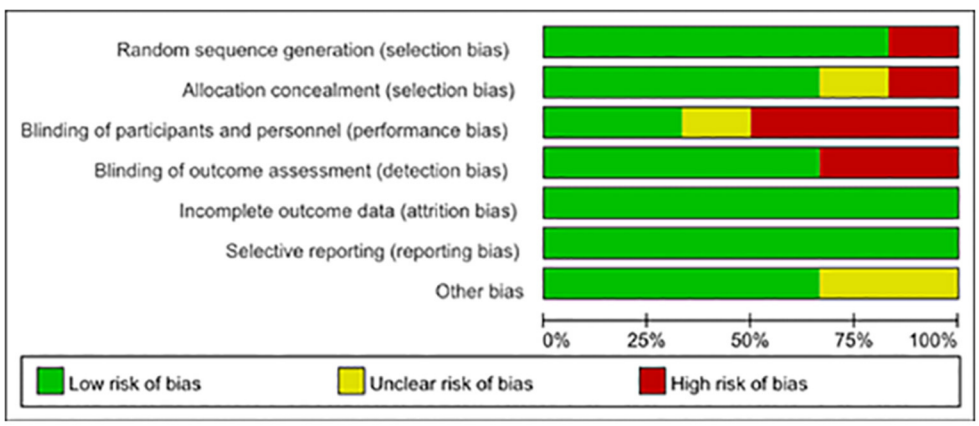

FIGURE 2 | Inclusion bias.

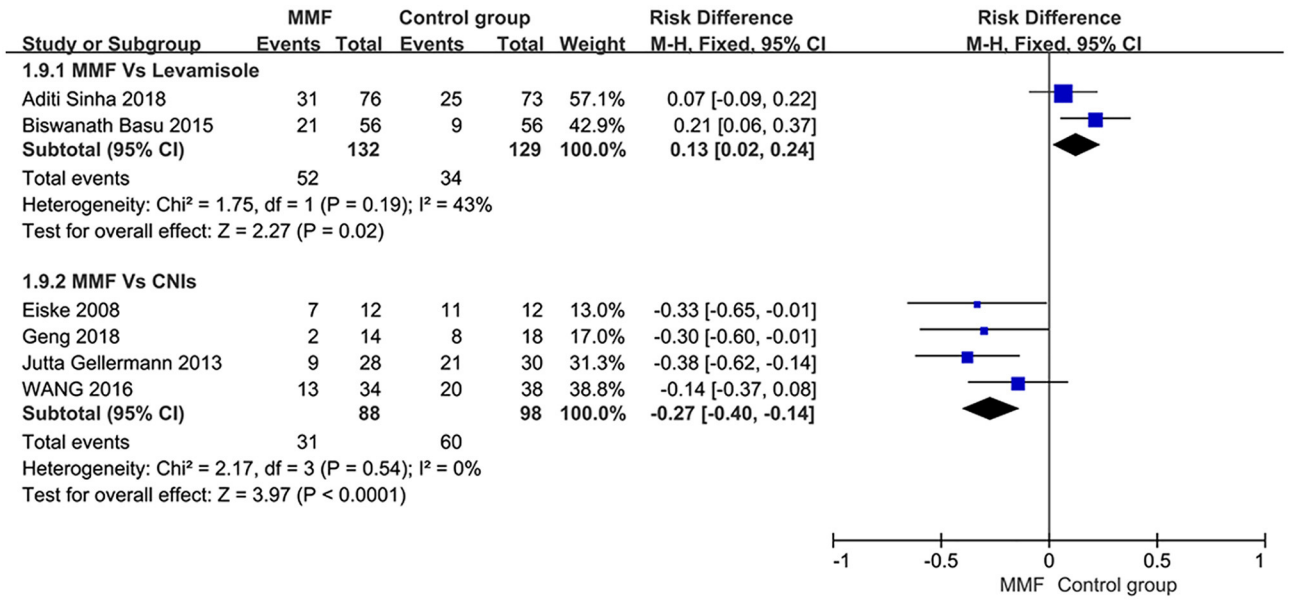

FIGURE 3 | Forest plot showing ratio difference (RD) of the 1-year relapse-free survival rate between the groups.

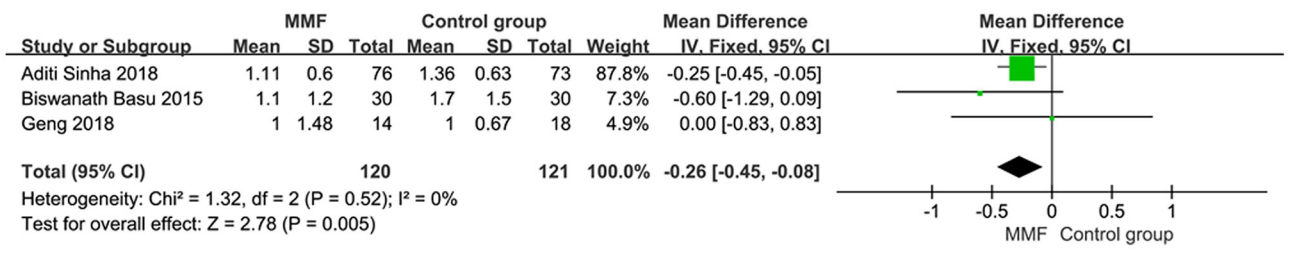

FIGURE 4 | Forest plot showing mean difference (MD) of the number of relapses within 1 year between the groups.

shown in **Figure 3** indicate that MMF was superior to levamisole but not to calcineurin inhibitors (CNIs), and the differences were statistically significant.

The Number of Relapses Within 1 Year

Four articles reported the number of relapses within 1 year. The total number of patients after treatment was analyzed. The results showed that there was heterogeneity among the studies. We have to find the source of heterogeneity by eliminating one by one. After removing the literature of Eiske2008, the heterogeneity was significantly reduced. Re-reading the literature, we found that the renal biopsy results of the subjects included in this study were minimal change diseases (MCDs). We thought it to be the source of heterogeneity, so the literature was deleted. The remaining three articles were analyzed by the fixed-effect model, and the specific results of the meta-analysis were as follows in **Figure 4** [MD = −0.26, 95%CI (−0.45, −0.08), $P = 0.005$]. The number of relapses within 1 year was statistically significant between the two groups. Thus, it was considered that the MMF group was superior to the control group in reducing the number of relapses within 1 year.

Cumulative Prednisone Dosage

Four articles reported the data of cumulative prednisone dosage. There was no obvious heterogeneity among the studies, so the fixed effect model was used for analysis. The specific meta-analysis results are shown in **Figure 5**. The difference in cumulative hormone usage between children with SDNS or FRNS treated with MMF and the control group was statistically significant [SMD = −0.32, 95%CI (−0.53, −0.11), $P = 0.003$], indicating that MMF is more effective in reducing cumulative prednisone dosage than the CNIs and levamisole.

Incidence of Adverse Reactions

Heterogeneity analysis was conducted on six articles reporting the incidence of adverse reactions. Then, the fixed-effect model was used for analysis. The results of meta-analysis are shown in **Figure 6**. There was no significant difference in the incidence of adverse reactions between the MMF group and the control group [RD = 0.02, 95%CI (−0.04, 0.09), $P = 0.46$]. We believe that

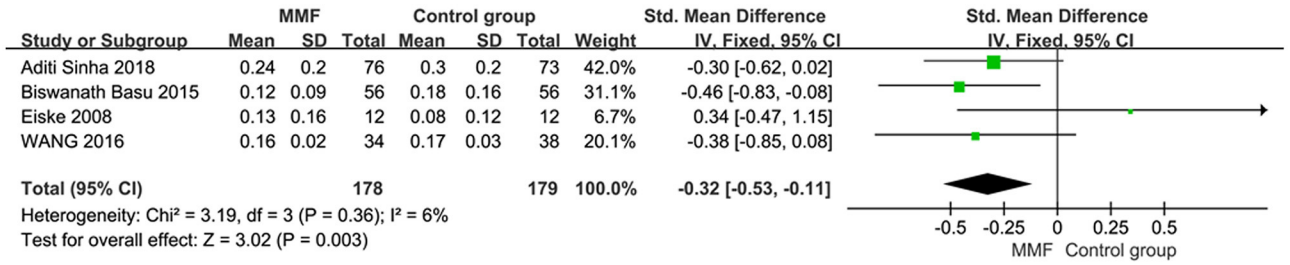

FIGURE 5 | Forest plot showing standardized mean difference (SMD) of the cumulative prednisone dosage between the groups.

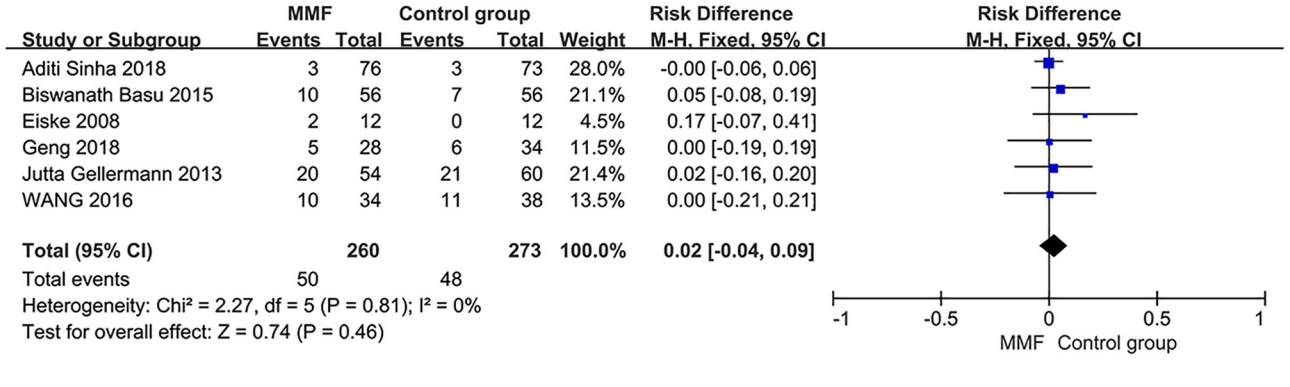

FIGURE 6 | Forest plot showing ratio difference (RD) of the incidence of adverse reactions between the groups.

there is no significant difference in safety between MMF and the control group.

DISCUSSION

The objective of this study was to evaluate the efficacy and safety of mycophenolate mofetil in the treatment of SDNS or FRNS in children by meta-analysis. Six prospective studies were included, with a total number of 447 children. The results show that when compared with other immunosuppressants (including tacrolimus, cyclosporine A, and levamisole), MMF has some advantages in reducing the number of relapses and cumulative prednisone dosage within 1 year. As for the relapse-free survival, MMF was superior to levamisole but not to CNIs. At the same time, the incidence of adverse reactions did not decrease, which was not consistent with previous research and clinical experience (17, 18). In addition, in order to explore the optimal dose of MMF, we conducted a subgroup analysis of different doses of MMF according to the RCTs included in the study. However, we did not find that the effects of different subgroups on the main outcomes were statistically significant.

We also used GRADE pro-GDT to evaluate the quality of the primary outcomes and secondary outcomes (**Table 2**). The results suggested that the quality of the evidence in the 1-year relapse-free survival rate was high, while the quality of the evidence for the cumulative prednisone dosage and the incidence of adverse reactions were moderate. However, the certainty of the number of relapses within 1 year was low.

One of the main outcomes of this study is the incidence of adverse reactions, which are mainly concerned with serious adverse reactions, such as severe infection and agranulocytosis. However, the mild adverse reactions such as rash and medicated fever may be ignored. Almost all kinds of immunosuppressants have varying degrees of side effects. This also has become one of the reasons why it is difficult for us to choose appropriate treatment plans in clinical work. It was reported that the side effects of MMF are cytopenia and diarrhea. Levamisole has an immunomodulatory function that is usually well-tolerated. Its main side effect is elevated liver enzymes. Calcineurin inhibitors have long been used in SDNS or FRNS. Their major side effects are hirsutism, gum hypertrophy, and nephrotoxicity, leading to interstitial kidney fibrosis and chronic kidney disease. Cyclophosphamide is an efficient treatment, but its gonadal toxicity is a major drawback to its use. More recent drugs such as rituximab are very effective but induce an increased risk of opportunistic infection, prolonged neutropenia, and anaphylaxis.

MMF can be used not only in the treatment of PNS but also in other kidney diseases in children. By comparing the different efficacy of MMF combined with steroid and steroid alone in the treatment of 76 children with Henoch–Schoenlein purpura nephritis (HSPN), Lu et al. (19) found that the

TABLE 2 | Evaluation of GRADE pro GDT of MMF or others for the treatment of SDNS or FRNS in children.

Certainty assessment							№ of patients		Effect		Certainty	Importance
Outcomes (No of studies)	**Study design**	**Risk of bias**	**Inconsistency**	**Indirectness**	**Imprecision**	**Other considerations**	**MMF**	**Others**	**Relative (95% CI)**	**Absolute (95% CI)**		
1-year relapse-free survival rate (6)	Random-ized trials (5/6)	Not serio-us	Not serious	Not serious	Not serious	None	80/220 (37.9%)	94/227 (41.4%)	RD −0.02 (-0.12 to 0.05)	-	⊕⊕⊕⊕ **HIGH**	IMPORTANT
The number of relapses within 1 year (3)	Randomized trials	Not serious	Not serious	Serious[a]	Serious[b]	None	120	121	-	MD 0.26 lower (0.45 lower to 0.08 lower)	⊕⊕◯◯ **LOW**	CRITICAL
Cumulative prednisone dosage (4)	Randomized trials (3/4)	Not serio-us	Not serious	Not serious	Serious[b]	None	178	179	-	SMD 0.32 lower (0.53 lower to 0.11 lower)	⊕⊕⊕◯ **MODERATE**	CRITICAL
Incidence of adverse reactions (6)	Randomized trials (5/6)	Not serious	Serious[c]	Not serious	Not serious	None	50/260 (19.2%)	48/273 (17.6%)	RD 0.02 (−0.04 to 0.09)	-	⊕⊕⊕◯ **MODERATE**	IMPORTANT

[a] *The mean and standard deviation of the article with largest weight are converted from the quartile notation by Luo's methods.*

[b] *Limited sample size.*

[c] *Different articles have different definitions of severe and mild adverse reactions.*

⊕, *Evidence quality symbol;* ◯, *Downgraded one level.*

combination of MMF and steroid was superior to steroid alone in relieving proteinuria. In the treatment of antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV). Hu et al. (20) found that MMF effectively ameliorates disease activity and considerably improves renal function in patients. Similarly, MMF also plays a significant role in renal transplant patients (21).

Due to the limited number of clinical researches in pediatric population and short application history of MMF, the data included in this study were insufficient. By consulting the Chinese Clinical Trail Registry (ChiCTR) and International Clinical Trails Registry Platform (WHO ICTRP), we found that some clinical projects in line with the theme of our research, including IRCT20130812014333n113, NCT04048161, CTRI/2019/04/018517, and JPRN-jRCTs051180081. We hope that our conclusions will be further confirmed after the successful completion of the above studies. In addition, some of the literature reports included in this study have shortcomings in the quality of methodology, such as unknown methods of randomization, unclear hidden distribution, and not using a blinded study design, as examples. In addition, this study has some incompleteness in obtaining literature data. For example, there are reports on the number of relapses within 1 year in the literature of Gellermann (11), but there are only the mean value and no standard deviation, without response through searching the original text or contacting the author. Thus, the study has to be excluded from the analysis of the outcome index. The authenticity of the research results may be affected. It is also suggested that when we carry out RCTs in the future, we should strictly abide by the above methodological requirements and report accordingly. In short, the treatment of mycophenolate mofetil in children with SDNS or FRNS still needs to be verified by more well-designed, large-sample, multicenter, long-term, and close follow-up RCTs.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

XX was responsible for the concept, design, definition of intellectual content, literature search, data acquisition, and statistical analysis. S-YQ was responsible for the manuscript preparation and editing. MW took responsibility for the manuscript review and integrity of the work as a whole from inception to published article. All authors contributed to the article and approved the submitted version.

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frontiers in Pediatrics

ORIGINAL RESEARCH
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Childhood Idiopathic Nephrotic Syndrome: Does the Initial Steroid Treatment Modify the Outcome? A Multicentre, Prospective Cohort Study

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Background: A great majority of children with idiopathic nephrotic syndrome will relapse after successful treatment of the initial episode. The possibility that different steroid dosing regimens at onset, adjusted for risk factors, can reduce the rate of relapse represents an interesting option to investigate.

Objectives: To evaluate the effect of the initial steroid regimen, adjusted for time to remission (TTR), on the frequency of relapses and steroid dependence, and to verify the influence of prognostic factors on disease course.

Methods: A multicentre, prospective, cohort study. Children with nephrotic syndrome, with TTR $\leq$10 days (Group A), were given a 20-week prednisone regimen (2,828 mg/m^2) and those with a TTR >10 days, a 22-week regimen (3,668 mg/m^2) (Group B). Previously published retrospective data from the same centers were also evaluated. Main outcomes were: relapse rate, number of frequent relapsers + steroid dependent children and total prednisone dose after induction.

Results: 143 children were enrolled. Rate of relapsed subjects (77 vs. 79%) and frequent relapsers + steroid dependent subjects (40 vs. 53%) did not differ between Groups A and B, or between the retrospective and prospective cohorts. The cumulative prednisone dose taken after the induction treatment was similar in both groups and in the retrospective and prospective cohorts. TTR was not associated with relapse risk. Age at onset and total serum protein were significantly lower in relapsing patients. At ROC analysis, the best cut-off was 5.3 years for age at onset and 4.2 g/dL for

total serum protein. According to these cut-offs, older children with higher total serum protein had a higher relapse free survival rate (58%) than younger children with lower total serum protein (17%).

Conclusions: TTR was not found to be a prognostic factor of relapse; because of this, different steroid regimens, adjusted for TTR, did not modify the relapse rate in any relevant measure. Conversely, younger age and low total serum protein were independent predictors of relapse risk, however this outcome was not modified by higher prednisone regimens.

Clinical Trial Registration: https://www.ClinicalTrials.gov/, identifier: NCT01386957 (www.nefrokid.it).

Keywords: childhood idiopathic nephrotic syndrome, steroid treatment, frequent relapsers, steroid dependency, prognostic factors, age at onset, total serum protein

INTRODUCTION

Steroid therapy is the first-line treatment for idiopathic nephrotic syndrome (INS), inducing remission in 80–90% of children (1–3). However, 75–80% of responders will relapse, and 40–50% will show frequent relapses or steroid dependence (4–9). No consensus exists regarding optimal dosing or treatment duration (10–16). In 2000, a Cochrane meta-analysis showed that 3 months of steroid treatment resulted in a lower relapse rate at 12 and 24 months, with an increased benefit being demonstrated for up to 7 months of treatment (17). While new trials focused on modifying the clinical evolution of INS, many study groups began looking for prognostic factors of relapse and steroid dependence [early age at onset (18–21), male gender (21), intrauterine growth retardation (22), and time to remission (23–25)]. Vivarelli et al. identified time to remission (TTR) >14 days as a main prognostic factor for relapse, with all subjects relapsing within 3 months, while 20% of subjects with a TTR ≤7 days were still in remission at 18 months.

On these bases, we designed an epidemiological, observational, prospective, multicenter study to evaluate the role of the initial steroid regimen as regards relapse occurrence and steroid dependence, and to verify the influence of potential prognostic factors on disease course (23–25).

Moreover, we took the clinical decision to differentiate the steroid regimen on the basis of TTR (≤10 or >10 days), with the aim of protecting later responders from the higher risk of relapse, as reported by some authors (23–25).

METHODS

This is an epidemiological, multicenter, prospective cohort study involving 49 Italian pediatric units, performed between 2011 and 2016 (ClinicalTrials.gov Id: NCT01386957). An additional cohort, compiled using retrospective data from children treated at the same centers, was used for further analyses (13). Ethics Committee approval from the participating hospitals and written informed consent were obtained.

Diagnosis

All children with a first episode of INS, defined as proteinuria >40 mg/m^2/h or urine protein/creatinine ratio (uPr/uCr) >2 mg/mg and albuminemia <2.5 g/dL, were enrolled. Inclusion criteria were age at onset >6 months and <18 years and a diagnosis of INS. Exclusion criteria were congenital and secondary forms of nephrotic syndrome and steroid resistance. During the treatment protocol, dipstick urinalysis was performed daily, in order to identify TTR (first day of negative/trace dipstick).Relapse was defined as 3 days of dipstick ≥2+, confirmed by uPr/uCr >2 mg/mg. Time to relapse was defined as the time elapsed since the start of treatment to the first relapse. At the end of the 24-month follow-up period, patients were classified as non-relapser (NR), infrequent relapser (IR), frequent relapser (FR), or steroid-dependent (SD) according to standard definitions (26). Non-relapsers and infrequent relapsers (NR + IR) and frequent relapsers and steroid-dependent (FR + SD) subjects were grouped and compared.

Therapy Protocol

Subjects with a TTR ≤10 days (Group A) received prednisone 60 mg/m^2/day for 4 weeks, those with a TTR >10 days (Group B) for 6 weeks. Patients not achieving remission within 6 weeks received 3 alternate-day pulses of iv methylprednisolone (1 g/1.73 m^2, max 1g), followed by alternate-day prednisone at 40 mg/m^2. Those who had not achieved remission after a further 2 weeks were classified as steroid-resistant (SR). Steroid tapering was identical for all patients: 4 weeks of alternate-day prednisone (40 mg/m^2), followed by 14 weeks of tapering. The first episode cumulative prednisone dose was 2,828 mg/m^2 (20 weeks) in Group A, and 3,668 mg/m^2 (22 weeks) in Group B. First relapses were treated with prednisone 60 mg/m^2/day, until proteinuria was negative for 5 consecutive days, then a single alternate-day 40 mg/m^2 dose (4 weeks). Subsequent relapses were treated according to each individual center's relapse protocol.

Abbreviations: INS, idiopathic nephrotic syndrome; TTR, time to remission; uPr/uCr, urine protein/creatinine; NR, non-relapser; IR, infrequent relapse; FR, frequent relapser; SD, steroid-dependent; SR, steroid-resistant; SDS, systolic and diastolic blood pressure; SS, steroid-sensitive.

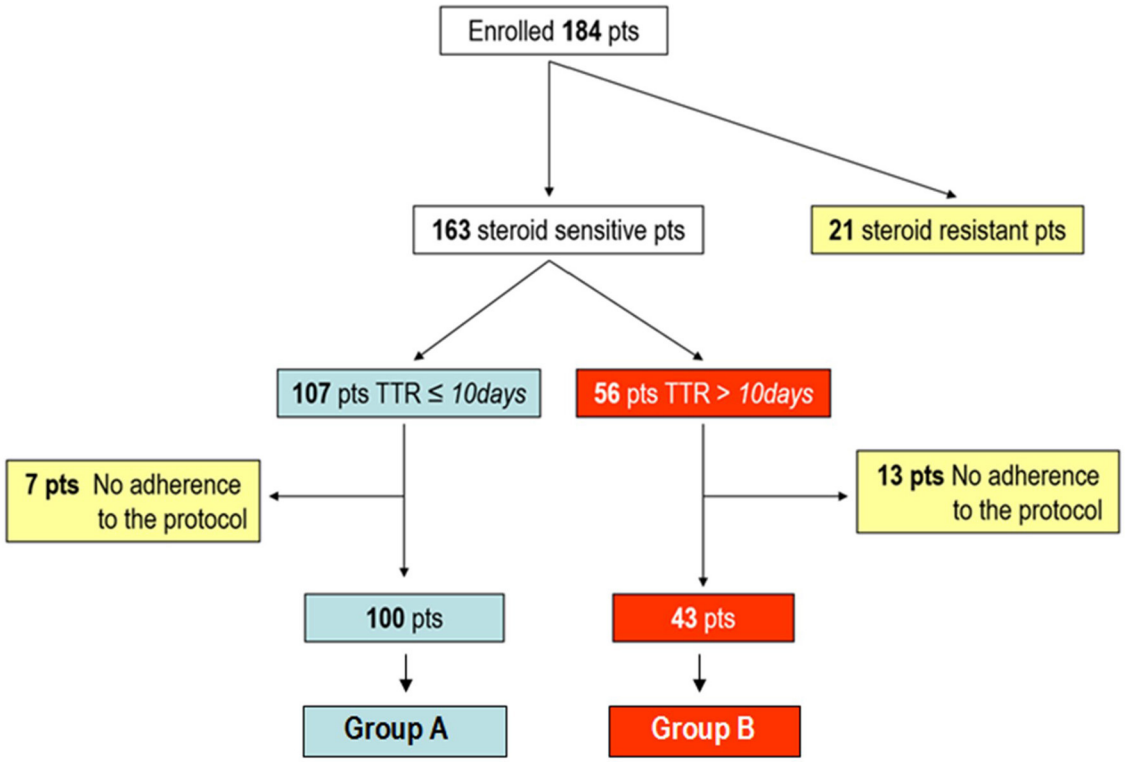


FIGURE 1 | Study flow chart.

Clinical and Laboratory Data

Height, weight, body mass index, systolic and diastolic blood pressure (SDS), complete blood count, urea, creatinine, uricemia, serum protein electrophoresis, albumin, total cholesterol, triglycerides, electrolytes, urinalysis, 24-h proteinuria, or uPr/uCr were recorded in an online database (www.nefrokid.it) at diagnosis, 12 and 24 months. Total number of relapses, time to relapse, total steroid dose at 12 and 24 months, and the use of other immunosuppressors were also recorded.

Additional Retrospective Cohort

We acknowledged that the original design of the prospective study was somewhat flawed. Therefore, we decided to get a wider perspective in order to draw more reliable conclusions (see Discussion). To that purpose, a previously published retrospective study of 144 INS children diagnosed between January 2007 and December 2009 and followed up for 24 months was used as an additional cohort for further analyses (13). Inclusion criteria were identical, while steroid treatment was not standardized. Steroid induction dose was 2,013 ± 617 mg/m^2, with a 5 (2.5–8) week duration. The first episode mean cumulative prednisone dose was 3,582 ± 881 mg/m^2, ranging from 1,904 to 6,035 mg/m^2, with a 21 (9–48) week duration.

Study Aims

(a) To evaluate and compare the clinical course of patients (Group A vs. Group B and prospective vs. retrospective cohort) at 24 months.

(b) To evaluate the prognostic relevance of the following factors: age and laboratory data at onset, TTR in continuous form, total prednisone induction dose (4 weeks of daily prednisone vs. 6 weeks).

Outcomes

(a) Relapse free survival, percentage of patients with at least one relapse at 24 months, number of relapses per patient and time to first relapse.

(b) Steroid sensitivity (prevalence of FR + SD subjects) at 24 months.

(c) Cumulative post-induction prednisone dose (cumulative prednisone dose administered from induction to 24 months).

Statistical Analysis

Statistical analysis was performed using the open source software R (27). The Chi-Square test of independence was used to analyze the association between categorical variables. Non-parametric tests (Wilcoxon, Kruskal-Wallis) were used to compare the distribution of a continuous variable in two or more different groups.

Linear regression models were used to analyse the association between continuous variables and the Kaplan Meier estimator to build relapse free survival curves. Cox hazard ratio models were used to evaluate significance of

TABLE 1 | Comparison of clinical and laboratory data at onset: Group A vs. Group B and prospective vs. retrospective cohort.

	Prospective cohort			Restrospective cohort	*p*-value			
	Total (143 pts)	Group A (100 pts)	Group B (43 pts)	Total (144 pts)	Group A vs. Group B	Adjusted (Holm's method)	Prospective vs. retrospective	Adjusted (Holm's method)
Clinical data								
Age (years)	4.55 ± 2.7	4.73 ± 2.53	4.14 ± 3.06	4.75 ± 3.06	0.01	0,21	0.885	1
Sex (Male)	93 (65.0%)	65 (65.0%)	28 (65.1%)	96 (66.7%)	0.989	1	0.771	1
Height (SDS)	−0.04 ± 0.87	−0.08 ± 0.81	0.05 ± 1.01	0.02 ± 1.00	0.412	1	0.481	1
Weight (SDS)	0.46 ± 0.89	0.4 ± 0.91	0.64 ± 0.84	0.55 ± 1.03	0.082	1	0.171	1
BMI (SDS)	0.65 ± 0.93	0.60 ± 0.92	0.79 ± 0.95	0.72 ± 0.89	0.207	1	0.541	1
sBP (SDS)	1.07 ± 1.12	1.01 ± 1.05	1.23 ± 1.27	0.86 ± 1.14	0.294	1	0.083	1
dBP (SDS)	1.34 ± 0.88	1.28 ± 0.76	1.5 ± 1.12	1.13 ± 0.91	0.529	1	0.150	1
SGA	16/143	10/100 (10%)	6/43 (13.8%)		0.491	1		
Laboratory data								
Hemoglobin (g/dL)	13.0 ± 1.2	13.08 ± 1.09	12.97 ± 1.34	13.3 ± 1.2	0.323	1	0.104	1
Urea (mg/dL)	29.3 ± 16.5	26.66 ± 13.25	35.26 ± 21.07	28.6 ± 13.2	0.01	0,21	0.936	1
Creatinine (mg/dl)	0.30 ± 0.15	0.29 ± 0.12	0.34 ± 0.21	0.35 ± 0.15	0.653	1	0.001	0.022
Uricemia (mg/dL)	4.23 ± 0.99	4.04 ± 0.93	4.65 ± 1.01	4.13 ± 1.09	0.004	0,088	0.546	1
Total proteins (g/dL)	4.23 ± 0.58	4.26 ± 0.59	4.18 ± 0.58	4.21 ± 0.67	0.323	1	0.734	1
Albumin (g/dL)	1.63 ± 0.50	1.64 ± 0.52	1.60 ± 0.46	1.40 ± 0.40	0.955	1	0.001	0.022
Tot. cholesterol (mg/dL)	403.7 ± 108.1	395.7 ± 108.05	421.09 ± 107.49	400.1 ± 102.1	0.049	0,784	0.665	1
Triglycerides (mg/dL)	197.20 ± 110.2	184.2 ± 92.12	225.86 ± 139.03	217.62 ± 138.5	0.074	1	0.331	1
Na (mmol/L)	136.8 ± 3.5	137.25 ± 3.54	135.84 ± 3.11	136.2 ± 3.4	0.03	0,522	0.240	1
K (mmol/L)	4.47 ± 0.50	4.42 ± 0.43	4.61 ± 0.52	4.51 ± 0.56	0.02	0,38	0.921	1
Ca (mg/dL)	8.18 ± 0.55	8.15 ± 0.61	8.24 ± 0.42	8.18 ± 0.70	0.439	1	0.652	1
P (mg/dL)	4.93 ± 0.82	4.84 ± 0.81	5.12 ± 0.82	5.08 ± 1.09	0.029	0,522	0.287	1
uPr/uCr (mg/mg)	13.39 ± 10.7	11.74 ± 9.41	17.44 ± 12.6	10.32 ± 7.22	0.002	0.046	0.056	1
Proteinuria (g/L)	9.32 ± 9.13	8.29 ± 8.27	11.91 ± 10.71	8.05 ± 8.17	0.088	1	0.230	1
Microhematuria	58 (40.6%)	37 (37.0%)	22 (51.2%)	59 (41.0%)	0.114	1	0.943	1
Steroid response								
TTR (days)	10.3 ± 7.8			14.8 ± 12.4			0.00025	0.00575

Mean ± SD for numerical variables, number (%) for categorical variables. BMI, body mass index; sBP, systolic blood pressure; dBP, dyastolic blood pressure; SGA, small for gestational age. The red color values represent the significant values.

prognostic factors in relation to survival curves and for multivariate analysis.

RESULTS

Clinical Course

Prospective Cohort

One hundered and eighty-four children (median age at diagnosis: 3.9, range 0.6–17 years; Male:Female 1.9:1) with INS were enrolled. One hundred and sixty-three (89%) were steroid-sensitive (SS), 21 (11%) were SR and were excluded alongside an additional 20 patients due to non-compliance. The final cohort comprised 143 subjects. Among them, 100 (70%) had a TTR $\leq$10 days and were administered the 4-week induction regimen (Group A), whilst the 43 with a later TTR were given the 6-week induction regimen (Group B) (**Figure 1**).

Retrospective Cohort

One hundred forty-four steroid sensitive patients from a retrospective study (13), followed-up for 24 months, were evaluated. Steroid induction dose was 2,013 ± 617 mg/m^2, with a 5 (2.5–8) week duration.

Data at Onset

Clinical characteristics and laboratory data are shown in **Table 1**. The two cohorts were similar, except for higher serum creatinine and lower serum albumin values in the retrospective cohort. Groups A and B were also similar, except for age at onset, which was significantly lower in Group B, while urea, uricemia, total cholesterol, Na, K, P, and PrU/CrU were significantly higher in Group B.

The median TTR was 8 (1–47) days in the prospective cohort, with 156 (96%) patients achieving remission within 4 weeks, and

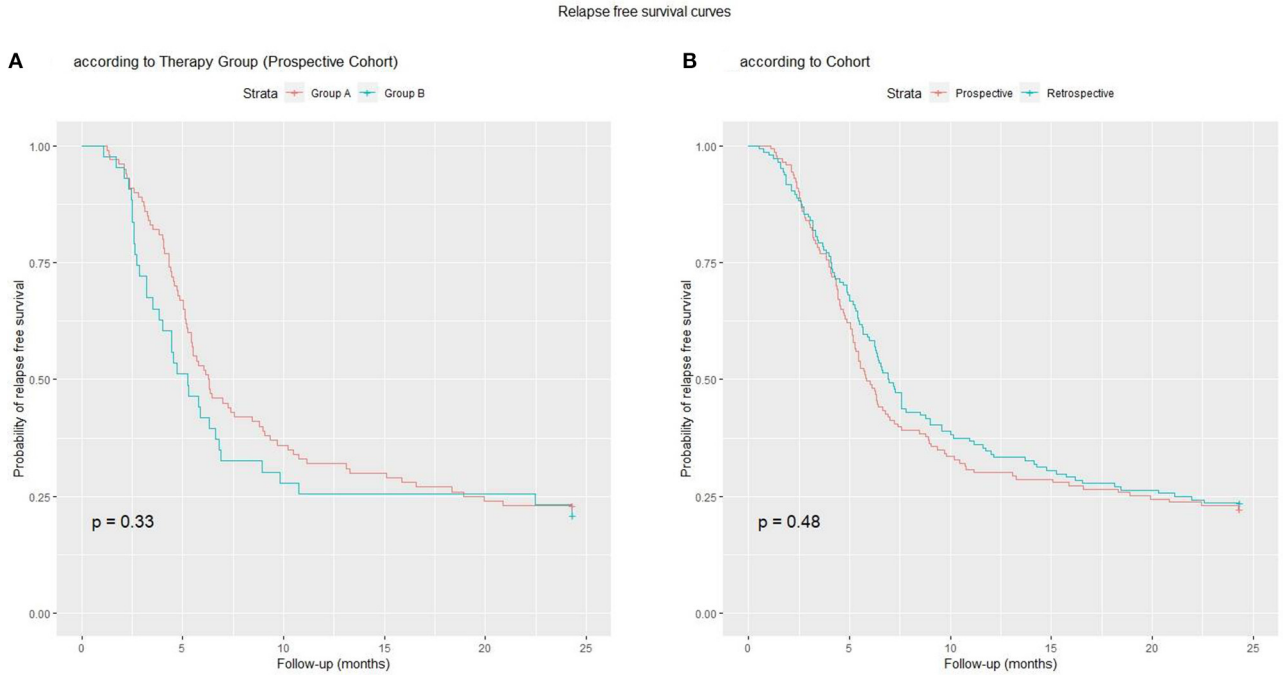


FIGURE 2 | Relapse-free survival curves: **(A)** Group A vs. Group B; **(B)** Prospective vs. Retrospective Cohort.

7 (4%) in more than 4 weeks (3 after iv methylprednisolone boluses). The median TTR was significantly longer (11; range 3–77 days) in the retrospective cohort. The mean total induction prednisone dose was 1,685 ± 107 mg/m^2 in Group A, 2,648 ± 426 in Group B, and 2,013 ± 617 in the retrospective cohort.

Relapses

In the prospective cohort, 111 (78%) subjects relapsed after a mean time of 188 ± 140 days from the start of treatment. At 12 months, 100 subjects (70%) had relapsed. There were a total of 268 relapses (182 in Group A, 86 in Group B), ranging from 1 (42 patients) to 7 (1 patient) per patient. The mean number of relapses per patient (1.8 vs. 2; $p = 0.43$), and the percentage of patients who had relapsed at 12 (68 vs. 74%; $p = 0.44$) and 24 months (77 vs. 79%; $p = 0.78$) did not differ between Groups A and B. The relapse rate in the retrospective vs. the prospective cohort (77 vs. 78% at 24 months, $p = 0.91$) and the number of relapses per patient (2.1 vs. 1.9, $p = 0.44$) did not differ. The time to first relapse was longer in Group A vs. Group B (mean = 205.6 vs. 166.8 days; $p = 0.05$). The relapse-free survival curve up to the end of follow-up did not differ between Groups A and B (p=0.30), or between the two cohorts ($p = 0.50$; **Figures 2A,B**).

Steroid Sensitivity

In the prospective cohort, 32 children (22%) were NR, 48 (34%) IR, 5 (3%) FR, 58 (41%) SD. The prevalence of FR + SD subjects was similar in Groups A and B (40 vs. 53%, $p = 0.14$) and in the two cohorts (43 vs. 44%, $p = 0.8$).

Cumulative Post-induction Prednisone Doses

The Group A induction dose was lower per protocol (4 vs. 6 weeks) and the cumulative dose remained significantly lower at 12 and 24 months ($p = 0.002$ and $p = 0.018$, respectively), compared to Group B. Therefore, the cumulative post-induction prednisone dose did not differ significantly ($p = 0.28$; **Figure 3A**). Steroid sparing agents were utilized more frequently in Group B than in Group A (42 vs. 25%, $p = 0.04$). When comparing the retrospective and prospective cohorts, the mean total induction prednisone dose (2013 vs. 1977, $p = 0.49$), the mean cumulative prednisone dose at 12 (5,656 vs. 5,355, $p = 0.3$) and 24 months (7,668 vs. 7,203, $p = 0.5$) and the mean cumulative prednisone dose administered from induction to 24 months (5,682 vs. 5,245, $p = 0.6$) did not differ (**Figure 3B**).

Prognostic Factors

The significance of prognostic factors for the whole population (prospective + retrospective cohort) is shown in **Table 2**, and separately for the prospective and retrospective cohorts in **Supplementary Tables 1, 2**, respectively.

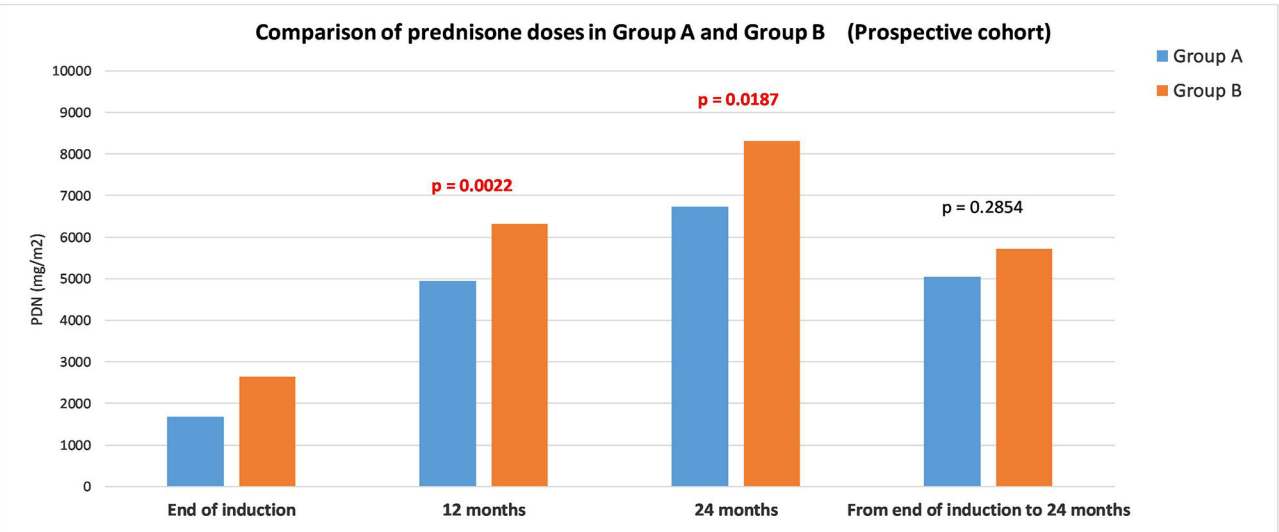
Age at Onset

Age at onset was significantly associated with all outcomes in the whole population (**Table 2**) and in the prospective cohort. In the retrospective cohort, only time to relapse and prednisone dose were not significantly associated with age.

TTR

In the whole population, TTR in continuous form was only significantly associated with a higher prevalence of FR-SD

A



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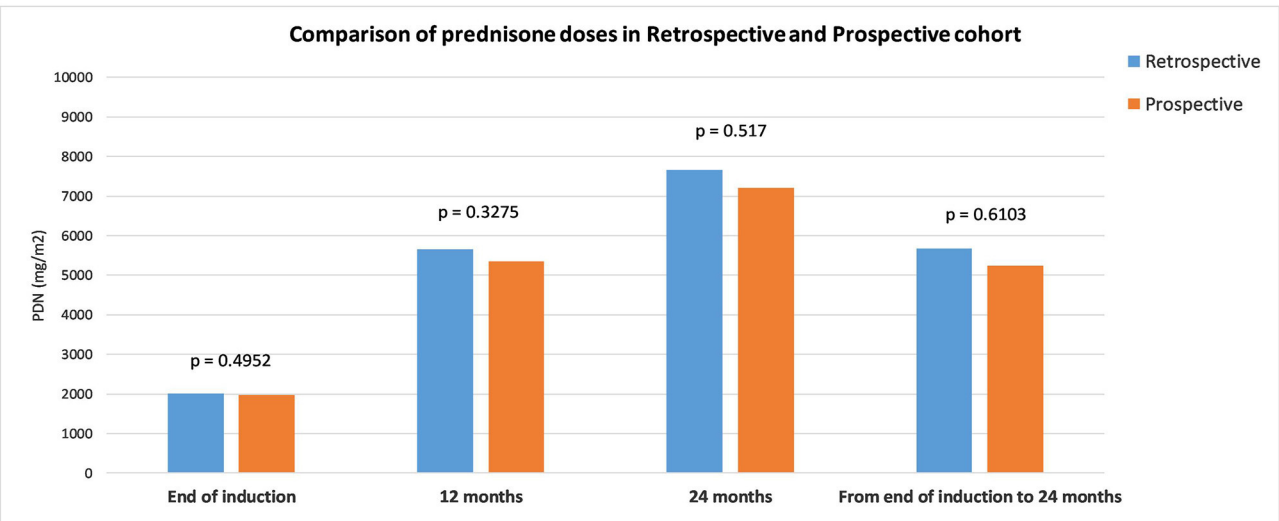

C

Total prednisone doses (mg/m2)				
	Induction	12 months	24 months	From end of induction to 24 months
Group A	1685±107	4953 ± 2152	6736 ± 3521	5048 ± 3242
Group B	2648±426	6316 ± 2287	8320 ± 3568	5717 ± 795
Retrospective	2013 ± 617	5656 ± 2355	7668 ± 4000	5682 ± 4011
Prospective	1977 ± 509	5355 ± 2270	7203 ± 3411	5245 ± 3348

FIGURE 3 | Cumulative prednisone doses. Total daily PRED dose, cumulative PRED dose at 12, 24 months and from the end of induction to the end of follow-up: Group A vs. B **(A)**, prospective vs. retrospective cohort **(B)**. All values are means ± SD (mg/m^2) **(C)**.

patients (**Table 2**). This association held true in the retrospective cohort, where a weak association with a higher relapse rate was also seen ($p = 0.02$). No associations were observed in the prospective cohort (**Supplementary Tables 1, 2**).

In order to exclude the confounding factor of a different induction therapy in the evaluation of TTR, we decided to perform a separate sub-analysis for Group A (induction prednisone dose 1,685 ± 107) and Group B (induction prednisone dose 2,648 ± 426) (**Figure 4**), categorizing patients as "lower TTR" and "higher TTR" in each group. In both groups, there was no detectable effect of TTR as a negative prognostic factor. When patients from the retrospective cohort were categorized for TTR in the same way as the prospective cohort, data did not show a significant prognostic role for TTR.

TABLE 2 | Prognostic factors evaluated in the whole population according to the different outcomes.

	Relapse	Time to relapse	*N.* of relapses	Prevalence of FR-SD	PDN after remission	Relapse-free survival	Relapse-free survival adjusted (Holm's method)
Whole population							
Age at onset (years)	0.00002	0.0006	0.0009	0.00005	0.046	0.0000001	0.000002
TTR (days)	0.088	NS	NS	0.003	NS	NS	NS
Prednisone dose in induction (mg/m2)	NS	NS	NS	NS	NS	NS	NS
Hemoglobin (g/dL)	NS	NS	NS	NS	NS	NS	NS
Urea (mg/dL)	NS	NS	NS	NS	0.005	NS	NS
Creatinine (mg/dl)	0.016	NS	0.012	0.042	0.017	0.0037	0.056
Uricemia (mg/dL)	NS	NS	NS	0.07	NS	0.096	NS
Total proteins (g/dL)	0.0003	0.009	0.005	0.019	0.00002	0.00001	0.00019
Albumin (g/dL)	0.058	0.004	NS	0.086	0.025	0.0009	0.0162
Alpha 2 globulins (g/L)	NS	NS	NS	NS	NS	NS	NS
Gamma globulins (g/L)	NS	NS	NS	NS	NS	0.068	NS
Tot. cholesterol (mg/dL)	0.049	NS	NS	NS	0.09	NS	NS
Triglycerides (mg/dL)	0.08	NS	NS	0.056	NS	0.059	NS
Na (mmol/L)	0.06	0.02	NS	NS	0.06	0.039	NS
K (mmol/L)	NS	NS	NS	NS	NS	NS	NS
Ca (mg/dL)	NS	NS	NS	NS	0.014	NS	NS
P (mg/dL)	NS	NS	NS	NS	NS	NS	NS
uPr/uCr (mg/mg)	0.039	0.016	NS	0.00016	0.026	0.001	0.017
Proteinuria (g/L)	NS	0.007	0.046	0.002	0.0006	0.001	0.017
Microhematuria	NS	NS	NS	NS	NS	NS	NS

p-values are in red if <0.05, in green if ≥0.05, and <0.10. All p > 0.10 are labeled as NS (not significant).

All total prednisone doses (taken by Group A, Group B, prospective and retrospective cohort) expressed as means ± SD (mg/m^2), are shown in **Figure 3C**.

Total Induction Prednisone Dose

In the whole population, induction dose was not associated with any of the outcomes (**Table 2**). The same was seen when the two cohorts were analyzed separately.

Laboratory Values at Onset (Table 2)

Total protein values were significantly associated with all outcomes in the entire population. Albumin behaved similarly for three outcomes. A lower total protein or albumin value was associated with higher relapse rate. The behavior was similar in the two cohorts (**Supplementary Tables 1, 2**). Among other values, only creatinine, proteinuria, and the PrU/CrU ratio showed a consistent association with most outcomes (**Table 2**). Creatinine showed a paradoxical association: higher values were associated with a better prognosis. As creatinine showed a strong collinearity with age, this association was not analyzed further. The other laboratory values were not consistently associated with the outcomes.

Relapse Free Survival Analysis

The last two columns in **Table 2** report the results of bivariate analysis for each prognostic factor using relapse free survival as outcome. Both raw *p*-values and adjusted *p*-values are shown. Time to first relapse was always computed from start of therapy, and follow-up was 24 months from start of therapy for all patients.

We chose to use relapse-free survival as outcome for our final multivariate analysis, because it includes information about both relapse rate and time to relapse.

Multivariate analysis was performed using a Cox proportional hazards model, including all the variables with a $p < 0.1$ in the bivariate analysis performed on the whole population. The following variables were excluded because of strong collinearity: albumin with total serum protein, creatinine with age, and PrU/CrU with proteinuria and age. In the final model, age at onset (coefficient = −0.128; $p = 0.000004$) and total serum protein (coefficient = −0.488; $p = 0.00002$) were the only significant independent predictors of relapse free survival (total likelihood ratio = 1.774e-10). Younger patients with lower total serum protein values at onset had a higher risk of earlier and more frequent relapses. None of the other risk factors were independent prognostic factors.

A ROC analysis showed that the best cut-offs (Youden's index) for age at onset (AUC = 0.681) and serum protein (AUC = 0.653) were 5.3 years and 4.2 g/dL, respectively. Using these cut-offs, children were categorized as follows: Group 1: not at risk for either total protein or age ($n = 41$, 15%), Group 2: at risk for total protein only ($n = 34$, 12%), Group 3: at risk

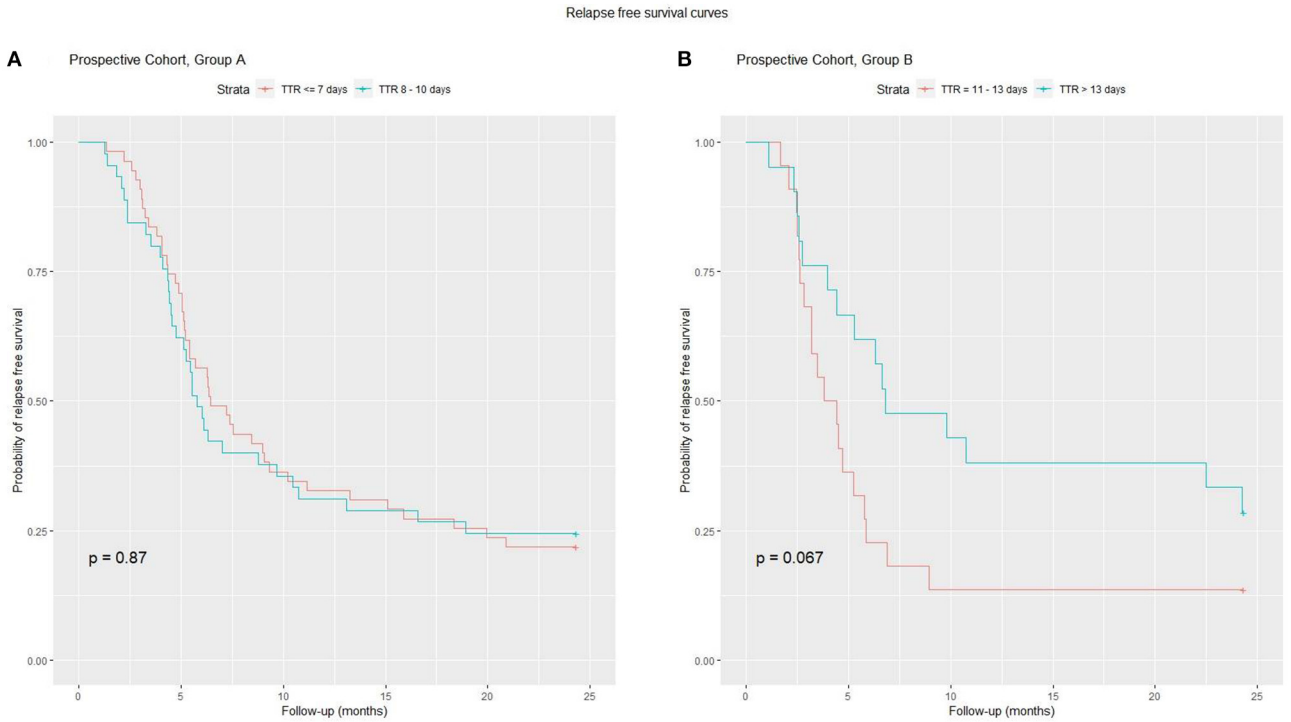

FIGURE 4 | A sub-analysis of TTR and relapse risk was performed on Group A and B. On the left **(A)** for Group A, the relapse-free survival curve did not differ between the two subgroups. On the right **(B)** for Group B patients with higher TTR have a better curve, but the difference is not significant.

for age only ($n = 78$, 29%) and Group 4: at risk for both total protein and age ($n = 120$, 44%).This categorization significantly predicted relapse free survival (**Figure 5A**). **Figure 5B** shows instead that total induction prednisone dose (threshold 2,000 mg/m^2) was not significant ($p = 0.70$). Even when analyzing induction prednisone dose in each of the abovementioned risk groups separately, no significant effect on relapse free survival was observed (**Figures 6A–D**).

These 4 groups were also good predictors of FR-SD prevalence ($p = 0.007$). In Group 1, 24% of patients were FR-SD vs. 54% in Group 4. Groups 2 and 3 showed similar behavior, with 38 and 41% of FR-SD patients, respectively.

DISCUSSION

This is the first published prospective study to use a steroid regimen adjusted for a prognostic factor. When this study was conceived, the Cochrane review (28) recommended prolonging the total prednisone dose and steroid protocol duration. In accordance with this, we prolonged and diversified our steroid regimen on the basis of TTR $\leq$10 or >10 days. The rationale was to administer a higher daily prednisone dose to children at risk of more frequent relapses or steroid dependence (23–25).

However, we do acknowledge that the original design of the prospective study was not really appropriate, for two important reasons:

1) It was based on the assumption that TTR is a strong negative prognostic factor for relapse. That assumption was based on rather limited evidence from the literature, and was not confirmed in successive studies. Moreover, our same results did not seem to support our initial assumption.
2) The lack of randomization and the association of TTR with different steroid therapy make it very difficult to distinguish between the possible effects of the two variables in our prospective data.

In particular, our results showed that a higher-dose and longer initial steroid therapy for childhood INS, diversified according to TTR, was not associated with a significantly different clinical course. At first glance, this result may appear to mean that treating the patients at higher risk of relapse with higher prednisone doses was successful, having reduced their relapse rate. Unfortunately, that conclusion holds only if the assumption of a strong negative prognostic role of TTR is confirmed.

To draw more reliable conclusions, we decided to expand our data by adding data from a previous retrospective cohort of patients, as described in Methods. At the same time, we performed specific sub analyses of our data aimed at differentiating, as much as possible, between the effect of TTR and the effect of therapy.

As a result of a more in-depth evaluation of our prospective data and a re-evaluation of all the prognostic factors in the whole population of our patients (prospective + retrospective), we found no evidence the TTR can be considered a major prognostic factor of relapse (**Figure 4**, **Supplementary Figures 1, 2**). Similarly, no detectable effect of different steroid doses (in the range of those tested) was found.

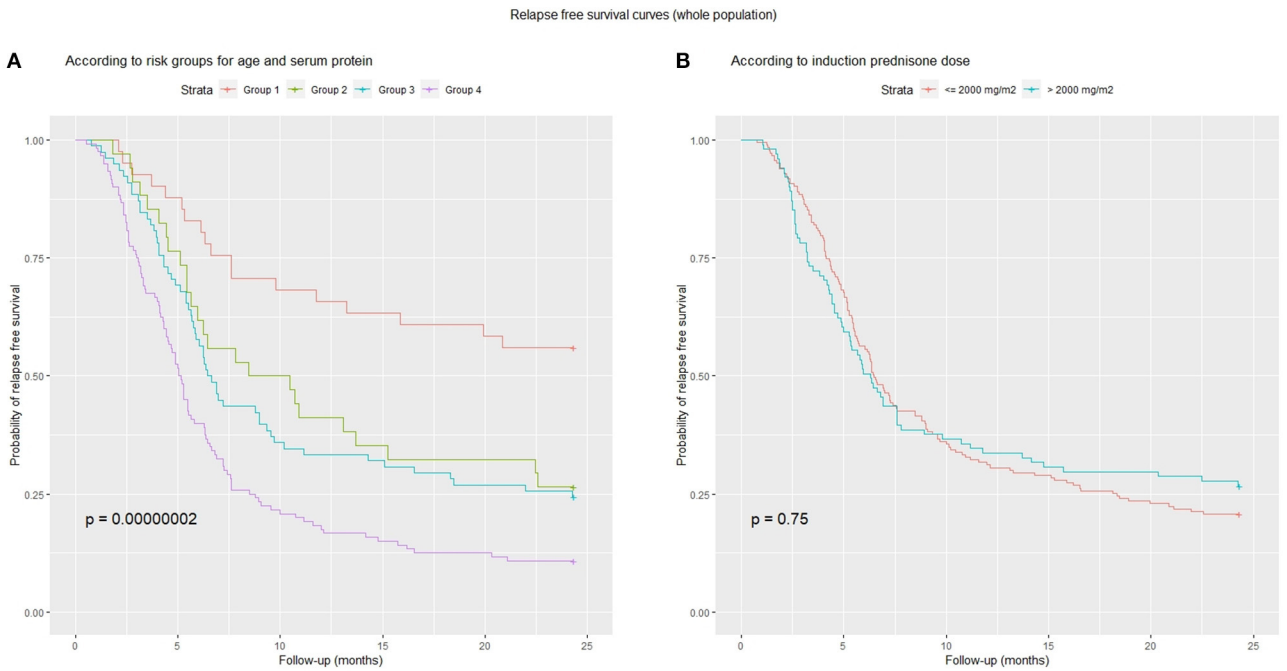

FIGURE 5 | Relapse-free survival curves: **(A)** combined risk groups according to age (cut-off 5.3 years) and serum protein (cut-off 4.2 g/dl): Group 1, low-risk for both; Group 2, high-risk for total proteins only; Group 3, high-risk for age only; Group 4, high-risk for both; **(B)** induction prednisone dose in binary form (threshold 2,000 mg/m^2).

Relapse Rate

Relapse rate at 24 months, time to relapse, the number of relapses per patient and the percentage of FR+SD subjects did not differ significantly either between Groups A and B, or between the prospective and retrospective cohorts, where no TTR-based categorization was applied.

Our data confirm the results of three RCTs, published when our study was ongoing, which showed that relapse rate was not modified by initial steroid regimen. In 2013, Teeninga demonstrated that extending initial prednisone treatment without increasing cumulative dose did not benefit clinical outcome (29). In 2015, Sinha et al. (30) and Yoshikawa et al. (31), comparing a 3-month (2,792 ± 287 mg/m^2) vs. a 6-month regimen (3,530 ± 399 mg/m^2), and a 2- vs. a 6-month regimen (2,240 vs. 3,885 mg/m^2), respectively, found no differences in relapse rate or the number of FR subjects (32). These findings were confirmed both by the authors of the 2015 Cochrane review (33) and the results of a recent RCT (34).

In particular, in our study, considering the total induction dose in binary form in the whole population, low-dose (<2,000 mg/m^2) was as effective as high-dose (>2,000 mg/m^2) in terms of relapse free survival (**Figure 5B**), and the same was true considering each risk group (based on age and total protein) separately (**Figures 6A–D**). Sinha obtained similar results in his RCT (30), comparing 3- vs. 6-month regimens.

As TTR was apparently associated with some differences in laboratory data at diagnosis, we suggest that it could be perhaps considered as a consequence of delayed diagnosis and more serious renal involvement at onset, rather than as a prognostic factor for a subsequent higher relapse rate.

On the contrary, both age and total serum protein concentrations at onset (but also proteinuria and uPr/uCr) were shown to be consistent predictors of relapse, independently of TTR and steroid induction doses.

Indeed, a more immature immunological status in younger patients could be related to a higher frequency of relapses and steroid dependency, as reported by many studies (18–21). Concurrently, a massive proteinuria causing a greater reduction of total serum protein concentrations, could be, at onset, a clinical prognostic sign of subsequent frequent relapses.

Cumulative Prednisone Doses

Another aspect we wanted to investigate in our study was the possible role of higher steroid dose at onset in reducing the need for steroids after remission. Our data definitely do not support that hypothesis, as shown by the evaluation of the mean cumulative doses taken from the end of the induction to 24 months (**Figure 3A**). This result cannot be attributed to a different use of steroid sparing agents.

Prognostic Factors

As previously discussed, our data did not confirm a major prognostic role for TTR, contrary to published studies (20, 22, 35–39).

Conversely, age at onset and total serum protein were significant and strong prognostic factors, associated with all the outcomes. A correlation between age at onset and risk of relapse

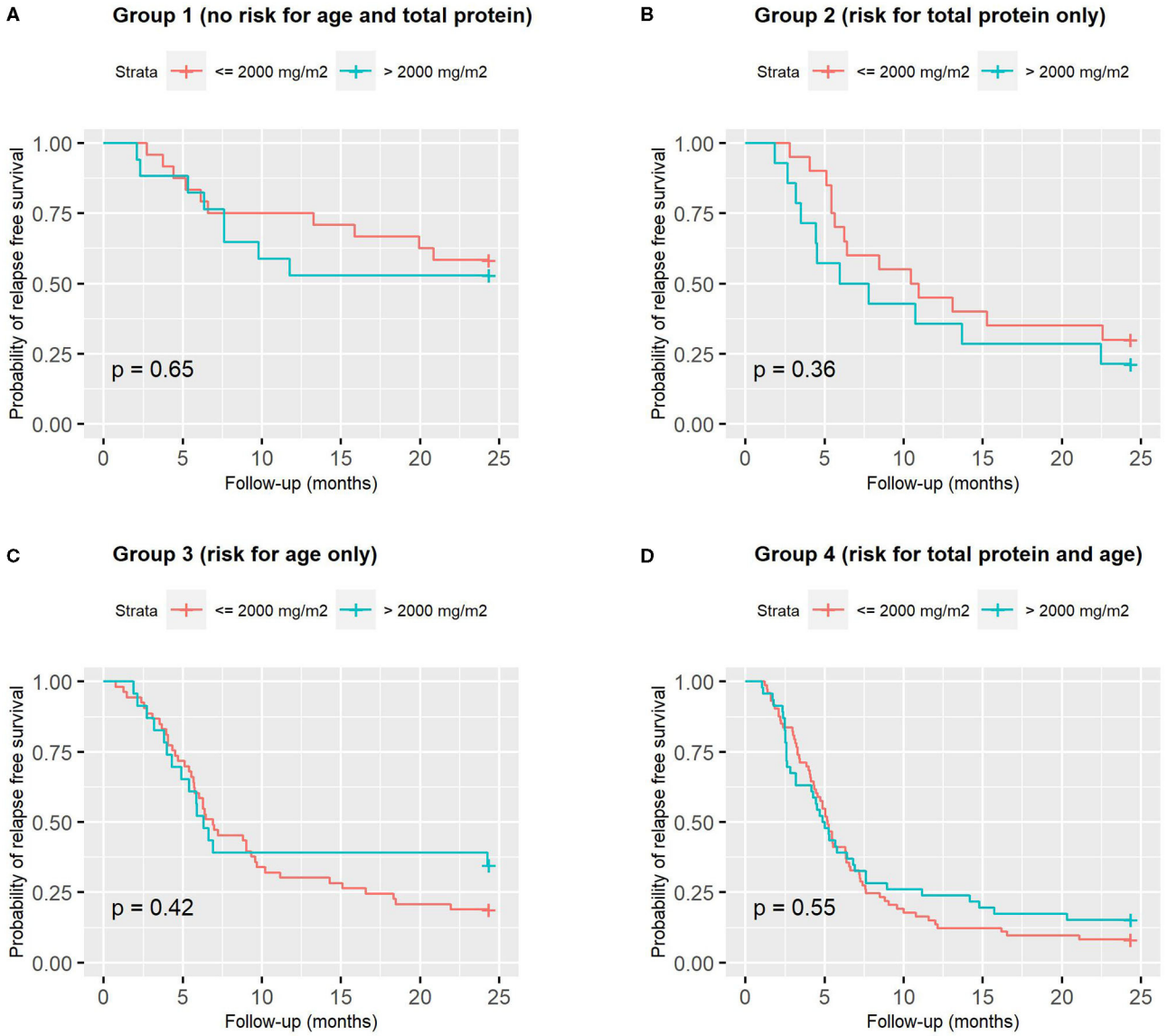

FIGURE 6 | Relapse-free survival curves according to induction prednisone dose in binary form (threshold 2,000 mg/m^2), analyzed in each of the 4 combined risk groups based on age (cut-off 5.3 years) and serum protein (cut-off 4.2 g/dl): **(A)** Group 1, low-risk for both; **(B)** Group 2, high-risk for total proteins only; **(C)** Group 3, high-risk for age only; **(D)** Group 4, high-risk for both.

has been shown by many authors (15, 16, 18, 30, 40, 41), but not by others (37). In 2003, an RCT comparing long vs. short-course prednisolone regimens (42) showed that younger children were more susceptible to relapse and benefitted from the long alternate-day regimen. In 2015, Sinha's *post-hoc* analysis (30) showed that age ≤3 years was associated with an increased relapse risk, but the number of FR children was not reduced by prolonged steroid therapy. The relapse rate and number of FR + SD subjects was not lower in younger children treated with higher prednisone doses.

In a multivariate analysis performed for relapse free survival in our whole population, total serum protein and age at onset were confirmed as the only independent prognostic factors. Based on the ROC analysis cut-offs (5.3 years and 4.2 g/dL), it was possible to categorize children into groups with different relapse risks (**Figure 5A**).

Nevertheless, a small percentage of younger subjects with lower serum protein never relapsed. Other factors are probably involved. Recent research has focused on the impact of genetic polymorphisms on glucocorticoid response (43–45).

Limitations

The non-optimal design of the original prospective study motivated us to add a retrospective cohort of patients to perform a more reliable analysis. Of course, that can be in itself a cause of bias and of difficulties in interpretation of the results. However,

we believe that that procedure allowed us to comprehensively analyse a large population of patients, especially for prognostic factor analysis. Our analysis shows that data from the two cohorts are comparable enough, and that our main conclusions are reliable and consistent across the two individual cohorts.

The choice of 10 days as a cut-off value for TTR was based on our retrospective study. In the prospective study, the distribution of TTR was shifted toward the left, with a median of 8 days, resulting in an unbalanced number of patients (100 vs. 43). Moreover, we did not confirm TTR as a significant predictive factor, as also suggested by recent studies.

Further limitations include the number of dropouts (20) for non-adherence.

CONCLUSIONS

Steroid doses, adjusted for TTR, did not modify either the relapse rate or the number of FR + SD children at 24 months. However, a more refined analysis of our data showed that TTR cannot be considered a major prognostic factor of relapse, differently from what was reported by some other authors.

Conversely, younger age and low total serum protein values at onset were reliable prognostic factors for relapse and steroid dependence, with no apparent effects of higher prednisone regimens at onset. According to our data, a 4-week steroid induction regimen does not require higher cumulative doses throughout 24 months of follow-up, and thus represents a valid option for pediatric INS. However, a deeper understanding of the pathogenetic mechanisms involved in INS will help us detect subjects at higher risk of relapse and choose new therapeutic options (46–50).

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comitato Etico Indipendente di Area Vasta Emilia Centro (CE-AVEC) della Regione Emilia-Romagna IRCCS Azienda Ospedaliero-Universitaria di Bologna, Policlinico S.Orsola-Malpighi. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

AP, AE, GMo conceptualized and designed the study, coordinated and supervised data collection, drafted the initial manuscript, reviewed, and revised the manuscript. GP carried out the initial analyses, drafted the initial manuscript, reviewed, and revised the manuscript. CB, LC, CC, GG, LG, MG, CL, CM, SM, AM, MM, FM, GMe, EM, WM, and PR collected data and reviewed and revised the manuscript. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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SUPPLEMENTARY MATERIAL

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Risk Factors for Poor Prognosis of Severe Infection in Children With Idiopathic Nephrotic Syndrome: A Double-Center, Retrospective Study

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Background: Infection is the most common complication of Idiopathic Nephrotic Syndrome (INS) and the main cause of INS recurrence, severe infection and even leading to mortality. The purpose of this study was to investigate the risk factors of severe infection in INS children and the clinical parameters influencing prognosis.

Methods: Totally 147 children with INS and concomitant infections were enrolled and classified into the severe infection group (SIG) and Non-severe infection group (Non-SIG). The clinical characteristics and auxiliary examination results were compared between the two groups, and the early-warning parameters for severe infection and risk factors for poor prognosis were evaluated.

Results: There were 49 patients in the SIG, 98 patients in the Non-SIG. In the SIG, the most common severe infections disease included severe pneumonia (63.6%), severe sepsis (30.6%), septic shock (4.1%). In SIG, Gram-positive bacteria (GPB) were more common, as was respiratory syncytial virus (RSV), and the three most common strains were Pseudomonas aeruginosa, Staphylococcus aureus (SA) and Staphylococcus epidermidis. There were more steroid-resistant nephrotic syndrome and combination of steroids and immunosuppressants in SIG, compared with the Non-SIG ($P = 0.000$). Patients in the SIG has lower complement 3 (C3, $\leq$0.55 g/L,) and absolute lymphocyte count (ALC, $\leq 1.5 \times 10^9$/L) ($P = 0.004$). Logistic regression analysis revealed that the independent risk factors for severe infections were the combined use of immunosuppressants [95% confidence interval (CI):1.569–463.541, $P = 0.023$], steroid resistance (95% CI: 4.845–2,071.880, $P = 0.003$), C-reactive protein (CRP) $\geq$8 mg/L (95% CI: 43.581–959, 935.668, $P = 0.001$), and infections caused by GPB (95% CI: 27.126–2,118, 452.938, $P = 0.002$), influenza (95% CI: 2.494–1, 932.221, $P = 0.012$) and RSV (95% CI: 5.011–24 963.819, $P = 0.007$). The patients in the SIG were classified into the survival group ($N = 39$) and the mortality group ($N = 5$). Logistic regression analysis showed that white blood cell count (WBC) $>15 \times 10^9$/L (95% CI: 1.046–2.844, $P = 0.033$) was an independent risk factor of poor prognosis for these patients.

Conclusions: Resistance to steroids, combined with steroids and IS agents, and GPB infections (especially SA) are high-risk factors for severe infection in children with INS. We should monitor CRP $\geq$ 8 mg/L, C3 $\leq$ 0.55 g/L and ALC $\leq$ 1.5 $\times$ 10^9/L to avoid developing severe infection. Accompanied by an increase in ANC, WBC significantly increased, suggesting a fatal infection.

Keywords: idiopathic nephrotic syndrome, severe infection, risk factor, prognosis, children

INTRODUCTION

Idiopathic nephrotic syndrome (INS) is one of the most common kidney diseases in children. It is characterized by heavy proteinuria, pitting edema, hypoalbuminemia, and hyperlipidemia. INS occurs in 1.15–16.9 per 100,000 children, and its incidence varies by ethnicity and region (1). Infection is the most common complication of INS; it hampers the treatment of the underlying kidney disease, leads to INS relapse, increases rates of unplanned hospitalizations, and even leads to increased mortality. Several recent reports have evaluated the clinical characteristics and risk factors in children with INS and infections (2, 3). However, few studies have assessed the risk factors for serious infections associated with INS and the risk factors for poor prognosis of infections. The purpose of this study was to investigate the risk factors of severe infections in children with INS and the clinical parameters leading to poor prognosis.

MATERIALS AND METHODS

Study Population

This study enrolled children aged 0–18 years with INS and concomitant infections who were hospitalized in the Children's Hospital of Chongqing Medical University and the Affiliated Hospital of Guizhou Medical University, from January 2013 to October 2019. Written informed consent was obtained from parents or legal guardians, and this study complied with the ethical principles of the Helsinki Declaration of the World Medical Association. All the patients met the following criteria: (1) An onset age from birth to 18 years. (2) The definition of nephrotic syndrome (NS) included: ① nephrotic- range proteinuria: urine protein/creatinine ratio (UPCR) $\geq$ 200.0 (mg/mmol) in spot morning urine or 24 h urine quantification $\geq$50 mg/kg/day ② hypoproteinaemia: serum albumin $<$ 25 g/L, ③ hyperlipidemia: serum cholesterol higher than 5.7 mmol/L and ④ varied degrees of edema. ① and ② are necessary for diagnosis. Children were excluded from congenital nephrotic syndrome, any other secondary NS, such as Henoch–Schoenlein purpura nephritis, lupus nephritis.

Clinical Data

The retrieved clinical data of children with INS who met the inclusion criteria were as follows: (1) Baseline characteristics, including sex, age, duration, usage, duration of steroid and immunosuppressive (IS) therapy. IS agents included cyclosporine, tacrolimus, mycophenolate, cyclophosphamide; (2) Laboratory data were collected, including complete blood count, urinalysis, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), serum urea nitrogen, serum creatinine, uric acid, serum albumin, serum potassium, serum sodium, serum chlorine, serum calcium, C-reactive protein (CRP), procalcitonin (PCT), total cholesterol (Tch), triglycerides, high-density lipoprotein (HDL), low-density lipoprotein (LDL), prothrombin time (PT), activated partial thromboplastin time (APTT), fibrinogen (Fib), D-dimer, immunoglobulin G (IgG), immunoglobulin A (IgA), immunoglobulin M (IgM), immunoglobulin E (IgE), complement 3 (C3), complement 4 (C4), and pathogens isolated by specimen culture (e.g., blood, urine, sputum, cerebrospinal fluid, and bronchoalveolar lavage fluid), antigen testing from blood, or histological material from a definite site of infection. Because complement detection helped to rule out nephropathy caused by autoimmune diseases (4), and children with INS were often accompanied by IgG deficiency, resulting in an increased risk of infection (5), almost all of the subjects in this study were tested for complement and immunoglobulin.

Definitions

The Standard definitions of the outcome of INS were in **Table 1** (6). Infection was defined as a suspected or proven infection caused by any pathogen or a clinical syndrome associated with a high probability of infection. Evidence of infection includes positive findings on clinical exam, imaging, or laboratory tests. We referred to the clinical manifestations, biochemical indicators, the definitions of severe infections in adults with INS (7, 8) and related diseases that cause INS infection in children (9–12). Finally, based on the International Guidelines for Management of Severe Sepsis and Septic Shock (2012) (13), the principle of severe infection was when children with INS have signs and symptoms of inflammation with following conditions occur: variations in general signs (at least two), variations in indicators of inflammation (at least one) and at least one of the variations in organ function, tissue perfusion or hemodynamic variations, as detailed in **Table 2**. The prognosis was determined based on the post-treatment conditions of the children, who were classified into good prognosis (survival group) and poor prognosis (mortality group) groups. The good prognosis was defined as symptoms and signs of inflammation are restored, without the manifestations of infection. The poor prognosis was defined as mortality.

Statistical Analysis

Statistical analysis was conducted using the SPSS 22.0 statistical software (IBM Corporation, NY, United States). In the

TABLE 1 | Relevant definitions in nephrotic syndrome.

	Definition
Term	
Relapse	Urine albumin 3+ or 4+ or proteinuria >40 mg/m ^{2} per h or urinary protein: creatinine ratio >2.0 (mg/mg) for 3 consecutive days
SSNS	Complete remission within 4 weeks of prednisone or prednisolone (PDN) at the standard dose (60 mg/m^2/day or 2 mg/kg/day, maximum 60 mg/day)
SRNS	Persistent proteinuria despite 60 mg/m^2 or 2 mg/kg for 8 weeks, after ensuring no infection or non-adherence to medication
SDNS	2 consecutive relapses occurring while weaning to alternate-day steroids or within 2 weeks of steroid discontinuation

SSNS, steroid-sensitive nephrotic syndrome; SRNS, steroid-resistant nephrotic syndrome; SDNS, steroid-dependent nephrotic syndrome.

TABLE 2 | General variables and definitions of severe infection.

General signs
Fever (>38.5°C)
Hypothermia (<35°C)
Heart rate more than two SD above the normal value for age
Tachypnea (Respiratory rate higher than WHO classification for age)
Altered mental status
Inflammatory variables
Leukocytosis (WBC count > 12,000 μL^{-1} or higher than the normal value for age)
Leukopenia (WBC count <4,000 μL^{-1} or below the normal value for age)
Plasma C-reactive protein more than two SD above the normal value
Plasma procalcitonin more than two SD above the normal value
Hemodynamic variables
Arterial hypotension (SBP less than two SD below normal value for age)
Organ dysfunction variables
Arterial hypoxemia (PaO_2/FiO_2 < 300)
Acute oliguria (urine output <0.5 mL kg^{-1} h^{-1} for at least 2 h despite adequate fluid resuscitation)
Coagulation abnormalities
Ileus (absent bowel sounds)
Tissue perfusion variables
Hyperlactatemia (Lactate above upper limits laboratory normal)
Decreased capillary refill or mottling

SD, standard deviation; WBC, white blood cell; SBP, systolic blood pressure; WHO, World Health Organization.

assessment of differences in clinical parameters, categorical data were evaluated using contingency tables and the chi-square test. Quantitative data with a normal distribution were analyzed using an independent sample *t*-test. On the contrary, quantitative data that did not follow a normal distribution were analyzed using the rank-sum test. Normality testing was performed using the one-sample Kolmogorov-Smirnov test. In the binary logistic regression analysis, parameters were entered following the "forward Wald" method. The sensitivity and specificity of each parameter were compared using a univariate receiver operating characteristic (ROC) curve. For significance criteria, values of $P < 0.05$ were considered statistically significant, and values of $P < 0.01$ were considered highly statistically significant.

Study Design

Patients who met the criteria and were complicated by infections and admitted to the Children's Hospital of Chongqing Medical University and the Affiliated Hospital of Guizhou Medical University, from January 2013 to October 2019 were into our research. According to the severity of the infections, the patients were divided into severe infection group (SIG) or Non-severe infection group (Non-SIG) (who didn't match the criteria of severe infection) and matched according to the ratio of 1:2. We retrospectively reviewed the medical data, including baseline characteristics, laboratory testing, pathogens. The flowchart of this study is shown in **Figure 1**.

RESULTS

Baseline Characteristics

In total, 147 children, including 111 (63.8%) boys and 36 (36.2%) girls, suffering from INS with concomitant infections were enrolled in this study. The youngest child was 6 months old, and the oldest was 16.4 years old. The median age was 5.25 years old, and there was no statistically significant difference between the two groups ($P > 0.05$) (**Table 3**). Among the children, the shortest and longest disease durations were 0.03 and 106.13 months, respectively, and the median was 0.67 months. There were 49 children in the SIG and 98 in the Non-SIG. The SIG primarily developed severe pneumonia ($n = 31$, 63.3%), severe sepsis ($n = 15$, 30.6%), septic shock ($n = 2$, 4.1%), and severe chickenpox ($n = 1$, 2.0%); whereas, the Non-SIG primarily developed pneumonia ($n = 81$, 82.7%), urinary tract infection (UTI) ($n = 12$, 12.2%), and upper respiratory tract infection (URTI) ($n = 5$, 5.1%).

Clinical Data Analysis

Through statistical analysis of clinical data, we found that there were many differences between SIG and Non-SIG. The percentage of relapsed cases and SRNS in SIG were higher than those Non- SIG (63.3 vs. 30.6%, 51.0 vs. 10.2%, respectively). The number of steroids (≥0.5 mg/kg) users and immunosuppressant users in SIG was significantly higher than that in the Non-SIG (46.9 vs. 16.3%, 36.7 vs. 4.1%). There were 18 cases using immunosuppressive agents, including 12 cases using tacrolimus, of which 2 cases were combined with mycophenolate mofetil and 1 case was combined with cyclophosphamide. The remaining 4 cases used mycophenolate mofetil alone and 2 cases used cyclophosphamide alone. Steroids and immunosuppressants for SIG were used longer than for Non -SIG. In the auxiliary examination, we also found that there were significant differences between the two groups. The ANC, PCT, PT, and APTT in

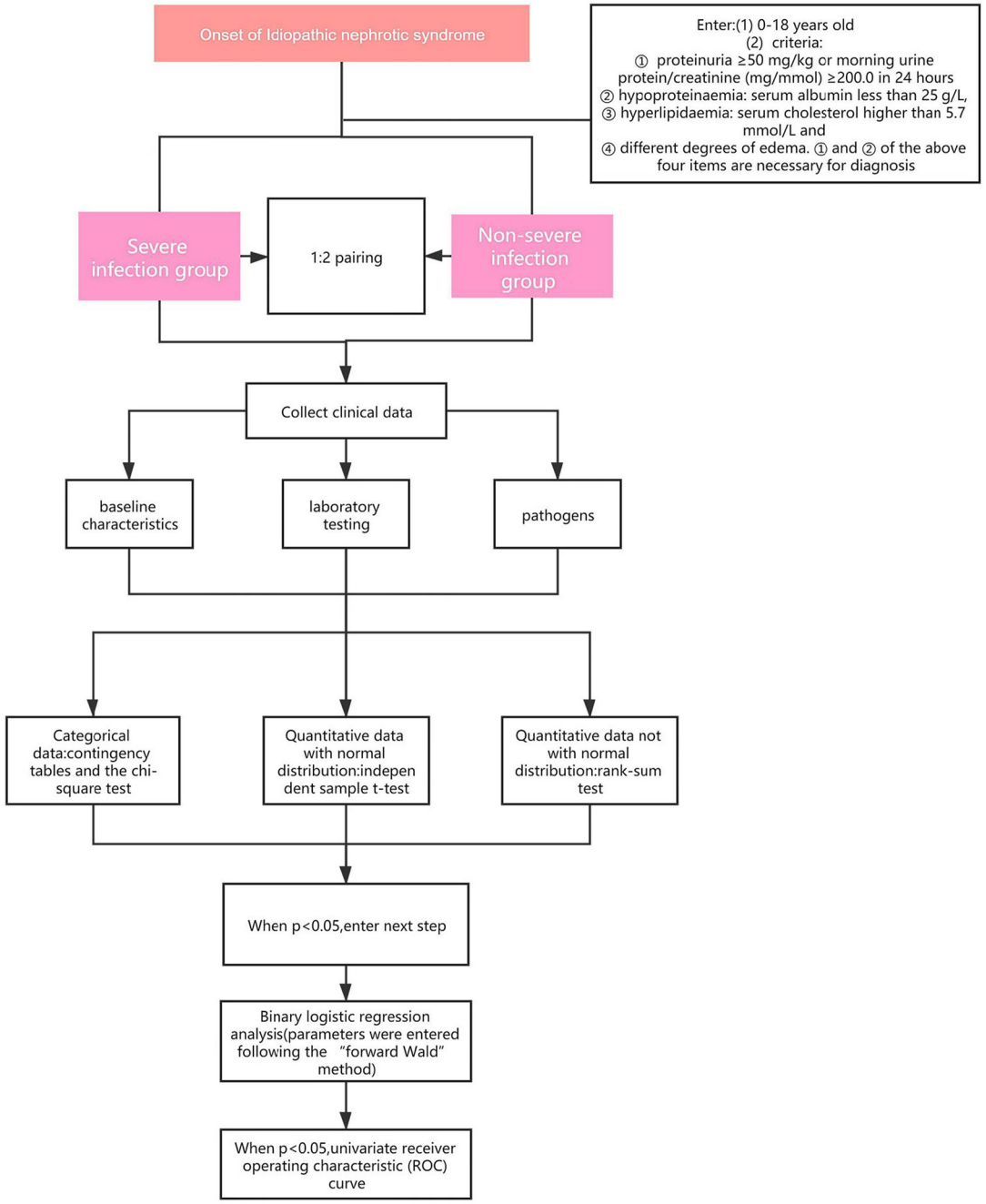

FIGURE 1 | Experimental design process.

the SIG were significantly higher than those in the Non-SIG, while ALC, Tch, LDL, and C3 were significantly lower than those in the Non-SIG. Other indicators were also significantly different, such as WBC, CRP, uric acid, urea nitrogen, and other indicators SIG are higher than Non-SIG. The SIG group's Fib, serum potassium, sodium, etc. were lower than the Non-SIG group (**Table 3**).

Analysis of Pathogens and Drug Resistance in the Non-SIG and SIG

Based on the data from blood, sputum, and urine cultures obtained from 147 children with INS and infections during hospitalization, 30 of them were detected and 46 strains were isolated. The three most common types in the SIG were Pseudomonas aeruginosa (three strains), Staphylococcus aureus

TABLE 3 | Demographic characteristics, laboratory data of children with INS in the severe and non-severe infection group.

	Non-SIG ($N = 98$)		SIG ($N = 49$)		X^2 value	p-value
	Summary	***N***	**Summary**	***N***		
Baseline characteristics						
Male	73 (74.5%)	98	38 (77.6%)	49	0.166	0.684
Female	25 (25.5%)	98	11 (22.4%)	49		
Duration (months)	0.37 (0.23, 1.78)	98	3.00 (0.67, 12.00)	49	$Z = -3.846$	0.000**
Age (years)	5.17 (2.52, 9.38)	98	6.00 (2.67, 10.00)	49	$Z = -0.321$	0.749
Disease conditions						
Initial episode	68 (69.4%)	98	18 (36.7%)	49	14.347	0.000**
Relapse	30 (30.6%)	98	31 (63.3%)	49		
SSNS	82 (83.7%)	98	19 (38.8%)	49	32.434	0.000**
SRNS	10 (10.2%)	98	25 (51.0%)	49		
SDNS	6 (6.1%)	98	5 (10.2%)	49		
Treatments before infection						
Duration of steroid (days)	0 (0, 0)	98	63 (0.362)	49	$Z = -5.135$	0.000**
MP pulse	1 (1.0%)	98	4 (8.2%)	49	3.132	0.077
Steroid dosage per day						
<0.5 mg/kg	82 (83.7%)	98	26 (53.1%)	49		
0.5–1.5 mg/kg	10 (10.2%)	98	15 (30.6%)	49	15.146	0.001**
>1.5 mg/kg	6 (6.1%)	98	8 (16.3%)	49		
IS therapy (*n*)	4 (4.1%)	98	18 (36.7%)	49	27.369	0.000**
Duration of IS (days)	0 (0, 0)	98	0 (0, 11)	49	−5.141	0.000**
Auxiliary examinations						
WBC ($\times 10^9$/L)	10.18 (8.25, 12.13)	98	13.25 (9.79, 17.34)	49	$Z = -3.850$	0.000**
ALC ($\times 10^9$/L)	3.27 (2.23, 4.71)	98	2.46 (1.56, 3.74)	49	$Z = -2.889$	0.004**
ANC ($\times 10^9$/L)	5.46 (3.92, 8.58)	98	9.99 (6.33, 12.33)	49	$Z = -4.442$	0.000**
PLT ($\times 10^9$/L)	390.0 (313.5, 459.5)	98	345.0 (244.0, 484.5)	49	$Z = -1.448$	0.147
Hb (g/L)	137 (125, 149)	98	122 (96, 144)	49	$Z = -3.276$	0.001**
CRP ≥ 8 mg/L (*n*)	1 (1.0%)	98	22 (44.9%)	49	47.651	0.000**
PCT (ng/ml)	0.05 (0.05, 0.10)	85	0.65 (0.10, 11.29)	46	$Z = -5.812$	0.000**
Serum albumin (g/L)	16.7 (14.7, 19.3)	98	17.2 (13.4, 22.7)	49	$Z = -0.072$	0.943
ALT (U/L)	16.0 (10.4, 23.8)	98	25.1 (14.8, 32.1)	49	$Z = -3.493$	0.000**
AST (U/L)	33.3 (24.7, 43.2)	98	31.2 (22.4, 51.3)	49	$Z = -0.557$	0.578
ALP (U/L)	163.6 (122.8, 199.3)	98	101.6 (77.3, 139.3)	49	$Z = -5.519$	0.000**
Serum uric acid (μmol/L)	326.5 (258.5, 374.0)	98	360.0 (234.0, 506.2)	49	$Z = -2.624$	0.009**
Serum urea nitrogen (mmol/L)	4.90 (3.78, 6.93)	98	7.64 (4.21, 12.32)	49	$Z = -2.750$	0.006**
Serum creatinine (μmol/L)	33.50 (25.68, 50.50)	98	45.90 (29.35, 116.25)	49	$Z = -1.354$	0.176
Serum potassium (mmol/L)	4.50 ± 0.71	98	4.04 ± 0.72	49	$t = 3.670$	0.000**
Serum sodium (mmol/L)	136.30 (132.55, 138.75)	98	132.20 (128.95, 137.10)	49	$Z = -3.048$	0.002**
Serum chlorine (mmol/L)	103.85 (100.20, 106.83)	98	101.50 (96.50, 105.05)	49	$Z = -2.554$	0.011*
Serum calcium (mmol/L)	1.94 (1.82, 2.05)	98	1.87 (1.73, 2.09)	49	$Z = -1.459$	0.145
Tch (mmol/L)	10.76 ± 3.10	98	8.86 ± 3.53	49	$t = 3.337$	0.001**
Triglyceride (mmol/L)	2.98 (2.01, 4.36)	98	3.14 (2.43, 4.17)	49	$Z = -0.489$	0.625
LDL (mmol/L)	7.86 ± 2.96	98	5.66 ± 3.23	49	$t = 4.112$	0.000**
HDL (mmol/L)	1.71 (1.23, 2.21)	98	1.50 (0.81, 2.16)	49	$Z = -1.724$	0.085
IgG (g/L)	2.24 (1.36, 3.02)	97	1.88 (0.83, 4.29)	48	$Z = -0.534$	0.594
IgA (g/L)	0.85 (0.63, 1.35)	97	0.89 (0.62, 1.30)	48	$Z = -0.089$	0.929
IgM (g/L)	1.76 (1.31, 2.40)	97	1.49 (1.09, 2.22)	48	$Z = -1.589$	0.112
IgE (g/L)	176.0 (46.2, 458.5)	97	76.3 (23.8, 372.0)	48	$Z = -1.790$	0.073

(Continued)

TABLE 3 | Continued

	Non-SIG ($N = 98$)		SIG ($N = 49$)		X^2 value	p-value
	Summary	N	Summary	N		
C3 (g/L)	0.99 ± 0.21	97	0.85 ± 0.30	48	$t = 2.947$	0.004**
C4 (g/L)	0.24 (0.19, 0.30)	97	0.23 (0.17, 0.27)	48	$Z = -0.854$	0.393
PT (s)	10.4 (9.7, 11.1)	87	11.2 (10.2, 13.3)	48	$Z = -3.151$	0.002**
APTT (s)	31.00 (26.90, 35.70)	87	36.75 (28.15, 53.23)	48	$Z = -2.882$	0.004**
Fib (g/L)	5.97 ± 1.70	87	5.31 ± 2.02	48	$t = 2.023$	0.045*
D-dimer	1.26 (0.58, 2.31)	87	1.83 (0.69, 5.80)	48	$Z = -1.836$	0.066

*$p < 0.05$, **$p < 0.01$. Continuous parameters were expressed as ($\bar{X} \pm s$) or P50 (P25, P75), and discrete parameters were expressed as a percentage (%). SIG, severe infection group; MP, methylprednisolone; IS, immunosuppressive; WBC, white blood cells; ALC, absolute lymphocyte count; ANC, absolute neutrophil count; PLT, platelets count; Hb, hemoglobin; CRP, C-reactive protein; PCT, procalcitonin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; Tch, total cholesterol; LDL, low-density lipoprotein; HDL, high-density lipoprotein; PT, prothrombin time; APTT, activated partial thromboplastin time; Fib, fibrinogen.*

(SA) (three strains), and Staphylococcus epidermidis (three strains). On the contrary, the three most common types in the Non-SIG were Haemophilus influenzae (three strains), Streptococcus pneumonia (SP) (three strains), and Enterococcus faecium (three strains). The detection rate of Gram-positive bacteria (GPB) was higher in the SIG than in the Non-SIG (**Table 4**). The pathogen profiles indicated that the SIG was more likely to be infected with GPB, respiratory syncytial virus (RSV) and influenza viruses (FLu), and fungi than the Non-SIG ($P < 0.05$). In total, 10 drug-resistant strains were detected in the two groups. The incidence of Methicillin-resistant strains was significantly higher in the SIG ($n = 4$) than in the Non-SIG ($n = 0$) ($P = 0.000$).

Risk Factor Analysis for Severe Infections in INS

To identify the risk factors of severe infections in children with INS, the aforementioned clinical parameters that were statistically different between the two groups were entered into the binary logistic regression analysis model. The results obtained using the "forward Wald" method were shown in **Table 5**. Binary logistic regression analysis showed that the combined use of immunosuppressants (OR: 26.969, 95% CI: 1.569–463.541), CRP ≥8 mg/L (OR: 6,468.029, 95% CI: 43.581–959,935.668), steroid resistance (OR: 100.191, 95% CI: 4.845–2,071.880), infection with gram-positive cocci (OR: 7,580.545, 95% CI: 27.126–2,118,452.938), infection with influenza virus (OR: 69.426, 95% CI: 2.494–1,932.221), and infection with respiratory syncytial virus (OR: 353.681, 95% CI: 5.011–24,963.819) were independent risk factors for severe infections in children with INS. ALC (increase $> 1.5 \times 10^9$/L) (OR: 0.356, 95% CI: 0.188–0.675) and complement C3 (increase > 0.55 g/L) (OR: 0.000, 95% CI: 0.000–0.276) were protective factors.

Through the ROC curve model analysis, it was found that when children with nephropathy were co-infected, the risk of infection progression often needed to consider the combined effect of multiple influencing factors at the same time. The sensitivity and specificity reflected by the area under the ROC curve (AUC) showed that when infection occurred in children with INS, when one or more factors including steroid resistance, IS treatment and increased CRP were combined, a more severe infection was very likely to occur (**Figures 2A,B**).

Analysis of Prognosis of INS Patients With Concomitant Severe Infections

In the SIG, 39 patients survived, five died, and five gave up treatment. Five patients who gave up treatment were excluded because of incomplete data and unclear prognosis. The causes of death included multiple organ failure syndromes (2 cases), brain herniation (1 case), and circulatory failure (2 cases). The patients were classified into the survival group ($N = 39$) and the mortality group ($N = 5$). The statistically significant parameters between two groups by univariate analysis entered into the binary logistic regression analysis model. There was no significant difference in baseline characteristics, treatment, and disease condition between the two groups. However, in the auxiliary examination, WBC, Serum chlorine, and ANC in the mortality group were significantly higher than those in the survival group, and ALT, AST, serum calcium, IgG, IgA, and C3 were significantly lower than those in the survival group (**Table 6**). Binary logistic regression analysis showed that WBC (increased $> 15 \times 10^9$/L) (OR: 1.725, 95% CI: 1.046–2.844) is an independent risk factor for the poor prognosis of INS children with severe infections, often accompanied by an increase in the ANC($>10 \times 10^9$/L), whereas serum calcium level (increase > 1.30 mmol/L) (OR: 0.000, 95% CI: 0.000–0.698) was a protective factor. The area under the ROC curve shows that serum calcium and WBC are highly specific and sensitive in predicting the risk of poor prognosis (**Figures 2C,D**).

DISCUSSION

Infection is one of the main complications of childhood idiopathic nephrotic syndrome, which can lead to frequent relapses of INS, treatment failures, and even death (14). Children with INS are more likely to have a poor prognosis after infection due to the primary disease and therapeutic drugs. At present, most studies on children's INS focus on the analysis of risk factors for infection, and there is a lack of large-scale reports on the risk factors and outcome factors

TABLE 4 | Pathogen spectrum and the analysis in the severe and non-severe infection group.

Pathogens	Non-severe infection group	Severe infection group	Total (*N*, %)	X^2 value	*p*-value
Gram-negative bacteria	12	14	26 (56.52)	2.577	0.108
Morganella	1	2	3 (6.52)		
Escherichia coli	3	1	4 (8.70)		
Acinetobacter baumannii	1	2	3 (6.52)		
Enterobacter cloacae	0	1	1 (2.17)		
Klebsiella pneumonia	0	2	2 (4.35)		
Pseudomonas aeruginosa	1	3	4 (8.70)		
Haemophilus influenzae	3	2	5 (10.87)		
Haemophilus parainfluenzae	2	0	2 (4.35)		
Elizabethkingia	0	1	1 (2.17)		
Pantoea agglomerans	1	0	1 (2.17)		
Gram-positive bacteria	7	13	20 (43.48)	10.256	0.001
Staphylococcus aureus	1	3	4 (8.70)		
Staphylococcus epidermidis	0	3	3 (6.52)		
Staphylococcus hominis	0	2	2 (4.35)		
Staphylococcus haemolyticus	0	1	1 (2.17)		
Streptococcus pneumoniae	3	2	5 (10.87)		
Enterococcus faecium	3	2	5 (10.87)		
Fungus	0	4	4	5.429	0.020
Virus	18	25	43		
Cytomegalovirus	8	5	13 (30.23)	0.011	0.918
Epstein-Barr virus	2	2	4 (9.30)	0.032	0.858
Adenovirus	1	0	1 (2.33)	0.000	1.000
Influenza virus	3	7	10 (23.26)	4.842	0.028
Parainfluenza virus	3	3	6 (13.95)	0.195	0.658
Respiratory syncytial virus	1	8	9 (20.93)	10.785	0.001
Mycoplasma pneumoniae	7	1	8	0.8100	0.368

TABLE 5 | Binary logistic regression analysis of risk factors of severe infections in children with INS.

	Coefficient (B)	Wald value	*p*	OR	95% Confidence interval for EXP (B)	
					Lower limit	Upper limit
Steroid Combined with IS	3.295	5.155	0.023	26.969	1.569	463.541
SSNS		9.375	0.009			
SRNS	4.607	8.886	0.003	100.191	4.845	2,071.880
SDNS	1.979	1.208	0.272	7.233	0.212	246.345
Increased ALC	−1.033	10.025	0.002	0.356	0.188	0.675
CRP ≥ 8 mg/L	8.775	11.831	0.001	6,468.029	43.581	959,935.668
Increased C3	−8.205	5.405	0.020	0.000	0.000	0.276
Infection with gram-positive bacteria	8.933	9.662	0.002	7,580.545	27.126	2,118,452.938
Infection with influenza virus	4.240	6.243	0.012	69.426	2.494	1,932.221
Infection with RSV	5.868	7.301	0.007	353.681	5.011	24,963.819
Constant	3.318	1.269	0.260	27.612		

IS, Immunosuppressant; ALC, absolute lymphocyte count; RSV, respiratory syncytial virus.

for severe infection. Referring to the definition of severe infection in adult INS and the indicators of severe infection in children (7, 8), we defined the manifestations of severe infection in children with INS and analyzed the relevant risk factors.

In this study, it was observed that pneumonia and urinary tract infection were dominant in Non-severely infective INS children, while severe pneumonia, sepsis was dominant in severely infective INS children. In Taiwan (15), pneumonia is the main type of infection in children with INS, which is consistent

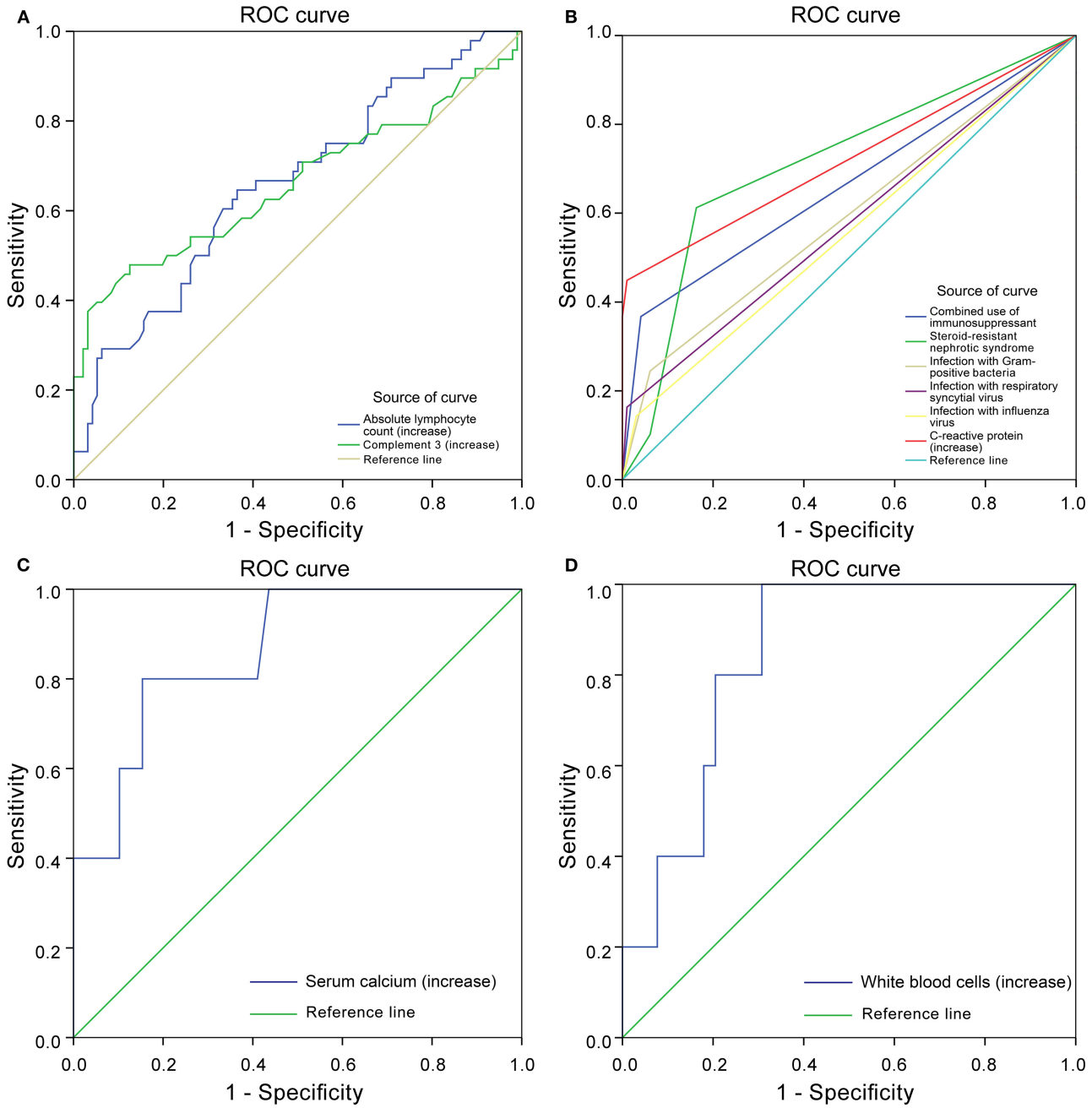

FIGURE 2 | Receiver operating characteristics (ROC) curve. Protective factors of severe infection **(A)**. Risk factors of severe infection **(B)**. Protective factors for poor prognosis of severe infection **(C)**. Risk factors for poor prognosis of severe infection **(D)**.

with the Non-SIG in our study, while in India, Saudi Arabia, and other regions (2, 16, 17), the upper respiratory tract infection is the main type, but these studies did not grade the infection. Although in Israel (18), the infections were mainly pneumonia and bacteremia/sepsis, but they defined the infection as a serious bacterial infection, which has limitations. From our research, it is found that both severe and Non-severe infections are mainly respiratory infections. Therefore, our treatment should be more active when pneumonia is complicated. We found that the pathogen spectrum of the two groups is significantly different, and GPB are an independent risk factor for severe infections. In SIG, the respiratory pathogens are mainly SA, while Non-SIG is mainly composed of SP and Haemophilus influenzae, and it is more common for SIG strains to have drug resistance. Therefore, when patients are infected with methicillin-resistant SA or other methicillin-resistant GPB, they are more likely to

TABLE 6 | Univariate analysis of risk factors for poor prognosis of SIG in children with INS.

	Survival group ($N = 39$)		Mortality group ($N = 5$)		X^2 value	*p*-value
	Summary	*N*	Summary	*N*		
WBC ($\times 10^9$/L)	12.75 (8.98, 15.99)	39	21.20 (15.78, 30.96)	5	$Z = -2.496$	0.013*
PLT ($\times 10^9$/L)	366 (298, 499)	39	155 (91, 461)	5	$Z = -1.498$	0.134
Hb (g/L)	126 (106, 145)	39	80 (68, 125)	5	$Z = -1.998$	0.046
ALC ($\times 10^9$/L)	2.36 (1.57, 3.43)	39	2.47 (1.38, 4.43)	5	$Z = -0.296$	0.767
ANC ($\times 10^9$/L)	9.27 (5.18, 11.39)	39	12.21 (10.84, 20.15)	5	$Z = -2.034$	0.042 *
CRP $\geq$ 8 mg/L (*n*)	17 (43.6%)	39	4 (80.0%)	5		0.176$^{\Delta}$
PCT (ng/ml)	0.43 (0.10, 8.65)	39	5.52 (0.62, 45.64)	5	$Z = -1.423$	0.155
Serum albumin (g/L)	17.06 (14.30, 23.80)	39	12.60 (10.20, 15.35)	5	$Z = -1.029$	0.303
ALT (U/L)	25.10 (15.10, 28.50)	39	13.40 (9.75, 67.75)	5	$Z = -2.663$	0.008**
AST (U/L)	31.20 (25.40, 46.50)	39	22.10 (18.65, 242.05)	5	$Z = -2.422$	0.015*
ALP (U/L)	102.60 (81.20, 137.50)	39	78.00 (63.05, 164.50)	5	$Z = -1.091$	0.275
Serum uric acid (μmol/L)	346 (224, 485)	39	360 (271, 449)	5	$Z = -0.185$	0.853
Serum urea nitrogen (mmol/L)	6.33 (3.70, 11.70)	39	8.20 (5.67, 28.08)	5	$Z = -1.183$	0.237
Serum creatinine (μmol/L)	40.60 (27.00, 74.50)	39	82.00 (37.00, 284.75)	5	$Z = -1.738$	0.082
Serum potassium (mmol/L)	4.11 ± 0.68	39	3.61 ± 1.04	5	$t = 1.440$	0.157
Serum sodium (mmol/L)	131.5 (129.2, 136.7)	39	132.4 (122.0, 141.0)	5	$Z = -0.129$	0.897
Serum chlorine (mmol/L)	101.9 (96.0, 104.1)	39	109.6 (101.1, 114.6)	5	$Z = -2.238$	0.025 *
Serum calcium (mmol/L)	1.91 (1.75, 2.09)	39	1.67 (1.29, 1.78)	5	$Z = -2.626$	0.009**
Tch (mmol/L)	9.24 ± 3.62	39	8.36 ± 2.69	5	$t = 0.519$	0.607
Triglyceride (mmol/L)	3.10 (2.38, 3.86)	39	3.73 (3.05, 10.27)	5	$Z = -1.110$	0.267
LDL (mmol/L)	5.89 ± 3.30	39	5.05 ± 3.10	5	$t = 0.541$	0.591
HDL (mmol/L)	1.53 (0.94, 2.04)	39	0.81 (0.56, 1.33)	5	$Z = -1.923$	0.054
IgG (g/L)	2.06 (0.96, 4.02)	39	0.66 (0.51, 0.86)	5	$Z = -2.644$	0.008**
IgA (g/L)	0.91 (0.66, 1.31)	39	0.48 (0.40, 0.62)	5	$Z = -2.570$	0.010*
IgM (g/L)	1.48 (1.08, 2.51)	39	1.74 (0.82, 1.85)	5	$Z = -0.481$	0.631
IgE (g/L)	81.30 (21.30, 410.00)	39	32.20 (27.66, 187.95)	5	$Z = -0.795$	0.427
C3 (g/L)	0.88 ± 0.30	39	0.63 ± 0.09	5	$t = 3.940$	0.001**
C4 (g/L)	0.22 (0.16, 0.27)	39	0.25 (0.23, 0.26)	5	$Z = -0.981$	0.326
PT (s)	11.05 (10.18, 12.83)	39	11.20 (10.00, 13.20)	5	$Z = -0.114$	0.909
APTT (s)	36.30 (26.78, 53.83)	39	37.50 (32.40, 44.20)	5	$Z = -0.265$	0.791
Fib (g/L)	5.56 ± 1.97	39	5.22 ± 1.99	5	$t = 0.369$	0.714
D-dimer	1.44 (0.55, 3.20)	39	3.10 (0.86, 32.25)	5	$Z = -1.137$	0.256

*$p < 0.05$, **$p < 0.01$. $^{\Delta}$*Fisher exact method. Continuous parameters were expressed as ($\bar{X} \pm s$) or P50 (P25, P75), and discrete parameters were expressed as a percentage (%). SIG, severe infection group; WBC, white blood cells; ALC, absolute lymphocyte count; ANC, absolute neutrophil count; Hb, hemoglobin; PLT, platelets count; CRP, C-reactive protein; PCT, procalcitonin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; Tch, total cholesterol; LDL, low-density lipoprotein; HDL, high-density lipoprotein; PT, prothrombin time; APTT, activated partial thromboplastin time; Fib, fibrinogen.*

cause serious infections. So, children with INS must vaccinate pneumococcal vaccine. Previous studies have confirmed that the use of high cumulative doses of steroids and IS agents (8, 17, 19–23) all increase the risk of INS infection. Besides, we found that children with SRNS or relapse are more susceptible to severe infection, and the risk of severe infection is higher when treated with steroids combined with immunosuppressive agents such as tacrolimus or mycophenolate mofetil. From the auxiliary inspection point of view, SIG has higher CRP and PCT, but their Tch and LDL are lower. The Tch and LDL of the two groups were higher than the normal range, but studies have shown that inflammation can cause a decrease in Tch (24), so the LDL of SIG is lower than that of Non-SIG. When the Tch and LDL of infected children with INS decrease, we need to be vigilant. SIG's PT and APTT are also higher, suggesting that when there is a problem with the coagulation function of Non-SIG, we need to be alert to the progress of the infection, and even cause diffuse intravascular coagulation. C3 (<0.55 g/L) and ALC ($<1.5 \times 10^9$/L) are predictors of severe infections, which had not been previously reported. We believe that this is because the use of high-dose steroids and IS agents and INS itself will cause ALC and C3 to decrease. Co-infection will further reduce the ALC and C3 (25–27). Therefore, When C3 $\leq$ 0.55 g/L or ALC $\leq 1.5 \times$

10^9/L, the infection should be timely and controlled to avoid further aggravation.

Regarding prognosis, we found that children in the mortality group had lower immunity. The serum calcium of the mortality group was significantly reduced, which is one of the factors of poor prognosis. In INS, hypoalbuminemia is often accompanied by a decrease in serum calcium. The cause of hypocalcemia may not only be caused by INS itself, but also infection. Serum calcium lower than 1.30 mmol/L may cause death. WBC ($>15 \times 10^9$/L) was the only independent risk factor for poor prognosis, no similar results have been reported in children with INS, whereas reports involving adults with INS have shown that hypotension and decreased platelet count were risk factors for a poor prognosis (14). Although decreased ALC is a risk factor for severe infection, however, due to an increase in ANC, the resulting increase in white blood cells became an early warning indicator of fatal severe infection, especially the increase in WBC increased ($>15 \times 10^9$/L).

Several limitations of this study should be mentioned. First, although we have found some risk factors of severe infection in children with INS, as a retrospective study, it might lead to the bias in data collection and uniform availability of information from the medical records. Second, the age span of children in this study was large, and the results could not well-reflect the clinical characteristics of children with INS complicated with severe infection in each age period. We tried to divide the children into infant, childhood and adolescence group based on tanner staging. However, due to the relatively small sample size, it was hard to perform statistical analysis. In the future, we will expand the sample size and design a prospective study based on the results of this study, so as to avoid poor prognosis of children with INS caused by severe infection, and to find more risk factors of severe infection in children with INS in different age period.

In summary, our double-center retrospective study showed that resistance to steroids, combined use of steroids and IS agents, and GPB infections (especially SA) are high risk factors for severe infection. We should monitor CRP $\geq$ 8 mg/L, C3 $\leq$ 0.55 g/L and ALC $\leq 1.5 \times 10^9$/L to avoid developing severe infection. The WBC of severely infected patients increases significantly, especially when ANC increases, which can lead to fatal infections.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Children's Hospital of Chongqing Medical University (reference number 2020-12, 2020-03-24). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

MW and XZ designed this study. HZ and SQ wrote the draft. CZ, LS, and TZ has participated in data collection and case follow-up. JL designed the statistical analysis of the study. All authors contributed to the article and approved the submitted version.

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SYSTEMATIC REVIEW
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Systematic Review and Meta-Analysis of Rituximab for Steroid-Dependent or Frequently Relapsing Nephrotic Syndrome in Children

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Objective: To explore the effectiveness and safety of rituximab (RTX) for steroid-dependent or frequently relapsing nephrotic syndrome *via* a systematic review and meta-analysis.

Methods: All the literature about RTX therapy for childhood nephrotic syndrome (NS) on PubMed, Web of Science, Cochrane Library, EMBASE, and Chinese biomedical literature database published before November 1, 2019, were conducted and selected according to the preset criteria. The Cochrane bias risk assessment tool was used to evaluate the quality of the literature included. The outcome data were analyzed by RevMan 5.3 software.

Results: There were six RCT studies that met the inclusion criteria with a moderate quality after evaluation. At the end of the treatment, the relapse rate of NS in the RTX group reduced significantly when compared with that in the control group [odds ratio (OR) = 0.11, 95% confidence interval (CI) (0.03, 0.43), $p = 0.001$]. The number of patients in the RTX group used less steroid or/and calcineurin inhibitors significantly than that in the control group [OR = 0.05, 95% CI (0.01, 0.28), $p = 0.0007$]. For children who were steroid-dependent, RTX treatment significantly reduced the dosage of the steroid, compared with that in control [standardized mean difference (SMD) = −1.49, 95% CI (−2.00, −0.99), $p < 0.00001$]. There was no significant reduction in protein excretion between the two groups [SMD = −0.33, 95% CI (−0.71, 0.04), $p = 0.08$]. Fewer serious adverse reactions of RTX in the six studies were reported and most adverse events were mild.

Conclusion: RTX is effective and safe for children with steroid-dependent or frequently relapsing nephrotic syndrome.

Systematic Review Registration: Identifier: CRD 42020150933. https://www.crd.york.ac.uk/prospero/. This review has been registered to the PROSPERO on 27 Feb 2020.

Keywords: rituximab, meta-analysis, systematic review, effect and safety, nephrotic syndrome

INTRODUCTION

The incidence of idiopathic nephrotic syndrome (NS) is 2–4 per 100,000 children (1). Prednisone is the core treatment for NS. There are still some patients who relapse when the steroid was withdrawn or tapering. According to their recurrence rate, these patients were classified as steroid-dependent nephrotic syndrome (SDNS) and frequently relapsing nephrotic syndrome (FRNS) (2, 3). The long-term use of steroids in children could result in serious adverse effects such as growth retardation, obesity, osteopenia, hypertension, and cataract (4). The SDNS or FRNS required a combined therapy of steroid and immunosuppressive agents, such as cyclophosphamide (5), cyclosporine A (CSA) (6), tacrolimus (TAC) (7), mycophenolate esters (8), and vincristine (VCR) (9). These immune suppressants brought the patients a higher remission rate, together with even more serious adverse reactions, such as renal toxicity, hyperglycemia, headache, and dyslipidemia (10).

Rituximab (RTX), a monoclonal antibody targeting the CD20 antigen of B lymphocytes, was first proposed to lymphoma (11) and rheumatoid arthritis (12). Recently, RTX was introduced to the relapsing NS (13–15) and brought the patients a long-lasting remission. Other findings suggested that RTX could also bring better benefits to children. In Guigonis's clinical trials (16), 22 patients were given RTX treatment. Nineteen children received remission. Additionally, there were some other case reports that showed that RTX induced remission in patients (17–20), whereas some studies reported no benefits of RTX to patients that were resistant to steroids and CNIs. Kari et al. (21) observed that in four children with steroid-resistant NS, after RTX protocol, only one child received a short remission and then relapsed, and the other three children showed little effects.

We aimed to evaluate the efficacy of RTX for SDNS or FRNS in children compared to the conventional treatment by meta-analysis and to summarize the adverse effects to evaluate the safety. The research was introduced as follows.

METHODS

Electronic Searches

A unified search strategy was adopted for the selected five databases to ensure consistency. The medical subject heading of the disease was restricted to Nephrotic Syndrome, the text words were Nephrotic Syndromes; Syndrome, Nephrotic; and Syndromes, Nephrotic; the medical subject heading of the intervention was RTX, and the text words were CD20 Antibody, Rituximab; Rituximab CD20 Antibody; Mabthera; IDEC-C2B8 Antibody IDEC C2B8 Antibody; IDEC-C2B8; IDEC C2B8; GP2013; and Rituxan. The subject heading of study type was Randomized Controlled Trial, and the text words were RCT; Random. Boolean operations were used to connect these subject words and text words to form search formulas. Two researchers checked the search results multiple times after independent searches and saved the search results in EndNote software.

Study Selection Criteria

The study type was restricted to the randomized controlled trial. We selected children with SDNS or FRNS who were younger than 18 years old. Two groups of patients were studied; one received any dose of RTX. The other was considered as the control, receiving conventional drugs, such as steroid and TAC/CSA. Since the use of RTX is only intended for non-genetic nephrotic syndrome, genetic nephrotic syndrome is not included in this study.

The main outcome was the relapse number. The secondary outcomes were the number of using steroids or/and calcineurin inhibitors, the dose of steroids, and the degree of proteinuria.

Literature Bias Evaluation

The risk of bias was evaluated by the Cochrane bias risk assessment tool of RevMan software. The risk of bias included selective bias, performance bias, detection bias, attrition bias, reporting bias, and other biases.

Data Extraction

Two researchers extracted relevant data to a pre-designed form independently. The disputed data were discussed with a third researcher. The extracted information included the following sections: (1) literature basic information, (2) baseline data, (3) interventions, and (4) outcomes. The extracted data were mostly represented by the mean and standard deviation (SD).

Data Analysis

The data analysis was performed by RevMan (version 5.3.5). For dichotomous variables, the odds ratio (OR) and 95% confidence interval (CI) were calculated. Continuous variables were analyzed by the standardized mean difference (SMD) and 95% CI. The Q-test and I^2 analysis were used to test the heterogeneity of the literature. When $p < 0.10$ in the Q-test or $I^2 > 50\%$ in I^2 analysis, the random-effect model was used; otherwise, the fixed-effect model was used. We then attempted to determine the source of heterogeneity, and subgroup analysis was continued if possible. $p < 0.05$ denotes that the difference was considered significant.

RESULTS

A total of 347 eligible articles were retrieved, namely, 42 from PubMed, 103 from Web of Science, 67 from the Cochrane Library, 103 from EMBASE, and 32 from the Chinese Biomedical Literature Database. A total of 146 duplicate articles were eliminated by Endnote software. After selecting the title and abstract, 190 articles were excluded. The remaining 11 articles were selected for further reading. Among them, studies from Webb et al. (22), Deble-Bertin et al. (23), and Sinha et al. (24) were excluded because these were cohort studies. Research from Basu et al. (25) was excluded due to the addition of TAC in the control group. The study of Kamei et al. (26) was a long-term follow-up observational study, which was excluded due to the lack of a control group. Finally, six articles (27–32) were included in our study, and the screening flow chart is shown in **Figure 1**. The general data of the study design is shown in **Table 1**, and the baseline data of the study patients are shown in **Table 2**.

After repeatedly reading the six articles, the Cochrane Bias Risk Assessment Tool was used to evaluate the RevMan software. The results of the included literature bias assessment are shown in **Figure 2**.

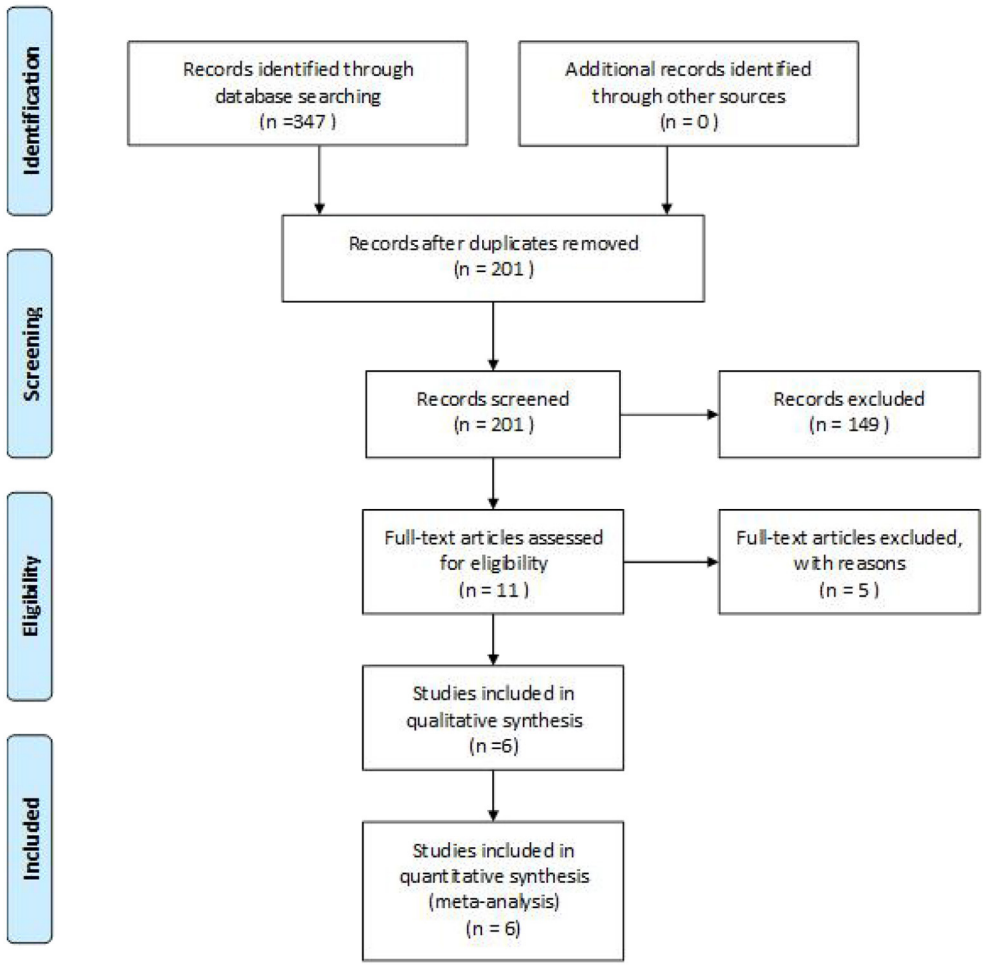

FIGURE 1 | The search results and selection details.

The Relapse Numbers

In this study, six studies reported the relapse number of NS in the RTX group and the control after the corresponding treatment. A total of 46 patients of 124 in the RTX group and 86 patients of 110 in the control relapsed, separately. RTX could significantly reduce the NS relapse during the observation period [OR 0.11, 95% CI (0.03, 0.43), $p = 0.001$]. The random-effect model was selected due to the large heterogeneity in this analysis ($I^2 = 66\%$, $p = 0.01$), and the results are shown in **Figure 3**.

The Number of Patients Using Steroids or/and CNI

In this study, there were two studies documenting the number of patients using steroids or/and CNI after the corresponding treatment. Twenty patients of 62 in the RTX group discontinued steroids or/and CNI and achieved remission. Only 1 patient of 43 in the control withdrew steroids or/and CNI. Thus, we concluded that RTX holds great potential of reducing steroids or/and CNI treatment [OR 0.05, 95% CI (0.01, 0.28), $p = 0.0007$]. The fixed-effect model was used according to the heterogeneity, which was acceptable ($I^2 = 14\%$, $p = 0.28$); the results are shown in **Figure 4**.

Steroid Dosage

Three studies recorded the steroid dosage after the corresponding treatment. Among them, the research from Ahn et al. (27) was excluded because of the large heterogeneity. The two remaining articles were included for further analyses and a total of 78 patients were studied, 39 in the RTX group and 39 in the control. The SMD was used for analysis due to the different units of steroids in the two research, which showed that the steroid dosage was significantly reduced after RTX treatment [SMD −1.49, 95% CI (−2.00, −0.99), $p < 0.00001$]. There was no obvious heterogeneity ($I^2 = 0\%$, $p = 0.77$) and the fixed-effect model was used; the results are shown in **Figure 5**.

Proteinuria Excretion

Three articles recorded urea protein excretion in 115 patients, of whom 58 were in the RTX group and 57 were in the

TABLE 1 | General information of the included studies.

References	Country	Histology RTX group	Histology con group	Treatment RTX group	Treatment con group	Follow-up
Ahn et al. (27)	Korea	MCD: 23 FSGS: 2 Unknown: 10	MCD: 8 FSGS: 1 Unknown: 7	RTX: a single dose of intravenous RTX (375 mg/m^2; maximum of 500 mg); PED; CNI	PED (60 mg/m^2/day, tapered by 25% every 4 weeks); CNI	12 M
Boumediene et al. (29)	France	MCD: 9 Unknown: 1	MCD: 11 FSGS: 1 Unknown: 1	RTX: two infusions at 1-week interval of Rituximab (375 mg/m^2); MMF: CNI; PED	PED (decreased every 2 weeks by 25%); MMF; CNI	6 M
Iijima et al. (28)	Japan	MCD: 21 FSGS: 2 Unknown: 1	MCD: 23 FSGS: 1	RTX: once weekly for 4 weeks (375 mg/m^2; maximum of 500 mg); MP; chlorpheniramine maleate; paracetamol	PED (60 mg/m^2/day, maximum of 80 mg/day, decreased every 2 weeks by 50%)	12 M
Magnasco et al. (30)	Italy	MCD: 3 FSGS: 10 Unknown: 2	MCD: 4 FSGS: 9 Unknown: 3	RTX: 375 mg/m^2, given intravenously twice (at randomization and after 2 weeks); PED; MP; CNI; chlorpheniramine maleate; paracetamol; ARB; ACEI	PED (median 0.42 mg/kg/day, after 30 days decreased every week by 0.3 mg/kg); CNI; ARB; ACEI	18 M
Ravani et al. (31)	Italy	MCD: 13 FSGS: 7 Unknown: 7	MCD: 6 FSGS: 10 Unknown: 116	Rituximab (375 mg/m^2) was given intravenously once or twice; ARB; MP; PED; CNI; ACEI; chlorpheniramine maleate; paracetamol	PED (tapered off by 0.3 mg/kg per week if proteinuria was <1 g/day); CNI; ACEI; ARB	12 M
Ravani et al. (32)	Italy	Unknown: 15	Unknown: 15	Rituximab (MabThera/RITUXAN;375 mg/m^2); PED; MP; chlorpheniramine maleate; paracetamol; ACEI; ARB; CNI; CYC	PED (tapered off by 0.3 mg/kg per week starting at 30 days); ACEI; ARB; CNI; CYC	12 M

MCD, minimal-change disease; FSGS, focal and segmental glomerular sclerosis; PED, prednisone; CNI, calcineurin inhibitors (cyclosporine or tacrolimus); MMF, mycophenolate mofetil; MP, methyl prednisone; ARB, angiotensin receptor inhibitor; ACEI, angiotensin-converting enzyme inhibitor; CYC, cyclophosphamide.

control group. Because the units of proteinuria were not uniform, the SMD was used for analysis. Compared with that in the control group, the proteinuria excretion in both RTX and control decreased, and the difference was not statistically significant [SMD −0.33, 95% CI (−0.71, 0.04), $p = 0.08$], as shown in the forest plot (**Figure 6**).

Safety

Adverse events, such as late-onset neutropenia, hypogammaglobulinemia, and increased risk of infections, have been rarely reported. Slight adverse reactions and infusion reactions were found in all included studies, which could be alleviated by reducing the speed of drug infusion or providing supportive treatment.

Publication Bias

In the main outcome of the relapse number, we used Stata software to evaluate publication bias. The results were as follows: $p = 0.060$ in the Begg test, $p = 0.022$ in the Egger test.

DISCUSSION

We included six randomized controlled trials. A total of 234 children were included and randomly assigned to the RTX group

TABLE 2 | Basic characteristics of included studies.

	Group	Ahn et al. (27)	Boumediene et al. (29)	Iijima et al. (28)	Magnasco et al. (30)	Ravani et al. (31)	Ravani et al. (32)
Sex (M/F)	RTX group	26/9	10/0	18/6	10/6	24/3	10/5
	Con group	13/3	6/7	16/8	9/6	19/8	11/4
Age (years)	RTX group	13.5 ± 5.0	11.1 ± 1.2	11.5 ± 5.0	8.5 ± 4.4	10.2 ± 4.0	6.9 ± 3.6
	Con group	12.5 ± 4.2	12.3 ± 1.0	13.6 ± 6.9	7.3 ± 3.7	11.3 ± 4.3	6.9 ± 3.1
Weight (kg)	RTX group	-	-	44.0 ± 18.6	31 ± 17	39.6 ± 15.2	30.0 ± 16
	Con group	-	-	47.5 ± 15.6	30 ± 17	45.5 ± 19.3	31.0 ± 41.0
Duration of NS (years)	RTX group	8.7 ± 4.9	-	7.9 ± 4.7	2.5 ± 8.4	5.7 ± 3.5	2.7 ± 2.4
	Con group	7.4 ± 4.9	-	8 ± 5.4	1.4 ± 5.6	7.8 ± 4.0	2.0 ± 2.5
Serum creatinine (μmol/L)	RTX group	-	-	39.8 ± 13.3	46.9 ± 30.9	48.6 ± 26.5	35.4 ± 17.7
	Con group	-	-	44.2 ± 15.9	48.6 ± 36.2	48.6 ± 17.7	36.2 ± 8.8
Serum albumin (g/dl)	RTX group	-	-	3.4 ± 0.6	2.4 ± 0.6	3.6 ± 0.9	3.8 ± 0.3
	Con group	-	-	3.4 ± 0.5	2.3 ± 0.5	3.2 ± 0.8	4.0 ± 0.4
Dose of prednisone (mg/kg/day)	RTX group	0.5 ± 0.4	-	-	0.5 ± 1.4	0.6 ± 0.4	0.6 ± 0.4
	Con group	0.3 ± 0.3	-	-	0.4 ± 0.8	0.6 ± 0.5	0.6 ± 0.5
CYC/TAC	RTX group	35/15	5/4	16/0	8/8	19/8	-
	Con group	16/6	6/3	16/0	7/8	19/8	-

CYC, cyclophosphamide; TAC, tacrolimus.

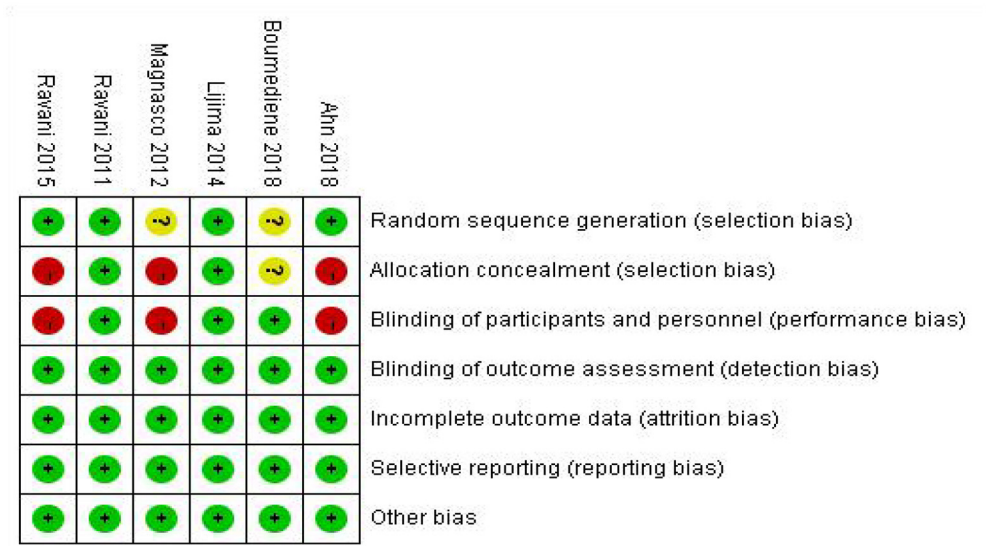

FIGURE 2 | Risk of bias summary.

(124 children) and the control group (110 children). We found that when compared to that in control, RTX could reduce the relapse number of NS, the use of steroids or/and calcineurin inhibitors, and the dosage of steroids, while RTX could not decrease the protein excretion. There were fewer reports of serious adverse reactions of RTX during the therapy, and most adverse events were mild and could be alleviated after stopping RTX or reducing the infusion speed.

As known, the immune disorder was an extremely important pathogenic factor for relapsing nephrotic syndrome (33). The T cell developed dysfunction and secreted some chemical mediators. These abnormal cytokines could change and damage the glomerular filtration membrane. The interest in B cell as a potential pathogenic factor for nephrotic syndrome resulted in reorganization in recent years. The B cells could express co-stimulatory molecules and cytokines, which induced activation and the dysfunction of T cell. RTX induced apoptosis and depletion of B cell, thus inhibiting the interaction between B cells and T cells and reducing the recurrence of nephrotic syndrome in children. Additionally, some research found that RTX had

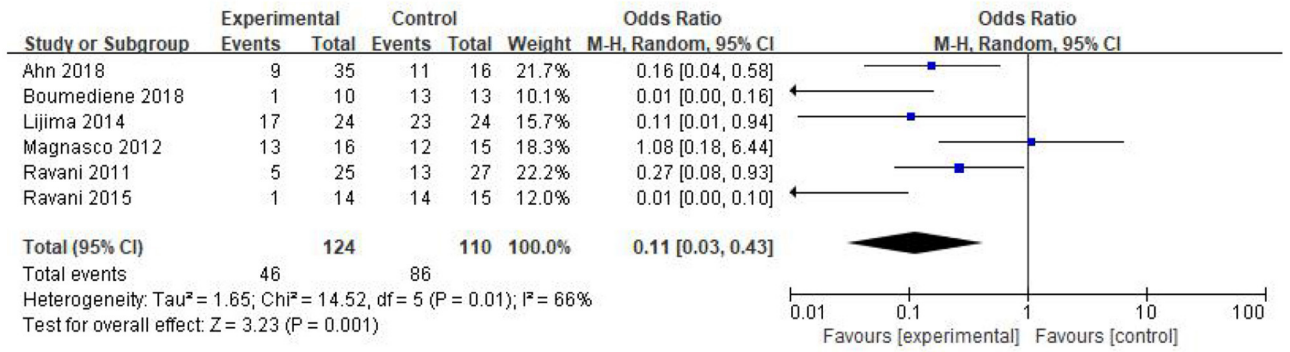

FIGURE 3 | Forest plot showing a meta-analysis for the RTX group vs. control group on the relapse numbers.

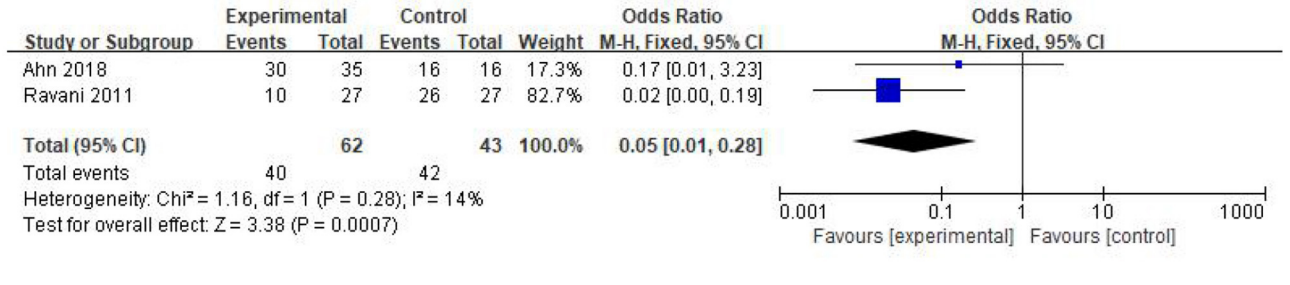

FIGURE 4 | Forest plot showing a meta-analysis for the RTX group vs. control group on the number of patients using steroids or/and CNI.

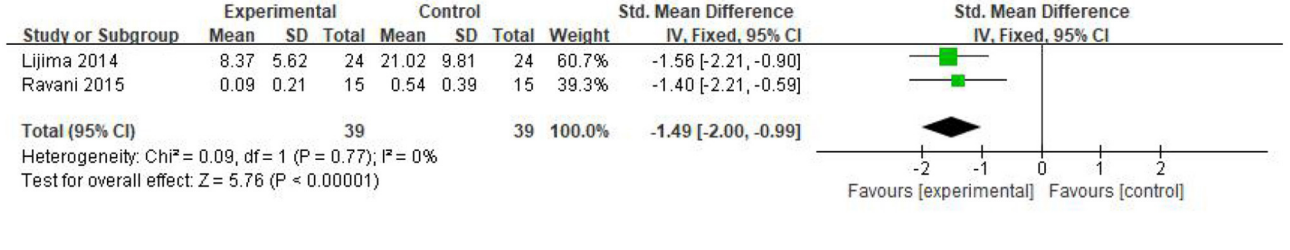

FIGURE 5 | Forest plot showing a meta-analysis for the RTX group vs. control group on the steroid dosage.

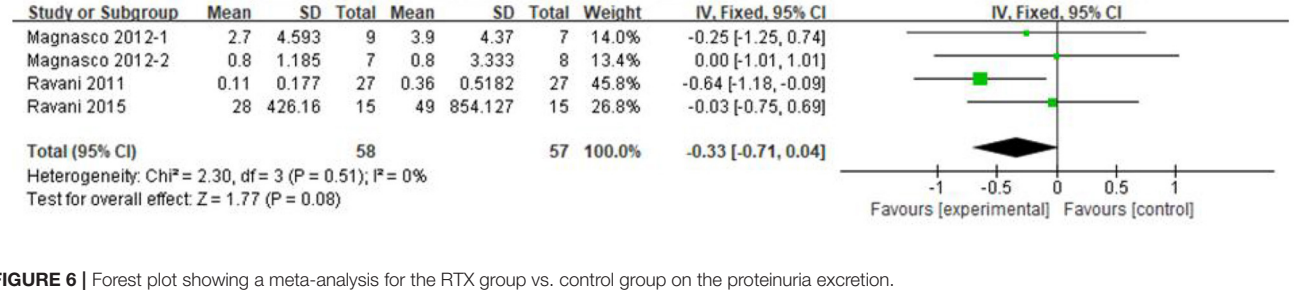

FIGURE 6 | Forest plot showing a meta-analysis for the RTX group vs. control group on the proteinuria excretion.

a protective effect on podocytes in a non-immune pathway (34). RTX could bind to the sphingomyelin phosphodiesterase acid-like 3b protein (SMPDL3b) in lipid rafts expressed on podocytes. The combination of RTX and SMPDL3b played a role in protecting the structure and function of podocytes because SMPDL3b was a key protein that regulated the cytoskeleton of the podocytes.

The research found that RTX holds great potential in reducing the relapses of NS compared with control. Kemper et al. (35) conducted a multicenter retrospective clinical trial and found that 69% of patients achieved long-term remission and 48% of patients stopped immune-suppressant treatments. Sun et al. (13) reported that RTX treatment showed an effective rate of 91.67%, and the number of relapses was significantly reduced ($p < 0.001$). Sellier-Leclerc et al. (19) showed that only 23% of the children relapsed during the treatment of RTX. After long-term observation, the number of relapses was only 37%.

We found a big heterogeneity in the number of relapses in the meta-analysis, with $I^2 = 66\%$, $p = 0.01$. We presumed that the different statistical time cutoffs could be the source of heterogeneity and performed a subgroup analysis by the different cutoffs, 3, 6, and 12 months. We did get a negative result; thus, considering the different cutoff could not be the source of the heterogeneity. We found another interesting difference among the studies included in the mate analysis; the dose of RTX was different. We hypothesized the dose of RTX as the variant and performed subgroup analysis, but could not find the sources of the heterogeneity, either. Last, we considered that the heterogeneity of the analysis would be caused by different experimental design methods, small sample sizes, and different outcome indicators.

Two articles in this study documented the number of patients who took steroids or/and CNI. The mate analysis showed a significant decrease in patients taking steroids or/and CNI after RTX treatment. In the research of Ito et al. (36), 41 of 53 (77%) SDNS/FRNS patients discontinued steroid successfully. Of 53 SDNS/FRNS patients, 17 (31%) stopped CSA. Guigonis et al. (16) found that 19 patients (85%) discontinued treatment with one or more immunosuppressive agents, with no recurrence and no additional immunosuppressive agents. These results indicated that RTX treatment could reduce the usage and the dosage of steroids and other immunosuppressants.

Three articles reported the dosage of steroids after the RTX therapy. In the research of Ahn et al. (27), a total of 61 patients were enrolled, 40 in the RTX group and 21 in the control, and the ratio was 2:1, which resulted in a huge heterogeneity, and the research was excluded. The heterogeneity could be reduced from 55 to 0%. The fixed-effect model was used to analyze the two remaining articles and the results showed the significantly reduced dosage of the steroid after RTX treatment. Sun et al. (13) recorded less steroid usage ($p = 0.014$) and less relapse ($p < 0.001$) in 12 children in the next 6 months after RTX treatment. Gulati et al. (37) studied 57 children, 12 of whom could discontinue one or more immunosuppressive agents. In the other eight patients, the dose of prednisone could be gradually reduced to 0.3–0.5 mg/kg every other day. Ahn et al. (27) and Iijima et al. (28) reported that the average days without steroid application were 141 and 211 days, respectively, and the toxic and side effects of long-term use of steroids for children were avoided.

As known, reducing proteinuria played a key role in NS treatments. Hofstra et al. (38) also found that proteinuria decreased significantly (2–3 g/day) within 2 weeks. Our analysis did not find a significant difference in the reduced proteinuria levels after RTX therapy. The following factors could be responsible for the result. First, the number of patients enrolled in the research was so limited and we needed a larger sample size for the evaluation of the effect of RTX. Secondly, there were big heterogeneities among the patients. Besides the differences in pathological types, children with early drug resistance and late drug resistance were enrolled in the research of Magnasco et al. (30). Last, the proteinuria was affected by a variety of factors. In the study of Dahan et al. (39), the serum albumin level can affect proteinuria.

Most patients tolerated the RTX treatment well. A review from Bonanni et al. (40) reported that the most common adverse event was rash, dyspnea, fever, cough, and infusion-related itching. All these adverse reactions could be well-controlled. Recently, anti-CD20 antibodies from human beings such as Ofatumumab were undergoing clinical research (41–43) and the adverse events remained to be further evaluated.

All the literature in our research showed good quality and held less bias in the Cochrane bias risk assessment. Stata software was applied to assess publication bias, and $p = 0.060$ in the Begg test and $p = 0.022$ in the Egger test. The Egger test result with a high $p = 0.022$ indicates that there was a publication bias, and the small size of the sample could be accounted for it.

There are some limitations. First, the length of remission before the relapse could be considered as an indicator of the drug efficacy. Second, the follow-up time of included studies was inconsistent and all were short. Third, the characteristics of baseline are different, and the age of the two studies (30, 32) is younger than other studies. Finally, RTX therapy has a higher cost than steroid and CNI treatment, and the economic benefits of RTX needed further evaluation. So, we needed much larger, long-term, comprehensive, and controlled studies to further evaluate the clinical value of RTX.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

XG and FZ: guarantor of integrity of entire study and manuscript final version approval. XG and YW: study concepts. YW:

study design, statistical analysis, manuscript definition of intellectual content, and manuscript revision/review. HD: literature research. ZX: data acquisition and data analysis/interpretation. HY: manuscript preparation and manuscript editing. All authors contributed to the article and approved the submitted version.

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CASE REPORT
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Case Report: A Novel Heterozygous Mutation of *CD2AP* in a Chinese Family With Proteinuria Leads to Focal Segmental Glomerulosclerosis

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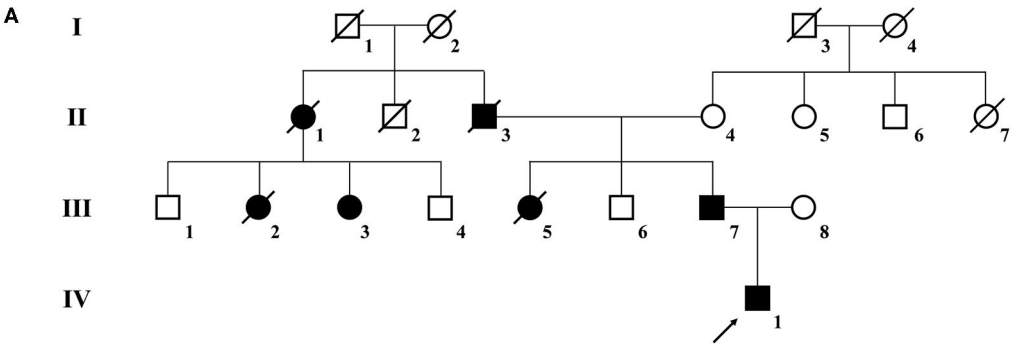
Idiopathic focal segmental glomerulosclerosis (FSGS) is a relatively frequent kidney disorder that manifest clinically as proteinuria and progressive loss of renal function. Genetic factors play a dominant role in the occurrence of FSGS. CD2-associated protein (CD2AP) is an adapter molecule and is essential for the slit-diaphragm assembly and function. Mutations in the *CD2AP* gene can contribute to FSGS development. Here, we describe a Chinese family of four generations with unexplained proteinuria. The proband, a 12-year-old boy, was diagnosed as FSGS. Whole-exome sequencing (WES) revealed an unknown frameshift insertion mutation (p.K579Efs*7) of *CD2AP* gene that leads to a truncation of CD2AP protein. Bioinformatics strategies predicted that the novel mutation was pathogenic. The mutation was absent in either healthy family members or our 200 healthy controls. In summary, we used WES to explore the genetic lesion of FSGS patients and identified a novel mutation in *CD2AP* gene. This work broadens the mutation spectrum of *CD2AP* gene and provides data for genetic counseling to additional FSGS patients.

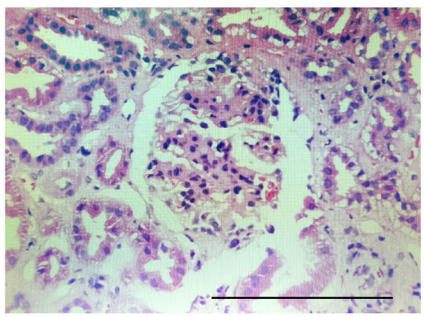
Keywords: FSGS, CD2AP, mutation, heterozygote, whole-exome sequencing

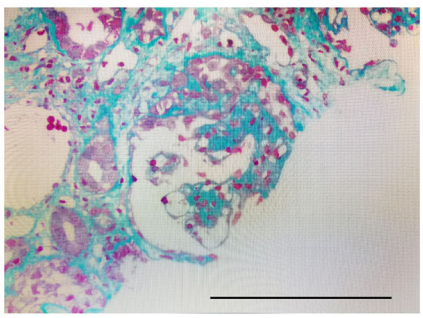
INTRODUCTION

Idiopathic focal segmental glomerulosclerosis (FSGS) is a relatively frequent kidney disorder that manifests clinically as proteinuria and progressive deterioration of renal function. FSGS is histologically characterized by focal and segmental glomerular sclerosis and foot-process effacement (1). As a leading cause of steroid-resistant nephrotic syndrome (SRNS), FSGS makes up about three quarters of the SRNS in children and adults and frequently leads to end-stage renal disease (2).

Genetic factors play a dominant role in the occurrence and development of FSGS. As a kind of podocytopathy, many FSGS-causing genes have been identified and are mainly expressed in glomerular podocytes. The proteins encoded by these genes are crucial for the maintenance of podocyte structure and function, including protein assembly of glomerular basement membrane (GBM) and podocyte skeleton (1). Over the past decades, at least 60 genes have been linked to







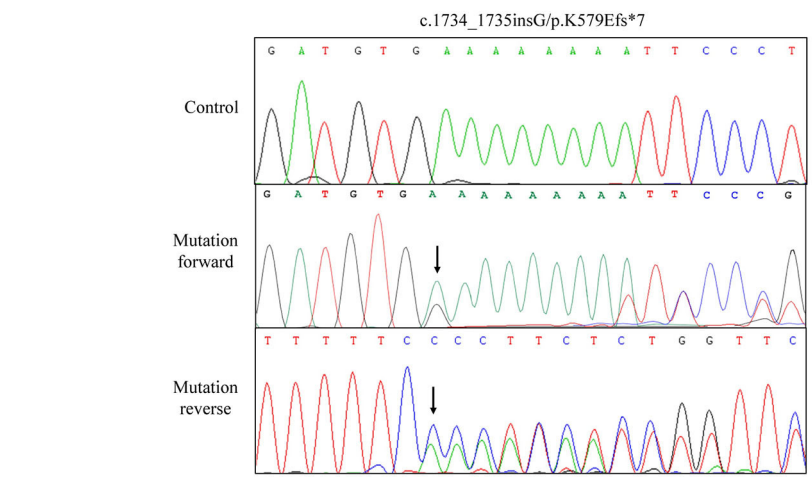


FIGURE 1 | The clinic and sequencing data of this FSGS family. **(A)** The pedigree of this FSGS family. Squares indicate male family members; circles, female members; black symbols, the affected members; white symbols, unaffected members; arrow, proband. **(B)** HE staining **(C)** Masson staining for renal biopsy of the proband (IV-1). **(D)** Sanger DNA sequencing chromatogram demonstrates a heterozygosity mutation (c.1734_1735insG/p.K579Efs*7) of *CD2AP* gene in affected members. **(E)** Alignment of multiple CD2AP protein sequences across species. Letters in red show the K579 site is evolutionarily conserved.

TABLE 1 | Clinical data of three patients in this family.

Subjects	IV-1 (proband)	III-3	III-7	Normal
Sex	M	F	M	/
Age (years)	12	49	40	/
Microscopic hematuria	1+	-	-	-
Proteinuria	2+	1+	1+	-
Uraemia	No	No	No	/
Blood creatinine (μmol/L)	149.0	128.3	168.5	M:<106; F:<86
Blood urea nitrogen (mmol/L)	8.81	11.04	8.52	1.8-7.1
Uric acid (μmol/L)	478.6	520.5	444.3	M:149-416; F:<89-357

F, female; M, male.

SRNS (3). Among them, ∼20 pathogenic genes have been identified in FSGS patients (4). Genes such as collagen α3-5 (*COL4A3–5*), anillin actin binding protein (*ANLN*), inverted formin 2 (*INF2*), paired box 2 (*PAX2*), transient receptor potential cation channel 6 (*TRPC6*), α-actinin-4 (*ACTN4*), and podocin (*NPHS2*) show a higher mutation rate in FSGS patients (5–10). Moreover, mutations of CD2 associated protein (*CD2AP*) and Rho GTPase activating protein 24 (*ARHGAP24*) can also contribute to FSGS development (11, 12). With the development of sequencing technology, more causative genes, such as IFT139 (*TTC21B*), LIM homeobox transcription factor 1β (*LMX1B*), Integrin subunit β4 (*ITGB4*), and Nuclear RNA export factor 5 (*NXF5*), were found in rare FSGS (13–15). On account of high genetic heterogeneity and complicated hereditary constitution, the genetic etiology of primary FSGS remains obscure in many cases.

Herein, we investigate a Chinese family with unexplained proteinuria. The proband was diagnosed as FSGS. Using whole-exome sequencing (WES) technology in combined with bioinformatics strategies, we detected a previously unreported heterozygous mutation in *CD2AP*.

CASE PRESENTATION

Clinical Features

The Chinese family with four generations including 13 persons was described in this research (**Figure 1A**). Three living cases (IV1, III-3, and III-7) among the seven patients were enrolled in this family. Two hundred unrelated healthy subjects were collected as control subjects to exclude polymorphisms. The information of the healthy controls group has been provided in our previous study (16).

The proband (IV1) was a 12-year-old boy from Hunan Province in China. He visited our hospital because of an abnormal urine test. Physical examination showed lower extremity edema and hypertension. Laboratory examination showed high proteinuria (2+), high serum creatinine (149 μmol/L), blood urea nitrogen (8.81 mmol/L), and uric acid (478.6 μmol/L). Microscopic urine analysis indicated microscopic hematuria (1+). Since persistent proteinuria after 6 weeks of prednisone treatment (60 mg/m^2/day), the patient was considered as steroid resistant. Renal biopsy was carried out and revealed glomerulomegaly, segmental podocytes proliferation, and hypertrophic. The GBM characterized segmental thickening. Masson staining mesangial area showed mesophilic deposition (**Figures 1B,C**). Thus, the patient was diagnosed as FSGS. Family history survey showed his father (III-7) also suffered from proteinuria. One aunt (III-3) investigated proteinuria and had a similar 3-year history of lower extremity edema and high blood pressure. The proband's mother refused further medical examination because of divorce. The relevant clinical data of the patients in this family are provided in **Table 1**.

Genetic Analysis

WES yielded 9.13 Gb of data with 99.7% coverage of the target region and 99.1% of the target covered over 10×. In total, about 4,995 variants were detected in the proband. Data filtering were performed as our previous study (17). A set of 11 variants in 11 genes were detected (**Table 2**) and were further analyzed. Information including the inheritance pattern, OMIM clinical phenotypes, Toppgene function (18), and American College of Medical Genetics Classification (19) of these 11 genes has been shown in **Table 2**. No variants in other known FSGS-related genes were detected. Sanger sequencing was carried out in all family members. Co-segregation analysis shown that only a previously not described heterozygous mutation (c.1734_1735insG/p.K579Efs*7) in exon 16 of the *CD2AP* gene was observed in all three affected patients (III-3, III7, and IV-1) and excluded in the healthy members (**Figure 1D**). Family screening showed that the frameshift mutation was inherited *via* the paternal allele (**Figure 1A**). The newly identified mutation was absent in our 200 healthy controls. Alignment of CD2AP amino acid sequences revealed the affected amino acid was evolutionarily conserved (**Figure 1E**). In addition, Swiss-Model software (https://swissmodel.expasy.org/interactive) was utilized to explore the spatial configuration of this CD2AP mutation. As the results showed, a loss of almost all of the C-terminal in the K579Efs*7 mutated CD2AP protein was observed, in comparison with the wild type, as marked by the red frame in the figure (**Figure 2A**).

DISCUSSION

In the current research, we described a Chinese family with unexplained proteinuria. The proband was diagnosed as FSGS. Employing WES combined with bioinformatics strategies, a newly heterozygous mutation (p.K579Efs*7) of the *CD2AP* gene was detected. The frameshift mutation (p.K579Efs*7) locates in the exon 16 that alters the lysine codon at position 579 to a glutamic codon, and is expected to form a premature stop codon, leading to a truncated protein. Sanger sequencing confirmed that all 3 affected members in this FSGS family, including the proband's father (III-7) and his aunt (III-3), harbored the heterozygous frameshift mutation in *CD2AP*. The late patients' DNA was not available. Moreover, this heterozygous variant did not exist in the remaining unaffected family members. Given the segregation of the frameshift mutation with the disease phenotype and the degree of protein structure alteration, it was

TABLE 2 | Variants identified by WES in this family.

Gene	Transcript variant	Protein variant	SIFT	Polyphen-2	Mutationtaster	GnomAD	OMIM clinical phenotype	ToppGene function	American college of medical genetics classification
SLC28A1	NM_004213.4; c.1-16C>G	-	-	-	D	0.0189213	AR, uridine-cytidineuria	Purine nucleobase transport	pvs1, pm3, pp3
NEUROD1	NM_002500.4; c.34G>C	p.G12R	T	B	D	0.0015766	AD, type 2 diabetes mellitus	Pancreatic A cell fate commitment	ps1, pm2
TGM6	NM_198994.2; c.1171G>A	p.V391M	D	B	D	0.0096227	AD, spinocerebellar ataxia	Peptide cross-linking	ps1, pm1, pp3
CIDEC	NM_022094.3; c.457C>T	p.Q153*	-	-	D	-	AR, lipodystrophy	Lipid droplet organization	pvs1, pm1, pm2
TREM2	NM_018965.3; c.574G>A	p.A192T	T	B	P	0.000435114	AR, Polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy	C-C chemokine receptor CCR7 signaling pathway	ps1, pm2, bp4
HOXA10	NM_018951.3; c.170A>G	p.Y57C	T	D	D	0.00121275	-	Proximal/distal pattern formation	ps1, pm2
CFTR	NM_000492.3; c.1666A>G	p.I556V	T	B	D	0.0471606	AR, Cystic fibrosis	Regulation of cyclic nucleotide-gated ion channel activity	ps1, pm1
PYGM	NM_005609.3; c.1860del	p.I621Sfs*37	-	-	D	-	AR, McArdle disease	Glucan catabolic process	pvs1, pm2
MCTP2	NM_018349.3; c.239del	p.S80Tfs*17	-	-	D	0.00364805	-	Calcium-dependent phospholipid binding	pvs1, pm2
XIRP2	NM_152381.5; c.3318_3319del	p.Y1107*	-	-	D	-	-	Muscle tissue morphogenesis	pvs1, pm2
CD2AP	NM_012120.2; c.1734dup	p.K579Efs*7	-	-	D	-	AD, Glomerulosclerosis, focal segmental	Transforming growth factor beta1 production	pvs1, pm2

B, begin; D, disease-causing; P, polymorphism; T, tolerated; AD, autosomal dominant; AR, autosomal recessive; bp, pathogenic benign; pp, pathogenic supporting; ps, pathogenic strong; pvs, pathogenic very strong; pm, pathogenic moderate.

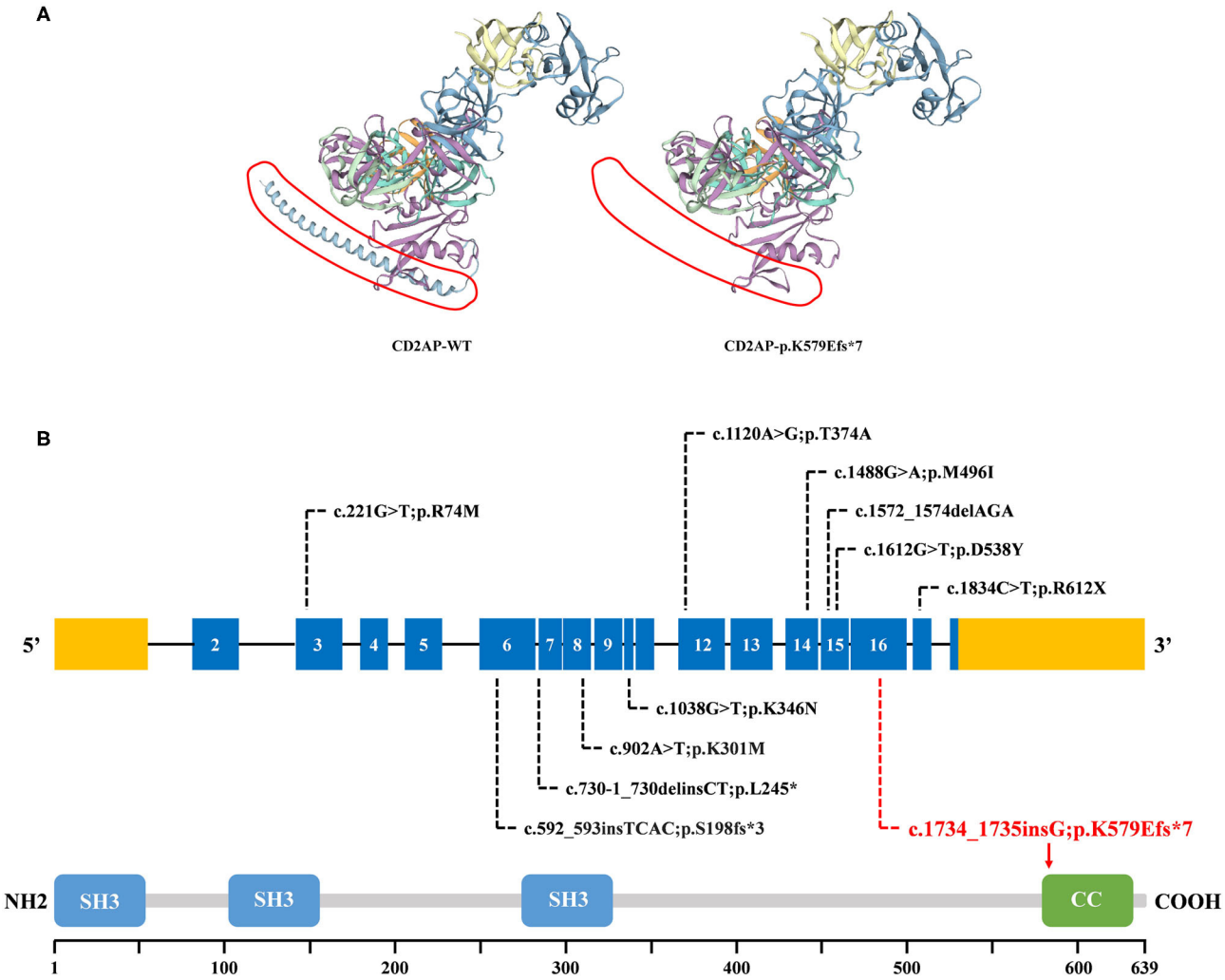


FIGURE 2 | The bioinformatics analysis of mutations. **(A)** Structure prediction of the mutant protein. The wild type CD2AP (CD2AP-WT) protein structure and the p.K579Efs*7 mutant CD2AP (CD2AP-p.K579Efs*7) protein structure were predicted by SWISS-MODEL online software. **(B)** Overview of all known and novel CD2AP mutations. The *CD2AP* gene is shown, with all known *CD2AP* mutations (black letters) and novel mutation (red letters). Blue rectangles indicate exons. Introns are not shown to scale. The CD2AP protein structure is shown. CC, coiled-coil domain; SH3, Src homology 3 domain.

highly considered that the mutation was responsible for the FSGS in this family.

FSGS is clinically characterized by proteinuria and progressive renal failure (1). In our study, all living patients (III-3, III7, and IV-1) in this family presented proteinuria, but definitive data on progression to end-stage renal failure were not available. In a previous report, Gigante et al. screened for changes in the *CD2AP* gene in a total of 80 Italian patients with idiopathic nephrotic syndrome. Three heterozygous mutations in *CD2AP* gene were found in three unrelated patients while there were no definitive data on renal failure in the reported patients (20). For the different molecular and pathogenesis bases of genetically associated FSGS and SRNS, the manifestation and prognosis are different (1). Thus, the genotype-phenotype relation between *CD2AP* gene and FSGS needs to be further investigated. Since most FSGS/SRNS patients with genetic factors do not respond to common treatment and show poor prognosis (1), the patients in this family were given the cyclosporine treatment according to the KDIGO 2012 guideline (21). And a follow-up visit has been scheduled in a few months to ensure the patients benefit from treatment.

CD2AP is prominently expressed in glomerular podocytes. It is an 80 kDa cytoplasmic protein which consists of four domains: three Src homology 3 (SH3) domains at the NH2 terminus and one coiled-coil domain at the COOH terminus (22). CD2AP was initially identified as a ligand for the T-cell-adhesion protein CD2. And it was also shown to bind to nephrin and podocin, thereby acting as an important component of the slit-diaphragm (SD) network (23). The three SH3 domains are essential for the interaction of CD2AP protein with CD2 (20). Recent reports

exploring that phosphorylation of tyrosine residues within the SH3–1 domain may modify interactions between CD2AP and its binding partners, including nephrin (24). Furthermore, it has been demonstrated that CD2AP directly interacts with nephrin at the C-terminal region between amino acids 428 and 600 (25, 26). As shown in **Figures 2A,B**, compared with 639 amino acids in the wild type, the resulting truncated protein is 580 amino acids in length with an abnormal binding domain of nephrin, and lacks the coiled-coil domain that promotes homodimerization. In this case, the truncated CD2AP protein in our study would fail to bind to nephrin. Similarly, Gigante et al. reported a frameshift mutation (p.delE525) in *CD2AP*, which is localized in the same region, affects the ultrafiltration functions of the SD network and might lead to proteinuria (20). Thus, we indicate that the frameshift mutation (p.K579Efs*7) identified in *CD2AP* gene may be a potential candidate factor for the development of FSGS, consistent with the previous research.

CD2AP was a strong candidate gene for nephrotic syndrome (NS). Animal studies have shown that *CD2AP* knockout mice suffer from severe NS and die of massive proteinuria in infancy (27). Moreover, the *CD2AP* heterozygous mouse is prone to proteinuria and presents a glomerular disease at old age with a histology pattern that is similar to human FSGS (28). The relevance of *CD2AP* in human renal pathology still remains largely unknown and the detailed molecule mechanisms involved requires further investigation (20). So far, ∼10 mutations of *CD2AP* have been reported in FSGS or NS patients. A brief review of these reported mutations was shown in **Figure 2B**, which may help for the genetic counseling and prenatal diagnosis of FSGS associated with mutation in the *CD2AP* gene. Although the pathogenic mechanism involved still requires further investigation, our findings offer more evidence that *CD2AP* gene variant is significant in FSGS. Remarkably, the mutation (c.1734_1735insG/p.K579Efs*7) identified in this study has not been published and, therefore, is considered novel.

CONCLUSION

We applied WES to explore the genetic lesion in a Chinese FSGS family. A novel heterozygous mutation (c.1734_1735insG/p.K579Efs*7) of *CD2AP* was identified. Our study broadens the mutation spectrum of *CD2AP* gene and provides data for the clinical management and genetic counseling respect to FSGS.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI BioSample; PRJNA739264.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Third Xiangya Hospital of Central South University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

Y-XL and A-QZ enrolled the family members. F-ML and YS performed DNA isolation and sanger sequencing. C-YW and YD performed genetic analysis. Y-XL and LF wrote the manuscript. LL supported the project. All authors reviewed the manuscript.

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CASE REPORT
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Case Report: The Monogenic Familial Steroid-Resistant Nephrotic Syndrome Caused by a Novel Missense Mutation of *NPHS2* Gene A593C in a Chinese Family

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Background: Pathogenic variants in the *NPHS2* gene encoding podocin in kidney podocytes are associated with autosomal recessive steroid-resistant nephrotic syndrome (SRNS) by disrupting podocyte function and the integrity of the glomerular filtration barrier. The outcome is generally poor by progressing into end-stage kidney disease (ESKD). With the help of gene diagnostics, we can further understand the role of podocin of podocytes in the development and progression of SRNS. However, the pathological mutation of *NPHS2* and clinical relevance remain further elusive.

Case Presentation: Two siblings, a 15-year-old girl and her 10-year-old younger brother from a consanguineous Chinese family, presented with nephrotic syndrome. Both of them developed progressive proteinuria starting from the 5-year-old of age. The renal pathological lesions for them revealed focal segmental glomerulosclerosis (FSGS). There was no response to the glucocorticoid, calcineurin inhibitors, and rituximab treatment. The female affected patient received the hemodialysis treatment due to ESKD in June 2020; the male patient was still in follow-up presenting with SRNS. The mutational screening of the two patients and their parents using Trio whole-exome sequencing showed the *NPHS2* gene *de novo* missense mutation in exon 5 (A593C), for which the two siblings were homozygous and their parents confirmed heterozygous asymptomatic carriers. No other SRNS-related gene variants with the SRNS were determined.

Conclusion: Pathological gene variants screening in children clinically suspected with SRNS might be helpful in the diagnosis as well as appropriate decisions on treatment strategies and prediction of prognosis.

Keywords: steroid-resistant nephrotic syndrome, mutation, *NPHS2*, genetic testing, focal segmental glomerulosclerosis

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INTRODUCTION

Nephrotic syndrome (NS) is a common glomerular disease characterized by massive proteinuria, hypoalbuminemia, hyperlipidemia, and edemas. NS can be classified into hereditary, primary, and secondary types based on their etiologies. Meanwhile, the NS can be clinically divided into two types depending on the response of patients to the steroid therapy, including the steroid-sensitive NS (SSNS) and steroid-resistant NS (SRNS) (1, 2). Podocyte plays a crucial role in maintaining the integrity and stability of the glomerular filtration barrier (3); in children-onset and early adult-onset SRNS, mutation of more than 50 genes in the podocyte have been associated with two-third of these patients, especially nephrin (*NPHS1*), and podocin (*NPHS2*) (4–6). The SRNS is the second most cause leading to end-stage kidney disease (ESKD) in children.

The *NPHS2* (OMIM number 604766) is localized on chromosome 1q25-q31, it was first reported and mapped by linkage analysis in families with autosomal recessive SRNS in 2000, in which 10 different *NPHS2* mutations were identified (7). *NPHS2* comprises of 8 exons and encodes the 42 KD protein podocin with a 383-amino acid in the podocyte (8). Podocin localizes at the slit diaphragm in podocyte and is required for the maintenance of the integrity of the glomerular filtration barrier by interacting with nephrin, CD2AP, and TRPC6 et al., thereby orchestrating the mechanosensation signaling, cytoskeleton organization, and cell survival (9). The mutation of *NPHS2* has been reported to occur in 13% of patients that manifested with SRNS before 25 years of age and accounted for the 34% of all 27 common genes in podocytes causing SRNS (10). To date, many novel *NPHS2* gene mutations have been discovered and more genotype-phenotype correlations have been published, such as deletion c.988_989delCT, c.725C>T, c622G>A and c.928G>A et al. (11–13). With the advent of the latest next gene screening technology and the development of molecular genome database platforms, more and more pathogenic genetic mutations contributing to pediatric SRNS have been identified in recent years (14–16). In this study, we presented a case of two siblings from a consanguineous Chinese family with *NPHS2* mutation, whose renal histology showed focal segmental glomerulosclerosis (FSGS) and had no response to glucocorticoid, calcineurin inhibitors, and rituximab therapy. To the best of our knowledge, this is the first case report of the novel missense mutation of *NPHS2* in exon 5 (A593C) contributing to the SRNS. Our reports may help to further understand the pathogenesis of hereditary glomerulonephropathy caused by *NPHS2* mutation in podocytes, may help the clinicians to choose the proper treatment strategies and genetic and reproductive counseling.

CASE PRESENTATION

A 15-year-old Chinese girl was first diagnosed with proteinuria (2+) during a regular check-up at the age of 5 years old in 2011 but remained untreated until the age of 12 years old in 2018 when she was diagnosed with nephrotic syndrome. The child was transferred to our hospital for further evaluation. There was no abnormal birth and past medical history. Physical examination revealed edema, no skin rash or arthralgia, or gross hematuria. Laboratory studies indicated nephrotic syndrome without hematuria. Blood pressure and serum creatinine levels were normal. Screening of serum and urine from the patient excluded the presence of systemic lupus erythematosus (SLE), antineutrophil cytoplasmic antibody-associated vasculitis (AAV) et al. Since the proteinuria and hypoalbuminemia were resistant to the steroid treatment, prednisone 2 mg/kg per day for 8 weeks, a renal biopsy for her at the age of 12 years old was performed showing FSGS (**Figures 1A,B**). Based on pathological lesion assessment, intravenous methylprednisolone pulse, prednisone plus FK506 (blood trough levels 1.7–2.5 ng/ml), cyclosporine A, and rituximab were administered for 2 years. However, the patient presented persistent nephrotic syndrome and progressive renal failure. At the age of 14 years old in 2020, the patient started on hemodialysis because of ESKD.

Her sibling, a 10-year old Chinese boy, was diagnosed with proteinuria at the age of 5 years old in 2016 during a regular check-up. He was diagnosed with nephrotic syndrome at the age of 8 years old in 2019 and he was admitted to the hospital because of the exacerbating edema in the abdomen. There was no abnormal birth and past medical history. Screening of serum and urine from the patient excluded the presence of SLE, AAV et al. The renal biopsy of the patient at the age of 8 years old was carried out revealing FSGS (**Figures 1C,D**). The proteinuria and hypoalbuminemia were unresponsive to the treatment with prednisone (2 mg/kg per day) and cyclosporin A for 8 weeks, following by the treatment with several pulses of cyclophosphamide and methylprednisone. The patient is presently on non-immunosuppressive antiproteinuric treatment including the optimized renin-angiotensin system (RAS) blockade under the outpatient follow-up, with a relatively stable kidney function determined by serum creatinine (16.5–19.2 μmol/L).

There was a consanguineous family history of father and mother being first cousins for the two siblings, the parents do not present any symptoms of kidney disease.

Because of the SRNS of these two siblings and the consanguineous family history, genetic testing was recommended. All patients and their parents gave their consent for inclusion in this study. Genetic analysis for these two siblings and their parents was carried out by next-generation sequencing (NGS)-based Trio whole-exome sequencing (WES) (Chigene translational medical research center Co. Ltd., Beijing, China). Briefly, genomic DNA was isolated from peripheral blood leukocytes with EDTA as the anticoagulant, sequences of exons were captured using IDT probes (IDT xGen Exome research pane 1 v1.0) and sequenced using NGS sequencer Genome analyzer (NovaSeq 6000, PE150, Illumina). SRNS-related target variants identified by Trio WES were verified by Sanger sequencing (ABI 3730 DNA Analyzer). This revealed a novel missense mutation in exon 5 c.593 A>C (p. E198A) of the *NPHS2* gene, the two siblings were found to be homozygous and their parents were both heterozygous for this mutation. The result of minor allele frequency (MAF) of the *NPHS2* c.593 A>C missense mutation was <0.0005 in the general population, a

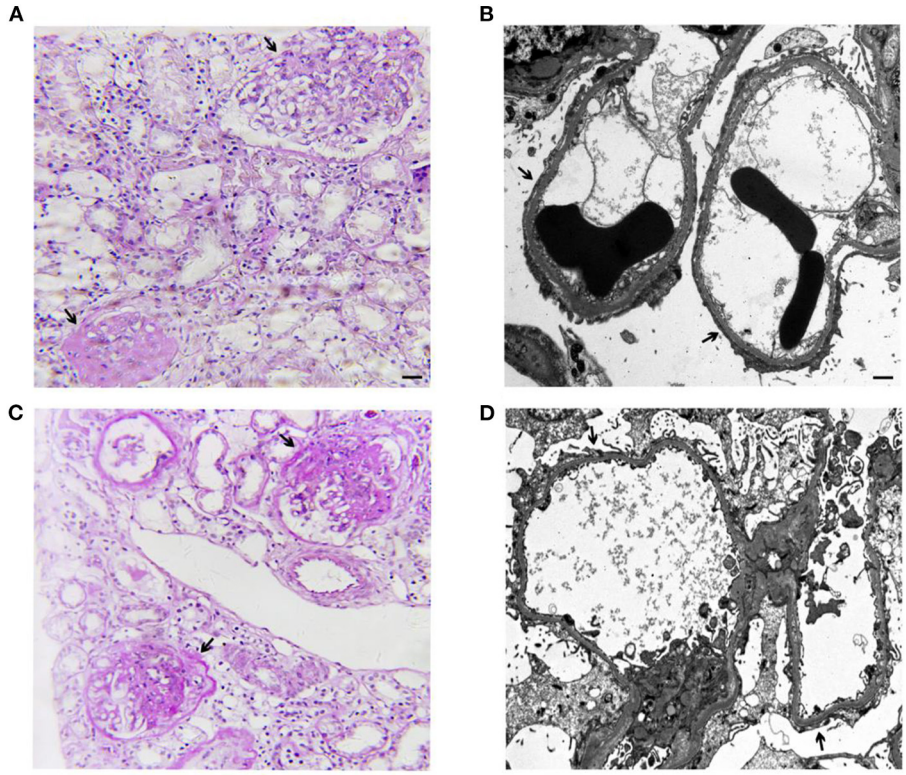

FIGURE 1 | Representative images of light microscopy and transmission electron microscopy (TEM) from the kidney biopsies of the elder sister at age 12 **(A,B)** and the younger brother at age 8 **(C,D)**. **(A)** Global or segmental glomerulosclerosis (arrow) in 23 of 38 glomeruli (Periodic Acid-Schiff staining. Scale bar: 50 μm). Immunofluorescence microscopy revealed negative for IgG, IgA, IgM, C3, C1q, kappa light chain, and lambda light chain in 2 glomeruli. **(B)** Representative TEM showed diffuse foot process effacement of the podocyte (arrow), partial irregularity of the glomerular basement membrane, foam cells, and no subendothelial and mesangial dense deposits (Scale bar: 1 μm). **(C)** Global or segmental glomerulosclerosis (arrow) in 7 of 33 glomeruli (Periodic Acid-Schiff staining. Scale bar: 50 μm). Immunofluorescence microscopy revealed mesangial IgM deposits (++) negative and negative results for IgG, IgA, C3, C1q, kappa light chain, and lambda light chain in 2 glomeruli. **(D)** Representative TEM showed diffuse foot process effacement of the podocyte (arrow), partial irregularity of the glomerular basement membrane, foam cells, and no subendothelial and mesangial dense deposits (Scale bar: 1 μm).

low-frequency mutation; the missense mutation results in the alanine instead of the glutamic acid in the 198th position of the amino acid sequence of the wild-type podocin (NM_014625). The missense mutation was not found in the normal control and was listed as "likely pathogenic" by ACMG (The American College of Medical Genetics and Genomics) 2015 guideline after analyzing the genotype-phenotype associated diseases database and genetic diagnostic platform. Novel missense mutation variants identified in this study were shown in **Figure 2**. The family cosegregation analysis of the phenotype-genotype for these two patients and their parents indicated the autosomal recessive inheritance type.

DISCUSSION

In this study, we described two siblings with SRNS who carried a novel homozygous missense mutation in exon 5 c.593 A>C (p. E198A) in the *NPHS2* gene, which encodes the podocin in the podocyte. No other mutations were found in the *NPHS2* gene or other SRNS-related genes such as *NPHS1*, *WT1*, *FN1*, *KANK2*, *BMP4*, and *PLOD1* et al. Their father and mother carried the same heterozygous missense mutation as the siblings. The parents showed no proteinuria, hematuria, or other abnormalities. It suggests that a missense mutation on both alleles in exon 5 c.593 A>C of the *NPHS2* gene is required for the development and progression of SRNS. The percentage of SNRS attributing to *NPHS2* variants varies from 12.7 to 29.5% (10, 17, 18). The *NPHS2* mutation usually presents the autosomal recessive form of hereditary SRNS, the phenotype is less severe than that of the SRNS caused by the *NPHS1* variants, which is one of the most common mutations resulting in the hereditary podocytopathy in children, and it encodes the nephrin in the podocyte. The onset age of patients with *NPHS1* mutation is at birth to 3 months, while it is later than 3 months at the onset age for the patients with *NPHS2* mutation (10, 19). The two siblings reported here both presented with SRNS and pathological lesions of FSGS, both of them were found proteinuria at 5 years old. Unfortunately, there were no medical records of their urinary analysis results in the past although their parent said that the two siblings were normal in appearance. Both of the two siblings

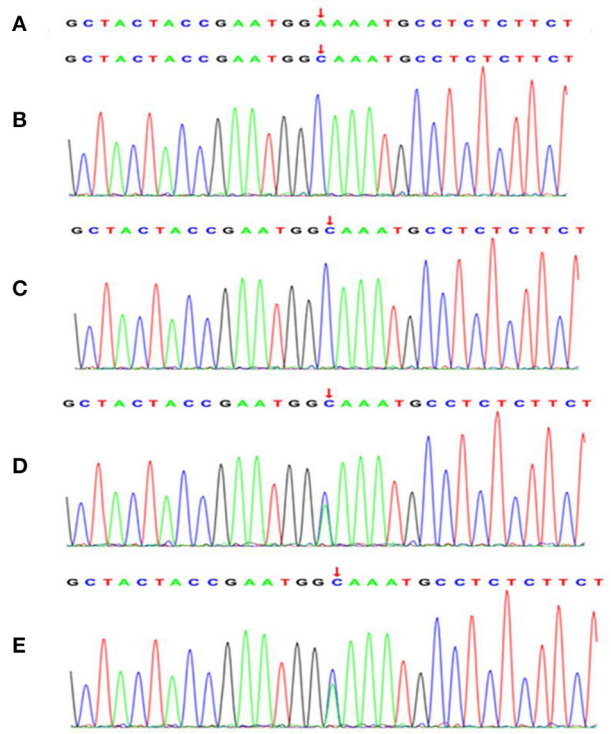

FIGURE 2 | A novel missense mutation in exon 5 (A593C) of the *NPHS2* gene was identified by Trio whole-exome sequencing in the affected individuals and family members. **(A)** normal control sequence. The affected elder sister **(B)** and the affected younger brother **(C)** carried the homozygous missense mutation in exon 5 (A593C) of the *NPHS2* gene. The father **(D)** and the mother **(E)** of the affected patients revealed the heterozygous missense mutation in exon 5 (A593C) of the *NPHS2* gene. The arrows indicate mutant positions.

failed to respond to steroid therapy and immunosuppressive agents, which did not have an impact on the etiological target of hereditary podocytopathy. It is worth noting, the longtime treatment with steroids and immunosuppressive agents causes side effects of drugs and increases the financial burdens to the family. The elder sister was started on hemodialysis at 14 years old due to ESKD, and the younger brother is currently on symptomatic treatment.

The underlying missense mutation of the *NPHS2* gene of patients in our study localized in the exon 5 encoding for the central part of the podocin consisting of 68 amino acids (from 179th amino acid to 246th amino acid), which suggests this area is of major importance for the proper function of podocin (20). Besides this mutation, two variants in exon 5 of the *NPHS2* gene have been reported to cause the SRNS (p.R229Q and p.A242V) in the population of Caucasians and Europeans (2). The c.593 A>C mutation of the *NPHS2* gene results in the alanine instead of the glutamic acid in the 198th position of the amino acid sequence of the podocin. Podocin is a membrane protein that is exclusively located in the slit diaphragm of the podocyte in the kidney (21). Podocin plays a critical role in the stability of the slit diaphragm and the maintenance of the integrity of the glomerular filtration barrier by forming a protein complex through co-localizing with other proteins such as nephrin, CD2-associated protein (CD2AP), zonula occludens-1(ZO-1), and transient receptor potential channel-6 (TRPC-6) et al., that ensure the stable anchorage of the membrane proteins of the slit diaphragm to the actin cytoskeleton in the podocyte (22, 23). These membrane multi-protein complexes localizing on the slit diaphragm in podocytes are capable of regulating the mechanosensation and cell signaling to maintain the integrity of the glomerular filtration barrier (23). The loss of the exon 5-coded central part, an essential component of the prohibitin homology domain, results in the podocin's inability to bind the cholesterol of the inner membrane of the cell (20) and abnormal distribution of podocyte-associated molecules (24). Nephrin is one of the most important components of the slit diaphragm in the podocyte, and nephrin exerts its role as the signal transducer through the phosphorylation of AP-1. The nephrin-activated AP-1 is augmented by podocin which is markedly attenuated by deletion of the prohibitin homology domain of podocin, thereby impairing the function of nephrin in podocytes (24, 25). Intriguingly, how does the replacement of glutamic acid in the 198th position of the amino acid sequence with the alanine lead to a significant loss of podocin function in podocytes, as observed in the family we described in this study, remains to be further determined.

SRNS caused by *NPHS2* mutation is believed to be a recessive inheritance disease requiring a mutation on both alleles coding for podocin, and these patients usually progress to an early onset of ESKD, such as the female patient reported here who started on hemodialysis at the age of 14 years old. Hence, it is very essential to adopt an appropriate therapeutic approach for these patients. Apart from the dialysis, burgeoning evidence has been furnished that renal transplantation may favor ESKD patients with genetic SRNS. In 2020, the results of 101 cases of kidney transplantation in children with SRNS in Italy reported that no disease recurrence was observed in all 41 genetic SRNS including *NPHS2* mutation compared to the idiopathic SRNS (59.5% recurrence) or unknown reasons SRNS patients (43.5% recurrence), during a median follow-up of 58.5 months (26). A cohort study in Germany involving 27 multiplex families (53 patients) and 25 patients with sporadic SRNS due to the *NPHS2* mutation showed these patients who underwent kidney transplantation did not develop recurrence of proteinuria (27). In the meantime, few reports suggested a risk of relapse in the SRNS due to *NPHS2* mutation (28, 29), however. the causative role of the *NPHS2* variants in these reports might be reconsidered with a further in-depth understanding of the pathogenic *NPHS2* variants in the SRNS; no anti-podocin antibodies have been detected in the tested *NPHS2* patients with disease recurrence after kidney transplantation (28, 30); many patients with disease recurrence showed an effective response to immunosuppression treatment (28, 29). The female patient with ESKD in this study has been recommended for kidney transplantation.

In conclusion, this paper reports an SRNS phenotype of familial NS with a pathological lesion of FSGS. Although the exact mechanism remains to be clarified, we offer evidence that the homozygous exon 5 c.593 A>C (p. E198A) of the

NPHS2 gene plays a significant role in proper podocin protein function and its pathogenesis. The nephrologists are encouraged to do the genetic testing for these early-onset SRNS patients especially those with a positive family history, as it helps in their right diagnosis, proposes the appropriate therapeutic strategies, predicts the prognosis, and assists genetic and reproductive counseling.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: The National Omics Data Encyclopedia (NODE), accession number: OEP002338.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical Ethics Committee of People's Hospital of Xinjiang Uygur Autonomous Region. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

LB, JZ, and HJ made clinical data collection and were actively involved in the clinical care of the patients. XT, CL, and HJ made substantial contributions to the conception of the manuscript. HJ and CZ evaluated the renal pathology of the patients. XT, LB, and HJ drafted and edited the manuscript. All authors read and approved the final manuscript.

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